

**REPROGRAMMING TUMOUR IMMUNE MICROENVIRONMENT OF
COLORECTAL LIVER METASTASES
WITH RENIN-ANGIOTENSIN INHIBITORS**

Dora Lucía Vallejo Ardila, MD

ORCID identification number: 0000- 0001- 9863- 608X

**Submitted in total fulfilment of the requirements of the degree of Doctor of
Philosophy**

**Department of Surgery
The University of Melbourne**

May 2021

ABSTRACT

Renin-angiotensin system inhibitors (RASi) have shown anti-tumour effects that may significantly impact the response to current cancer immunotherapies and the prognosis in patients with colorectal liver metastases (CLM). RAS components are expressed by various immune cells and adult hematopoietic cells. The mechanisms by which RASi reprogram the tumour immune microenvironment toward an immunostimulatory milieu involves modulating the function of immune T lymphocytes, myeloid cells and cancer-associated fibroblasts.

Experimental Design: Liver metastases were established in a mouse model using an autologous colorectal cancer cell line. RASi (captopril 750mg/kg) or carrier (saline) was administered to the mice daily via intraperitoneal injection, from day one post-tumour induction to endpoint (day 15 or 21 post tumour induction). At the endpoint, tumour growth was determined, and lymphocyte and myeloid-derived infiltration and composition in the tumour and liver tissues were analyzed by flow cytometry and immunohistochemistry. At endpoint day 15, livers and tumour tissues were collected, and tissue lysates were prepared and analyzed using liquid chromatography-tandem mass spectrometry proteomic techniques to determine relative changes in protein abundance. The proteomics results were analyzed using open-source software such as MaxQuant and Perseus.

A 7-plex OPALTM protocol was used to assess the composition and spatial distribution of T cell markers CD3, CD8, FoxP3 and CD103 and the epithelial to mesenchymal transition markers α -SMA and E-cadherin. The protocol was manually optimized and validated in two independent cohorts of formalin-fixed, paraffin-embedded CLM patient tissues using well-established antibodies, a single spectral library, negative controls, and biological controls corroborating the staining pattern of immune infiltrates.

Results: Captopril significantly decreased tumour viability and impaired metastatic growth, increased the infiltration of CD3⁺ T cells into both tissues, and the primary contributing phenotype to this influx is a CD4 and CD8 double-negative T cell subtype, while CD4⁺ T cells decreased and CD8⁺ T cells remained unchanged. Furthermore, clustering and functional enrichment showed that captopril modulates the expression of some of the immunoproteasome subunits, including PSMB8, PSMB10, PSMB5 and

PSME1. Also, T lymphocyte phenotypes' spatial distribution was seen in significantly higher levels in the invasive tumour margin compared to their density within the liver parenchyma or the tumour centre in human CLM. Thus, high CD3⁺ density in the tumour core or high CD8⁺ density in the invasive tumour margin predicts a significantly better overall survival. Moreover, high CD3⁺ T cell density at the adjacent liver parenchyma and a high CD8⁺ density at the tumour core segment influenced patient survival after CLM resection in patients who received RASi antihypertensive medication. In the case of myeloid cells, a significant decline in myeloid cells expressing CD31⁺ was induced by captopril, while the relative proportion of CD31⁻ cells was preserved when comparing between the liver and the tumour. RASi significantly reduces a CD31⁺monocyte-derived subset F4/80⁻ Ly6C^{intermediate/High}, which may have a pro-angiogenic and immunosuppressive function.

Conclusion: The work of this thesis highlights the mechanism by which RAS inhibitors decreased tumour viability and impaired metastatic growth by effectively restoring the immunogenicity of CLM toward an immunostimulatory milieu.

Keywords: renin-angiotensin system, tumour microenvironment, anti-tumour immunity, Proteomics, Colorectal liver metastases, Immunotherapy, and multiplex IHC

DECLARATION

This declaration is to certify that the thesis comprises only my original work towards the PhD degree, except for that outlined in the preface section.

The thesis is less than 100,000 words in length, exclusive of tables, references, and appendices.

No part of this thesis has been submitted for examination towards attaining any other qualification at The University of Melbourne or any other institute.

Dr. Dora Lucia Vallejo-Ardila

Department of Surgery,
The University of Melbourne Austin Hospital,
Heidelberg Victoria, 3084, Australia

PREFACE

The candidate performed most of the work described in this thesis under the supervision of Prof. Christopher Christophi, Dr Theodora Fifis and Dr Katrina A Walsh at the Department of Surgery, Austin Health. The University of Melbourne, from 2018-2020.

The candidate submitted this thesis under the supervision of Dr Sophie Marie Paquet-Fifield at the Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity. The University of Melbourne.

This document is a thesis with publications. Chapter 1 and 3 are author-accepted versions of manuscripts according to University of Melbourne guidelines. The corresponding published papers are included as Appendices.

Chapter 1

Dr Theodora Fifis, Dr Louise M Burrell (Department of Medicine, Austin Health), Dr Katrina Walsh, Prof. Christopher Christophi discussed and commented on the literature review.

Chapter 2

Dr Theodora Fifis and Dr Rita Paolini (Melbourne Dental School, The University of Melbourne) revised mouse surgical procedures and IHC protocol. Dr Ching-Seng Ang (Bio21 Institute, The University of Melbourne) revised the Proteomic Mass Spectrometry protocol. Dr Katrina Walsh assisted and revised the FACS protocol. Candani Tutuka (Olivia-Newton John Cancer Research Institute) assisted and revised multiplex IHC protocol and inForm® analysis.

Chapter 3

Dr Rita Paolini (Melbourne Dental School, The University of Melbourne), George Kastropis (Department of Surgery, Austin Health), Dr Katrina Walsh, Dr Theodora Fifis assisted with the animal experiments. Rita Paolini (Melbourne Dental School, The University of Melbourne), George Kastropis (Department of Surgery, Austin Health)

and Katrina Walsh assisted with the FACS experiments. Theodora Fifis assisted with the interpretation of IHC. Prof. Christopher Christophi, A/Prof. Marcos Vinicius Perini, Dr Theodora Fifis and Dr Katrina A Walsh discussed the results and commented on the manuscript.

Chapter 4

Dr Rita Paolini (Melbourne Dental School, The University of Melbourne), George Kastrapis (Department of Surgery, Austin Health), Dr Katrina Walsh, Dr Theodora Fifis assisted with the animal experiments. Rita Paolini (Melbourne Dental School, The University of Melbourne), George Kastrapis (Department of Surgery, Austin Health) and Katrina Walsh assisted with the FACS experiments. Theodora Fifis assisted with the interpretation of IHC. Dr Sophie Paquet-Fifield (The Peter Doherty Institute for Infection and Immunity) assisted with the manuscript preparation and submission.

Chapter 5

Dr Rita Paolini (Melbourne Dental School, The University of Melbourne), Dr Ching-Seng Ang (Bio21 Institute, The University of Melbourne) and George Kastrapis (Department of Surgery, Austin Health) assisted with the Proteomic Mass Spectrometry experiments.

Chapter 6

Dr Theodora Fifis assisted with the study design. Marco A. De Bastiani assisted with spatial engagement and cell density analysis. Dr David Williams performed the histological evaluation and classification of growth patterns. Dr James Tai (Department of Surgery, Austin Health) collected and verified clinical and surgical data from histopathological reports of the specimens included in the study. Dr Jonathan Banting and A/Prof. Vijayaragavan Muralidharan provided whole tissue FFPE slides and survival information of the patients included in the study. Candani Tutuka (Olivia-Newton John Cancer Research Institute) provided multiplex IHC staining procedures and inForm® analysis training. Dr Sophie Paquet-Fifield (The Peter Doherty Institute for Infection and Immunity) assisted with the manuscript preparation and submission.

Chapter 7

Dr Theodora Fifis assisted with the study design. Marco A. De Bastiani provided the customized R code for spatial engagement, cell density and cell distribution analysis. Dr David Williams performed the histological evaluation and classification of growth patterns. Victorian Cancer Biobank collected and verified clinical and surgical data of the RASi group specimens included in the study. Dr Jonathan Banting and A/Prof. Vijayaragavan Muralidharan provided whole tissue FFPE slides (control group) and survival information of the patients included in the study. Dr Theodora Fifis, Dr Katrina A Walsh and discussed the results and commented on the chapter.

The candidate was the primary author of all of these manuscripts, conceptualized the studies, analysed the data, and wrote the manuscripts' first draft. All the other authors contributed their expertise from different aspects, revised all the manuscripts critically for important intellectual content, and final approval of the version to be published.

The candidate carried out no work prior to enrolment, and no work has been submitted for other qualifications. No third-party editorial assistance was used for the formatting of this thesis.

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to Dr Sophie Paquet-Fifield; without her support and encouragement, the completion of this work would not have been possible. I feel honoured to have a brilliant scientist and a wonderful human being as a principal supervisor. Thank you for sharing your love for cancer research and your wisdom.

First and foremost, Professor Christopher Christophi, I would like to sincerely thank you for allowing me to undertake my PhD at the Department of Surgery, Austin Health. I appreciate your intellectual inspiration; I will continue working on your research legacy in my pursuit of becoming an academic surgeon. Simultaneously, I believe that you and Dr Theodora Fifis gave me plenty of room to develop my hypothesis. I cherished the memories of our lab meetings.

Dr Theodora Fifis, thank you for your support and guidance. Your patience and assistance over the past three years have been invaluable. Although there were difficult times, your advice and your support meant a lot to me.

I would like to acknowledge all the Liver Research group members and the Department of Surgery for your help, both past and present. A special mention to co-supervisor Dr Katrina Walsh, my sincerest gratitude for sharing your expertise. Dr Rita Paolini and Mr George Kastrapis, thank you for your help in the laboratory, dedicated to working at the Bioresources facility every weekend. A big thank you for all your laughs.

Translational research involves personnel across multiple departments within the Austin Health precinct. I would like to thank the Bioresources Facility members, the Victorian Cancer Biobank, the Olivia Newton-John Cancer Research Institute (Candani Tutuka and Associate Professor Sarah Ellis). Additionally, I would like to acknowledge the support from the Department of Pathology, Austin Health.

I would like to dedicate my thesis to the most generous and beautiful human being, my wonderful wife, Viviana Andrea Arteaga. Thank you for believing in me, for standing by me over the past six years and across the journey through six different countries and against all odds, my darling, we did it. Of additional importance, I would like to dedicate every awaken minute to finish this thesis to my beloved mother, Maria Dora and brother José Leonardo and my academic soulmate, Dr Marco Antonio de Bastiani; your unrelenting support and encouragement can never be put into words.

FUNDING AND AWARDS

I would like to acknowledge and thank the following funding bodies for their financial support.

Personnel funding

- The Department of Science, Technology, and Innovation (COLCIENCIAS-Colombia) (PhD overseas 679 program)
- The University of Melbourne, Faculty of Medicine, Dentistry and Health Sciences (Postgraduate Research Scholarship 2017-2021).

Research funding

- Austin Medical Research Foundation: Walsh -2018, Perini -2018, Fifis -2019.
- Austin Medical Research Foundation: Walsh/Vallejo- 2020.

SCIENTIFIC PRESENTATIONS

Some of the work in this thesis has been presented at scientific conferences by me.

- Oral presentation as Best of the Best poster, Australasia Gastrointestinal Trial Group 21st scientific meeting, Adelaide, Australia, 2019. *Immunomodulatory effects of Renin-angiotensin system inhibitors on T lymphocytes in mice with Colorectal Liver Metastases.*
- Poster presentation at the Austin Research Festival, Austin Medical Research Foundation, Heidelberg, Australia, 2019. *Immunomodulatory effects of Renin-angiotensin system inhibitors on T lymphocytes in mice with Colorectal Liver Metastases.*
- Poster presentation 21st EMBL PhD. Symposium. Facing the future: Perspectives and challenges of life sciences in the 21st century, 28th–30th November 2019 EMBL Heidelberg, Germany. *Renin-Angiotensin System inhibitors modulate the immunoproteasome expression in the tumour and liver tissues of mice with colorectal liver metastasis.*
- E-poster presentation at the Australasian Gastro-Intestinal Trials Group 22nd, Annual Scientific Meeting 2020. *Prognostic Value of Immune Profiling Colorectal Liver Metastases Follow Spatial Distribution.*
- 2020 University of Melbourne Competition: Visualise your thesis, August 26, 2020. Audio-visual Media publication: <https://vimeo.com/449180218>
- Poster presentation at the 14th World Congress of the Hepato-Pancreato-Biliary Association (IHPBA 2020), November 27- 29, 2020. **Vallejo Ardila DL**, et al. *Immune Profiling of Histologic Growth Patterns of Colorectal Liver Metastases: Spatial Distribution and Phenotyping of T Lymphocytes, Cytotoxic T Cells and Tissue-resident Memory T Cells.* HPB. 2021;23:S126.

AWARDS

I feel honoured to have received the following nominations and awards during my candidature:

- Nomination as *Best of the Best poster award*, Australasia Gastrointestinal Trial Group scientific meeting, Adelaide, Australia, 2019.
- Nomination as *Best Poster Austin Research Festival award*, Austin Medical Research Foundation, Heidelberg, Australia, 2019.
- Advance Clinical Trial Design Scholarship, Australia, 2019.
- Nominated for the Olivia Newton-John Cancer Research Scholarship, Australia, 2020.

TABLE OF CONTENTS

CHAPTER 1	2
1.1 Introduction.....	6
1.2 Regulation of tumour angiogenesis using renin-angiotensin inhibitors impacts cancer progression.....	9
1.3 Targeting Ang II/AT1R axis to impair hypoxia and acidosis within the tumour stroma	11
1.4 Renin-angiotensin inhibitors regulate inflammatory signalling pathways and oxidative stress in tumour biology.....	11
1.5 Renin-angiotensin inhibitors modulate the function of immune cells within the tumour microenvironment	12
1.6 Targeting tumour non-cellular components using Renin-angiotensin inhibitors may enhance antitumour immunity	15
1.7 The cross-regulation of the kallikrein-kinin system and Renin-angiotensin system by angiotensin-converting enzyme has pleiotropic effects in the tumour microenvironment.....	16
1.8 Perspectives and significance	18
1.9 Brief description of the physiology of RAS and therapeutic relevance of targeting the conventional axis.....	19
CHAPTER 2	21
2.1 Animals and experimental model of colorectal liver metastasis.....	24
2.2 Primary cancer cell line MoCR maintenance	24
2.3 Intrasplenic Colorectal Liver Metastases induction in CBA mice	25
2.4 Kinetics of the tumour growth in the MoCR mouse model	26
2.5 Treatment protocol.....	27
2.6 MoCR-CLM model Tissue collection	28
2.7 Macroscopic Stereology.....	29
2.8 Hematoxylin and Eosin staining.....	31
2.9 Immunohistochemistry in Mouse liver and tumour tissues.....	31
2.10 Image caption and analysis algorithm	33
2.11 Flow cytometry procedure and dimensionality reduction analysis.....	36
2.12 Global proteomics and bioinformatic analysis	43
2.13 Multiplex IHC staining of Mouse liver and tumour tissues	45
2.14 Statistical analysis for Mouse CLM studies.....	48
2.15 Human Colorectal Liver Metastases immune profiling.....	48
2.15.1 Human Tissue collection	48
2.15.2 Inclusion and exclusion criteria	49

2.15.3 Histological investigation of growth patterns.....	49
2.15.4 Multiplex IHC staining using human CRL.....	49
2.16 Image analysis using inForm®	51
2.16.1 Tissue segmentation using Machine learning-based algorithms.....	51
2.16.2 Cell segmentation and cell phenotyping using Machine learning-based algorithms	51
2.16.3 Pixel-based colocalization analysis.....	52
2.16.4 R analysis of Spatial distribution and spatial engagement.....	53
2.16.5 Cell density analysis	53
2.16.6 Survival analysis	54
2.16.7 Statistical Analysis	54
2.16.8 Code availability.....	55
CHAPTER 3	59
3.1 Background	62
3.2 Materials and Methods.....	63
3.2.1 Animals and experimental model of colorectal liver metastasis.....	63
3.2.2 Experimental design	64
3.2.3 Treatment protocol.....	64
3.2.4 Stereology	64
3.2.5 Immunohistochemistry	65
3.2.6 Image caption and analysis algorithm	65
3.2.7 Flow cytometry	66
3.2.8 Statistical analysis.....	66
3.3 Results.....	67
3.3.1 Captopril inhibits metastatic growth and reduces tumour viability	67
3.3.2 Captopril treatment modulates spatial and temporal infiltration of tumour Lymphocytes expressing CD3 ⁺ and CD4 ⁺	69
3.3.3 Captopril treatment does not alter the tumour PD-L1 expression	70
3.3.4 Captopril treatment differentially modulates T cell subpopulations infiltrating into the tumour and liver tissues.....	71
3.3.5 Captopril treatment differentially modulates the activation of T cell subtypes in the tumour and liver tissues.....	73
3.4 Discussion.....	76
3.5 Conclusion	79
CHAPTER 4	82
4.1 Introduction.....	84
4.2 Methods	87

4.2.1 Statistical analysis.....	87
4.3 Results.....	88
4.3.1 Captopril treatment subtly modifies the liver or tumour expression of myeloid populations identified with CD11b and CD11c markers.....	88
4.3.2 Captopril treatment modulates the spatial and temporal distribution of F4/80+ macrophages in the liver and tumour	94
4.3.3 Captopril treatment differentially modulates myeloid cell subpopulations expressing CD31 into the tumour and liver tissues	98
4.3.4 Captopril treatment decreases the proportion CD31 ⁺ F4/80 ⁻ subpopulation within the tumour	100
4.3.5 Captopril treatment reduces PD-L1 expression on CD31 ⁺ F4/80 ⁺ and CD31 ⁻ F4/80 ⁺ myeloid subpopulations in the liver	102
4.3.5 Captopril reduces the proportion of Ly6C ^{high} monocyte-derived subpopulation in the liver and tumour tissues	103
4.3.6 t-SNE analysis identifies and stratifies a discrete monocyte-derived subset targeted by Captopril in the tumour	104
4.4 Discussion.....	108
4.5 Conclusion	112
CHAPTER 5	116
5.1 Introduction.....	118
5.2 Methods	119
5.3 Results.....	120
5.3.1 Impact of captopril treatment on the global proteome of liver tissues at day 15 post-tumour induction	120
5.3.2 Functional enrichment analysis of differentially regulated proteins in liver and tumour tissues of captopril treated mice	124
5.4 Discussion.....	129
5.5 Conclusion	132
CHAPTER 6	136
6.1 Introduction.....	138
6.2 Methods	140
6.2.1 Survival analysis and Kaplan-Meier (KM) curves.....	140
6.2.2 Statistical Analysis for tissue segmentation distribution (cell count/HPF)	141
6.2.3 Code availability.....	141
6.3 Results.....	141
6.3.1 Spatial tumour region delineation and distribution of positively stained cells.....	141

6.3.2 Spatial and phenotypical characterization of CLM infiltrating immune cells within the tumour and adjacent liver parenchyma	148
6.3.3 Marker predictive value for CLM patient survival integrating frequency of tumour immune infiltrates and spatial distribution	149
6.3.4 Spatial expression patterns of α -SMA and E- cadherin in CLM	151
6.3.5 Prognostic value of E-cadherin expressing CD8 ⁺ T cells	154
6.3.6 Spatial near neighbour relationships of T Lymphocytes influence the survival of CLM patients	155
6.3.7 Histologic growth patterns influence the spatial distribution of infiltrating immune cells and EMT markers	159
6.3.8 Correlation Between Frequency and Spatial Distribution in the histologic growth patterns	162
6.3.9 Spatial Engagement Profile of Desmoplastic and Pushing Pattern Differed from Replacement Pattern	164
6.4 Discussion	166
6.5 Conclusion	170
CHAPTER 7	174
7.1 Introduction	176
7.2 Methods	178
7.3 Statistical analysis	178
7.3.1 Cell density analysis	178
7.3.2 Survival analysis and KM curves	179
7.3.3 Code availability	179
7.4 Results	180
7.4.1 Demographic and clinical characteristics	180
7.4.2 Spatial and phenotypical characterization of CLM infiltrating immune cells within the tumour and adjacent liver parenchyma in patients using RASi medications	181
7.4.3 The effect of RASi on Human CLM alters cell density and spatial distribution of T cell populations	184
7.4.4 The influence of RASi on CLM patient survival integrating T- cell density and their spatial distribution on the liver-tumour interface	187
7.4.5 The effect of RASi on Human CLM modifies cell density and spatial distribution of non-T cells expressing CD103	194
7.4.6 The effect of RASi on spatial expression patterns of E- cadherin and α -SMA	198
7.4.7 The changes of spatial engagement profile of immune cells and EMT markers after RASi	204

7.4.8 RASi influences cell density of immune cells and EMT markers in the different histologic growth patterns	206
7.5 Discussion.....	209
7.6 Conclusion	214
CHAPTER 8	217
GENERAL DISCUSSION AND FUTURE DIRECTIONS.....	217
8.1 Contributions of the thesis using the MoCR-CLM mouse model.....	219
8.2 Contributions of the thesis using high-dimensional immune-profiling of human CLM	223
8.3 Clinical translation potential of MoCR-CLM mouse model to benefit patients with CLM.....	227
8.4 Conclusions and Future directions	229
CHAPTER 9	232
SUMMARY.....	232
CHAPTER 10	237
REFERENCES	237

LIST OF FIGURES

Figure 1. The pro-tumour and anti-tumour mediated effects of the conventional and alternative RAS axis.....	7
Figure 2. Renin-angiotensin inhibitors reprogram tumour immune microenvironment.	9
Figure 3. Renin-angiotensin system components intersecting with the kallikrein-kinin system.....	17
Figure 4. Kinetics of the tumour growth in the MoCR mouse model.	27
Figure 5. Workflow diagram of experimental design.....	29
Figure 6. Quantitative stereology for calculating the percentage of tumour burden..	30
Figure 7. Tumour viability analysis.	31
Figure 8. Histological quantitation strategy.....	35
Figure 9. Gating strategy T lymphocytes and DNT.....	38
Figure 10. Gating strategy for analysis of Myeloid-derived population.....	39
Figure 11. Identification and stratification strategy for t-SNE-guided gating analysis of myeloid population and subpopulations.	42
Figure 12. mIHC based on Tyramide signal amplification (TSA) using Opal chemistry.	47
Figure 13. Tissue and Cell segmentation and phenotype scoring algorithm.....	52
Figure 14. Pixel-based colocalization.....	53
Figure 15. Control of tumour growth and viability by RASi treatment.	68
Figure 16. Tumour infiltrating lymphocytes are modulated by RASi.	70
Figure 17. Temporal and spatial PD-L1 ⁺ expression in tumour cells.....	71
Figure 18. RASi differentially alters the proportions of CD3 ⁺ subpopulations of lymphocytes in the liver and tumour.	73
Figure 19. RASi differentially changes the proportions of CD3 ⁺ subpopulations expressing PD1 ⁺ and tissue-resident CD8 ⁺ lymphocytes in the liver and tumour.	75
Figure 20. The effect of RASi on CD11b and CD11c populations in the liver and tumour tissues.....	90
Figure 21. The cell density of CD11c ⁺ and CD8α ⁺ in the tumour region.....	92
Figure 22. The effect of captopril on inguinal lymph node morphology and CD11b and CD11c populations.....	93
Figure 23. Temporal changes of F4/80 ⁺ expression in the adjacent liver parenchyma.	95
Figure 24. Temporal and spatial changes of F4/80 ⁺ expression in the tumour.	97
Figure 25. RASi differentially alters the proportions of CD11b ⁺ populations in the liver and the tumour.	99
Figure 26. Captopril differentially alters the proportions of F4/80 expression of CD11b ⁺ CD31 populations in the liver and the tumour.....	101
Figure 27. Captopril differentially alters PD-L1 expression on CD31 ⁺ F4/80 ⁺ and CD31 ⁻ F4/80 ⁺ myeloid subpopulations.	103
Figure 28. Captopril differentially alters the proportions of myeloid-derived suppressor cells in the liver and the tumour.....	104
Figure 29.	108
Figure 30. Quantitation of the changes induced by captopril in the global proteome of the liver.....	122

Figure 31. The pattern of expression of PSMB5 and PSMB8 in liver and tumour tissues.	127
Figure 32. The effect of ACEi in the expression of PSMB8 (LMP7) in the tumour region. Representative microphotographs of IHC LMP7	128
Figure 33. Representative microphotographs of the workflow diagram for analysis using Aperio ImageScope™, Vectra® imaging and inForm® cell phenotyping	145
Figure 34. Spatial distribution of T Lymphocytes (CD3 ⁺ CD8 ⁻ CD103 ⁻), CD8 ⁺ T cells (CD3 ⁺ CD8 ⁺ CD103 ⁻), TRM (CD3 ⁺ CD8 ⁺ CD103 ⁺), and non-lymphocytes expressing CD8 ⁺ or CD103 ⁺ in the CLM cohort.	147
Figure 35. CLM patient survival post-liver resection univariate analysis using the median distribution of cell count per HPF and T cell subpopulations' spatial distribution.	150
Figure 36. CLM patient survival post-liver resection univariate analysis using the median distribution of cell count per HPF and spatial distribution of non- T cell subpopulations.	151
Figure 37. Spatial expression patterns of α-SMA and E- cadherin in CLM and colocalization of E-cadherin expression on CD8 ⁺ T cells	153
Figure 38. Nearest neighbour spatial relationships of T Lymphocytes and their influence on patient survival. a Schematic representation of T lymphocytes subpopulations in the TME and their nearest neighbour (NN) cells.....	159
Figure 39. Comprehensive immune profile characterization of the three dominant histologic growth patterns of CLM relative to their distribution.	161
Figure 40. Correlation between cell phenotypes in the three dominant tumour growth patterns.	163
Figure 41. Immune cell spatial engagement in the three dominant tumour growth patterns.	165
Figure 42. Overall survival between RASi and Control groups.	181
Figure 43. Representative microphotographs of the tissue segmentation using Vectra® imaging and inForm® pathology view analysis.	182
Figure 44. Cell density calculated for the RASi group relative to their distribution at the liver-tumour interface, including FoxP3 cell phenotypes.	184
Figure 45. Comparison between T-cell density and spatial distribution in the control and RASi group.	186
Figure 46. The influence of RASi on CLM patient survival post-liver resection univariate analysis using CD3 ⁺ T-cell density and spatial distribution.	190
Figure 47. The influence of RASi on CLM patient survival post-liver resection univariate analysis using CD8 ⁺ T- cell density and their spatial distribution.....	192
Figure 48. The influence of RASi on CLM patient survival post-liver resection univariate analysis using T _{RM} CD103 ⁺ T- cell density and their spatial distribution in the IM.	194
Figure 49. Comparison between cell density and spatial distribution of CD103 ⁺ (CD3 ⁻) in the control and RASi group.	195
Figure 50. The influence of RASi on CLM patient survival post-liver resection univariate analysis using CD103 ⁺ (CD3 ⁻) cell density and their spatial distribution.	198
Figure 51. Comparison between cell density and spatial distribution of E-cadherin and α-SMA in the control and RASi group.	199
Figure 52. The influence of RASi on CLM patient survival post-liver resection univariate analysis using E-cadherin cell density and spatial distribution.	202

Figure 53. The influence of RASi on CLM patient survival post-liver resection univariate analysis using α -SMA cell density and spatial distribution.....	204
Figure 54. Nearest neighbour spatial relationships of T Lymphocytes and non-T lymphocytes.....	207
Figure 55. The effect of RASi on cell density of immune cells and EMT markers in the different histologic growth patterns.....	208

LIST OF TABLES

Table 1. IHC panel of antibodies for mouse studies.	33
Table 2. FACS panel of antibodies for mouse studies.	37
Table 3. Multiplex IHC antibody panel for mouse studies.	48
Table 4. Multiplex IHC antibody panel for Human CLM studies.	50
Table 5. Differentially regulated proteins in the captopril liver tissues compared to saline liver tissues	121
Table 6. Functional enrichments of proteins differentially regulated in captopril treated compared to saline liver tissues.	123
Table 7. Reactome pathway analysis of proteins differentially regulated in captopril treated compared to saline liver tissues.	125
Table 8. Clinical, histopathological, and surgical characteristics of the CLM study cohort	143
Table 9. List of antihypertensive medications taken in the Group RASi before liver metastasectomy	180

LIST OF SUPPLEMENTARY FIGURES

SUPPLEMENTARY FIGURES

Fig. S1 Histological quantitation strategy.....	288
Fig. S2 Gating strategy for analysis of lymphocyte subpopulations.	289
Fig.S3 Captopril treatment effect the number and size of metastatic foci.....	290
Fig. S4 Correlation matrix using Pearson r to determine the distribution of tumour infiltrating.....	290
Fig.S5 Histological quantitation of CD11b ⁺ and CD11c ⁺	291
Fig.S6 Histological quantitation of F4/80 ⁺	292
Fig.S7 Methodology for the spatial delineation of tumour regions and spatial immune profiling in CLM.	293
Fig.S8 Study flow diagram	294
Fig.S9 Individual patient's immune profiles were compared with sclerosed hemangioma and normal liver	295
Fig.S10 Colocalization analysis of CD3 ⁺ , CD8 ⁺ , E-cadherin and FOXP3 ⁺	296

LIST OF SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLES

Supplementary Table 1. FACS panel for mouse studies.....	298
Supplementary Table 2. Descriptive statistics of T Lymphocytes (CD3 ⁺ T-cells), CD8 ⁺ T cells (CD3 ⁺ CD8 ⁺ CD103 ⁻), T _{RM} (CD3 ⁺ CD8 ⁺ CD103 ⁺), and non-lymphocytes expressing CD8 ⁺ or CD103 ⁺ in the CLM cohort (<i>cell count per HPF</i>).	298
Supplementary Table 3. Survival information of the CLM study cohort.....	299
Supplementary Table 4. Survival information of the control and RASi CLM study cohort.....	300

ABBREVIATIONS

ABBREVIATION	DEFINITION
ACE	Angiotensin-Converting Enzyme
ACEH	Angiotensin-Converting Enzyme Homolog
ACEi	Angiotensin-Converting Enzyme Inhibitors
AGT	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
ARBs	Angiotensin II Receptor Blockers
AT1R	Angiotensin II Type 1 Receptor
AT2R	Angiotensin II Type 2 Receptor
B1R	Kinin Receptor 1
B2R	Kinin Receptor 2
BK	Bradykinin
CAF	Cancer-Associated Fibroblast
CAFs	Cancer-Associated Fibroblasts
CapL	Captopril Treated Liver
CapT	Captopril Treated Tumour
CEA	Carcinoembryonic Antigen
CL	Control Liver
CLM	Colorectal Liver Metastases
COX-2	Cyclooxygenase 2
CRC	Colorectal Cancer
CRP	C- Reactive Protein
CRS	Clinical Risk Scoring
CT	Control Tumour
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
DAB	Diaminobenzene
DC	Dendritic Cells
dMMR	Deficient Mismatch Repair Pathways
DNT	Double Negative T Cells
DOCA	Deoxycorticosterone Acetate
EC	Endothelial Cells
EGFR2	Epidermal Growth Factor Receptor 2
EMT	Epithelial-Mesenchymal Transition
FACS	Fluorescent-Activated Cell Sorting
FACs	Flow Cytometry Analysis
FDA	Food And Drug Administration
FFPE	Formalin-Fixed Paraffin-Embedded
FoxP3	Forkhead Box P3
FunRich	Functional Enrichment Analysis Tool
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
G-MDSCs	Granulocytic-Myeloid Derived Suppressor Cells
GO	Gene Ontology

HCC	Hepatocellular Carcinoma
H&E	Hematoxylin And Eosin Staining
HIER	Heat-Induced Epitope Retrieval
HIF	Hypoxia Inducible Factor
ICOS	Inducible T Cell Co-Stimulator
ICI	Immune-Check Point Inhibition
IHC	Immunohistochemistry
IL-1a, IL-1B, IL-6, IL-8	Interleukin 1 Alpha (IL-1 α); Interleukin 1 Beta (IL-1 β); IL-6 (Interleucina-6); Interleucina-8 (IL-8)
IM	Invasive Tumour Margin
IFNs	Interferons
IFN- γ	Interferon Gamma
IP	Intraperitoneal
ISAT	Inter-Cellular Spatial Analysis Tool
KC	Kupffer Cells
KKS	Krein Kinin System
LC-MS/MS	Liquid Chromatography With Tandem Mass Spectrometry
LCMV	Lymphocytic Choriomeningitis Virus
LM	Liver Metastases
LMP2	Low Molecular Mass Protein 2
LMP7	Low Molecular Mass Protein 7
LN	Liquid Nitrogen
LP	Liver Parenchyma
LPS	Lipopolysaccharide
M-CSF	Macrophage Colony-Stimulating Factor
MDSC	Myeloid-Derived Suppressor Cells
MECL-1	Multicatalytic Endopeptidase Complex Subunit
MHC-II	Major Histocompatibility Complex I
MHC-I	Major Histocompatibility Complex II
mIHC	Multiplex Immunohistochemistry
M-MDSCs	Monocytic-Myeloid Derived Suppressor Cells
MRGD	MAS-Related G Protein Couple Receptor D
M Φ	Macrophages
NEP	Neutral Endopeptidase
NK	Natural Killer
NN	Nearest Neighbour
OS	Overall Survival
PD-1	Programmed Cell Death-1
PDAC	Pancreatic Ductal Adenocarcinoma
PD-L1	Programmed Cell Death Ligand-1
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PPI	Protein-Protein
RAS	Renin-Angiotensin System
RASi	Renin-Angiotensin System Inhibitors
RFS	Relapse-Free Survival

ROS	Reactive Oxygen Species
rpm	Revolution Per Minutes
STRING	Search Tool For Retrieval Of Interacting Genes
TAM	Tumour Associated Macrophages
TAMs	Tumour-Associated Macrophages
TANs	Tumour-Associated Neutrophils
TBST	Tris Buffered Saline
TC	Tumour Core
TEAB	Tetraethylammonium Bromide
T _H	T Helper
TIL	Tumour Infiltrating Lymphocyte
TM	Tumour Margin
TME	Tumour Microenvironment
TNF α	Tumour Necrosis Factor
tRAS	Tissue Renin Angiotensin
TRM	Tissue-Resident Memory T
VCB	Victorian Cancer Biobank
VEGF	Vascular Endothelial Growth Factor
α -SMA	A-Smooth Muscle Actin

Chapter 1

Literature review

This chapter aims to gain an in-depth understanding of the existing research about the mechanisms by which inhibition of the renin-angiotensin system reprograms the tumour immune microenvironment.

This chapter is published in the journal *Oncotarget*, as per the following reference:

- Vallejo-Ardila, D. L., Fifi, T., Burrell, L. M., Walsh, K., & Christophi, C. (2018). Renin-angiotensin inhibitors reprogram tumour immune microenvironment: A comprehensive view of the influences on anti-tumour immunity. *Oncotarget*, 9(84), 35500-35511.

Due to copyright obligations, the author accepted version of this manuscript is presented in Chapter 1 of this thesis.

The copyedited, typeset published version of this manuscript can be found at <https://doi.org/10.18632/oncotarget.26174>

CHAPTER 1

LITERATURE REVIEW

Research Perspective

Title: Renin-angiotensin inhibitors reprogram tumor immune microenvironment: a comprehensive view of the influences on anti-tumor immunity

Authors:

Dora L Vallejo-Ardila¹, Theodora Fifi¹, Louise M Burrell^{2,3}, Katrina Walsh¹, Christopher Christophi¹

¹Department of Surgery, Austin Health. University of Melbourne, Melbourne, VIC 3084, Australia.

²Department of Medicine, Austin Health. University of Melbourne, Melbourne, VIC 3084, Australia.

³Department of Cardiology, Austin Health. University of Melbourne, Melbourne, VIC 3084, Australia.

*Corresponding author:

Dr. Dora L Vallejo-Ardila, M.D. and General Surgeon. Ph. D research scholar. Department of Surgery, Austin Health. University of Melbourne, Melbourne, VIC 3084, Australia. Phone: (+61) 03 9496 3672; FAX: (+61) 039496 4800; e-mail: dvallejo@student.unimelb.edu.au

Summary:

Renin-angiotensin system inhibitors (RASi) have shown potential anti-tumor effects that may have a significant impact on cancer therapy. The components of the renin-angiotensin system (RAS) including both, conventional and alternative axis, appear to have contradictory effects on tumor biology. The mechanisms by which RASi impair tumor growth extend beyond their function of modulating tumor vasculature. The major focus of this review is to analyse other mechanisms by which RASi reprogram the tumor immune microenvironment. These involve impairing hypoxia and acidosis within the tumor stroma, regulating inflammatory signalling pathways and oxidative stress, modulating the function of the non-cellular components and immune cells, and regulating the cross-talk between the kallikrein-kinin system and RAS.

Keywords: renin-angiotensin system, tumor microenvironment, anti-tumor immunity, kallikrein-kinin system

1.1 Introduction

The main conventional axis of the renin-angiotensin system (RAS) [such as angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE), angiotensin I (Ang I), angiotensin II (Ang II)], functions as an intricate peptide signalling network through several receptors [including angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R)] (Carey & Siragy, 2003). AGT is produced and released into circulation by the liver, then AGT is hydrolysed by renin, which is produced by the juxtaglomerular cells of the kidney to form Ang I (Carey & Siragy, 2003). Afterwards Ang I is hydrolysed by ACE in the endothelial cells of the lungs, to produce the biologically active Ang II. Ang II interacts with two different receptors, AT1R and AT2R (Carey & Siragy, 2003). The alternative axis comprises ACE2, which is known as ACE-related carboxypeptidase or angiotensin-converting enzyme homolog (ACEH). ACE2 is mostly found in the vascular endothelial cells and renal tubular epithelium (Donoghue et al., 2000). ACE2 cleaves Ang II to Ang-(1-7), whereas ACE produces Ang-(1-7) by cleaving Ang-(1-9). Ang-(1-7) commonly acts via mitochondrial assembly receptor (Mas receptor or MasR) (Lautner et al., 2013). Another peptide alamandine is generated either by the cleavage of Ang A or Ang-(1-7), by ACE and decarboxylase, respectively. Alamandine binds through MAS-related G protein couple receptor D (MRGD) (Lautner et al., 2013). Several other truncated bioactive peptides have been previously characterized including Angiotensin III (Moeller et al., 1998), Angiotensin IV (Mustafa et al., 2001), and Ang A (Walmor De, 2014), as part of the RAS network (**Fig. 1**).

In the mid-1970s it became possible for the first time to therapeutically target the components of Ang II/AT1R axis using the angiotensin-converting enzyme inhibitor, captopril (Erdos, 1976), and followed by selective blockade of the AT1R with losartan (Bhardwaj, 2006). Renin-angiotensin system inhibitors (RASi) includes two classes of pharmacological agents, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) (Bhardwaj, 2006). A third class of RASi, a direct renin inhibitor such as aliskiren is also included in the list of RASi approved by the U.S. Food and Drug Administration (FDA) (Müller & Luft, 2006).

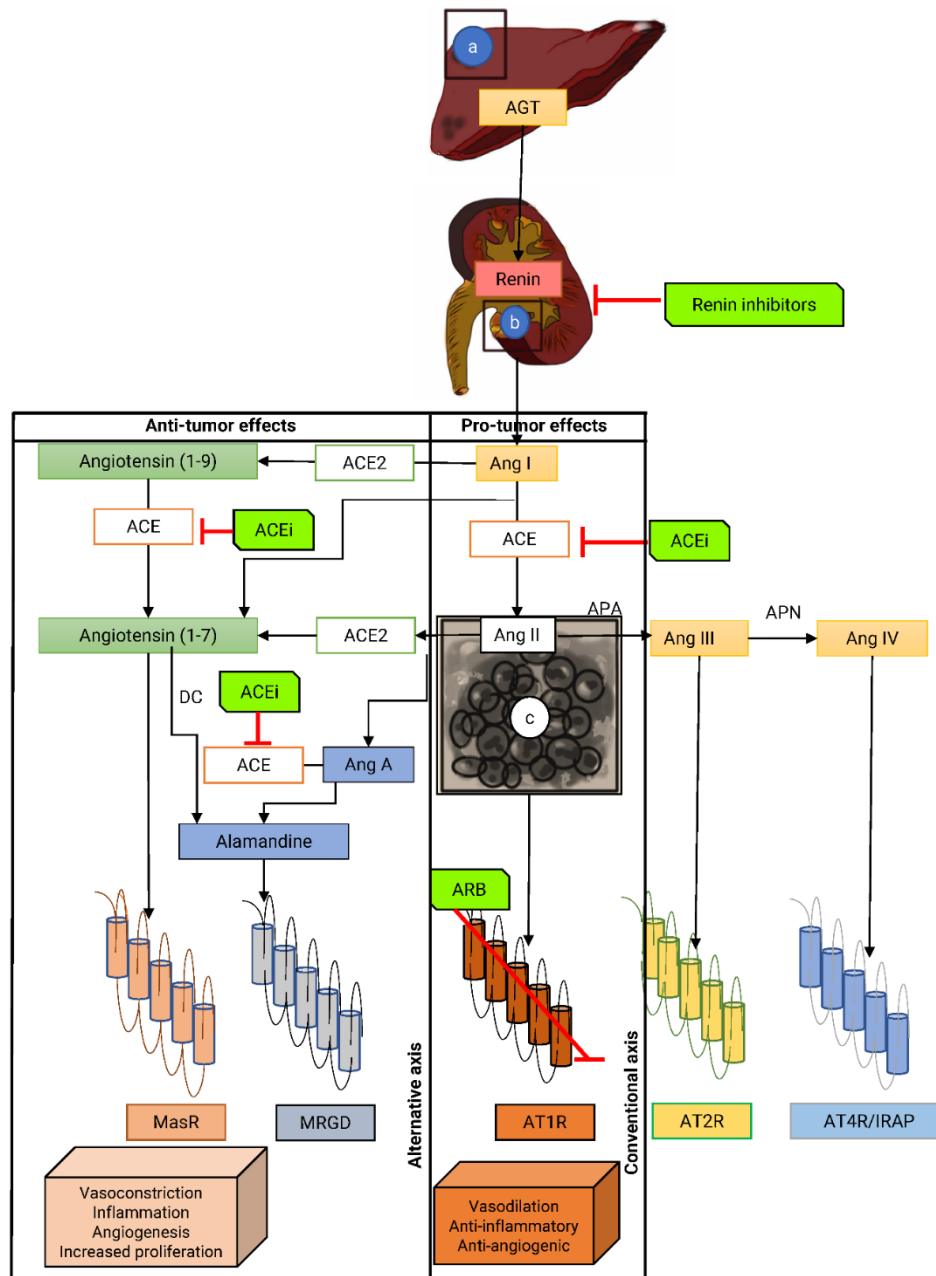


Figure 1. The pro-tumour and anti-tumour mediated effects of the conventional and alternative RAS axis. Angiotensinogen (AGT) is produced and released into circulation by the liver (a), then AGT is hydrolysed by renin. Renin is produced by the juxtaglomerular cells of the kidney (b) to form Angiotensin I (Ang I). Afterwards Ang I is hydrolysed by angiotensin-converting enzyme (ACE) in the endothelial cells of the lungs (c), to produce the biologically active angiotensin II (Ang II). Ang II interacts with two different receptors, angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). ACE2 cleaves Ang II to Angiotensin (1-7) (Ang (1-7)), whereas ACE produces Ang (1-7) by cleaving Angiotensin (1-9) (Ang(1-9)). Ang (1-7) commonly acts via mitochondrial assembly receptor (Mas receptor or MasR). Alamandine is generated either by the cleavage of Ang A or Ang (1-7), by ACE and decarboxylase (DC) respectively. Alamandine binds through MAS-related G protein couple receptor D (MRGD). Angiotensin II is metabolized to Angiotensin III (Ang III) by aminopeptidase A (APA), whereas Ang III is metabolized to Angiotensin IV (Ang IV) by aminopeptidase N (APN). AT4R or Insulin-regulated membrane aminopeptidase (IRAP) is activated by Ang IV. NEP, neutral endopeptidase; PEP, prolyndopeptidase. The inhibition of the conventional axis by ACEi or ARB reduces inflammation, vasoconstriction, and angiogenesis means by which also inhibits tumour growth. Opposite effects have been associated with the activation of the alternative axis including vasodilatation, anti-inflammatory and anti-angiogenic effects.

RASi are widely used to treat heart failure (Yusuf et al., 1991), myocardial infarction (Okura et al., 2016), and systemic hypertension (Daïen et al., 2012) and have proved to be effective in preventing cardiovascular (Tardif & Gregoire, 2013) and renal associated comorbidities (Daïen et al., 2012). Additionally, RASi have shown potential anti-tumour effects that promise a significant impact in cancer therapy (Pinter & Jain, 2017). Several studies have yielded diverging findings on the role of RASi on the incidence of recurrence, metastasis and survival in cancer patients (Sun et al., 2017). To comprehend the potential benefit of RASi in cancer patients consideration should be given to factors such as tumour heterogeneity, tumour stage, hormone receptor status, human epidermal growth factor receptor 2 (EGFR2) over-expression, and (neo)adjuvant treatment regimen (Pinter & Jain, 2017). A meta-analysis including 55 studies suggested that RASi may improve the survival of cancer patients depending on cancer type and class of RASi (Sun et al., 2017).

Differences between the mediated effects of the RAS conventional and alternative axis have been previously described. In general, the over-expression of RAS components within the Ang II/AT1R axis (such as ACE and AT1R) is associated with tumour growth and with more aggressive tumour features in several types of human cancer, including breast cancer, ovarian cancer and renal cancer (Arrieta et al., 2008; Arrieta et al., 2015; Ino et al., 2006), whereas Ang II/AT2R and Ang(1-7)/MasR showed opposite effects (Xu et al., 2017). Nonetheless, there are also conflicting evidence, which may suggest tumour-type specific differences (Arrieta et al., 2015). Similarly, ACE2/Ang-(1-7)/MasR axis dysregulation has been shown to be up-regulated or down-regulated depending on the type of cancer (C. Yu et al., 2016; Zhao et al., 2010; Zheng et al., 2015) (**Fig. 1**).

The role of the various RASi in modulating pathological processes involving cell inflammation (Husain et al., 2015; Pacurari et al., 2014), fibrosis (G. Kim et al., 2016; Saber et al., 2018; Zhu et al., 2016), and tumour growth (Ager et al., 2008; Neo et al., 2010; Sugimoto et al., 2012; Wegman-Ostrosky et al., 2015), has been well-documented, but its influence on anti-tumour immunity is uncertain. The aim of this literature review is to analyse current evidence on the effects of RASi on anti-tumour immunity by reprogramming the tumour microenvironment (TME).

The pathological processes and cellular functions inside the TME which appear to be influenced by RASi include tumour angiogenesis, hypoxia and acidosis within the tumour stroma, inflammatory signalling pathways, oxidative stress, immune cell modulation and the role of kallikrein-kinin system (KKS) (**Fig. 2**).

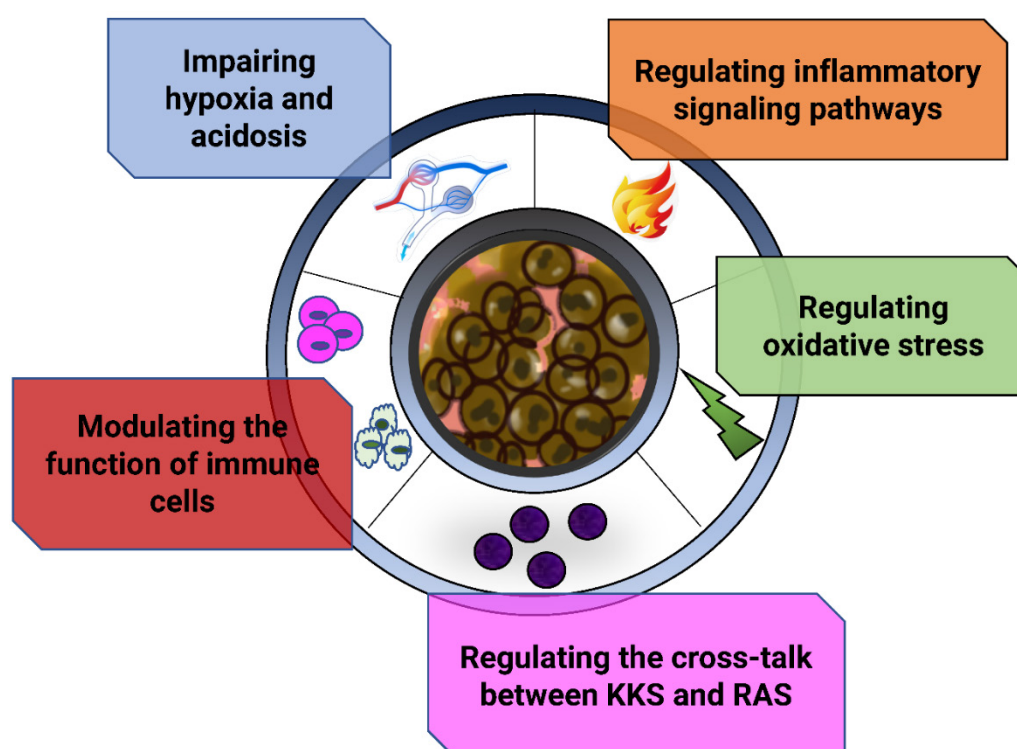


Figure 2. Renin-angiotensin inhibitors reprogram tumour immune microenvironment. The pathological processes and cellular functions inside the tumour microenvironment which appear to be influenced by RASi include tumour angiogenesis, hypoxia and acidosis within the tumour stroma, inflammatory signalling pathways, oxidative stress, immune cell modulation and the role of kallikrein-kinin system (KKS).

1.2 Regulation of tumour angiogenesis using renin-angiotensin inhibitors impacts cancer progression

Conclusive evidence has shown that targeting Ang II/AT1R impairs neovascularization and vascular permeability and decreases microvessel density by reducing vascular endothelial growth factor (VEGF)-expression (Carbajo-Lozoya et al., 2012; Ino et al., 2006; Shirotake et al., 2011; Usui et al., 2008). Angiogenesis is initiated by the disruption of the endothelial cells (EC) monolayer and their invasion into the

surrounding stroma. The recruitment of pericytes along the basement membrane into the new vessels provides both, mechanical stability and molecular cross-talk with EC, which induces VEGF production and survival signalling (Mittal et al., 2014; Senger & Davis, 2011). The cellular infiltrating populations into the TME can originate either in the blood vessels, the stroma or the bone marrow (Mittal et al., 2014). For example, cancer-associated fibroblast (CAF) can be derived from both, tumour stroma and from bone marrow precursors (Mittal et al., 2014). This cellular plasticity is mediated by the epithelial-mesenchymal transition (EMT) (Mittal et al., 2014). Many of the different cell types involved in tumour vasculature, and/or are part of the TME such as pericytes (Berg et al., 2013; Stefanska et al., 2016) and CAF (Nakamura et al., 2018) express RAS components. Regulating angiogenesis using RASi may extend beyond the converging point of VEGF-response. ACE2 acts as potent counter-regulator against ACE and Ang II activity (Patel et al., 2016; Xu et al., 2017). ACE2 overexpression or ACE2-Ang-(1-7)-MasR activation may suppress angiogenesis either by inhibiting the production of VEGFa in non-small cell lung cancer (NSCLC) (Cheng et al., 2016), or VEGF receptors attenuation in nasopharyngeal carcinoma (Pei et al., 2016), respectively.

The use of RASi concomitant with anti-VEGF therapy was associated with better survival in metastatic renal cell carcinoma (Izzedine et al., 2015; Keizman et al., 2014; Keizman et al., 2011; McKay et al., 2015), metastatic CRC (Osumi et al., 2015), advanced HCC (Pinter et al., 2017) and glioblastoma (Levin et al., 2017). Increased resistance has been reported to VEGF inhibition (Jain et al., 2009; Vasudev & Reynolds, 2014). The tumour vasculature interacts with the TME via several other mechanisms offering potential new therapeutic targets (Schaaf et al., 2018). The regulation of TME by emerging anti-angiogenic therapies are aiming to counteract tumour progression and to achieve greater tumour destruction. Rather than acute vessel disrupting strategies, there is now a focus on anti-inflammatory regimens. For instance, cabozantinib inhibits the activity of VEGFR, c-MET, AXL and other tyrosine kinases, thereby leading not only to impair angiogenesis but also the disruption of other processes inside the TME (Tannir et al., 2017).

1.3 Targeting Ang II/AT1R axis to impair hypoxia and acidosis within the tumour stroma

Local RAS works synergistically and independently of systemic RAS in a paracrine fashion (Campbell, 2014). Ang II mediates effects that reduce tumour perfusion and oxygenation, resulting in hypoxia and subsequent acidosis within the tumour stroma (Thews et al., 2000). For example, local Ang II predominantly exists in hypoxic regions of nasopharyngeal carcinoma and breast cancer cells, where it is autocrinally produced by chymase-dependant rather than ACE dependent mechanism (Xie et al., 2017). In which case the action of ACEi will not be effective to inhibit tumour growth. Tumour hypoxia and acidosis trigger a cascade of up-regulation of transcription factors, growth factors and cytokines, including hypoxia-inducible factor (HIF), VEGF and Transforming growth factor-beta (TGF- β) that together promote immunosuppression within the TME (Dayan et al., 2008; Riemann et al., 2017). An immunosuppressive microenvironment includes an impaired function of T cells (Anderson et al., 2017), dendritic cells (DC) (Shurin et al., 2013) the accumulation of M2-like macrophages (Chanmee et al., 2014) and myeloid-derived suppressor cells (MDSC), but also increased expression of immune checkpoint molecules such as [cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), and its ligand (PD-L1)] on tumour / stromal cells and the immune cells (Alsaab et al., 2017). In the liver, the local RAS is upregulated in response to tissue injury and hypoxia (Neo et al., 2010). The main effector is Ang II, which normally maintains tissues homeostasis, in this scenario can stimulate the expression of pro-angiogenic VEGF via AT1R signalling, leading to wound healing in tissue injury or to tumour neovascularization in hypoxic tumours (Walther et al., 2003). ACEi mechanism of action is to reduce the production of Ang II, while ARBs selectively block the action of AT1R receptors inhibiting tumour-associated angiogenesis (Sun et al., 2017).

1.4 Renin-angiotensin inhibitors regulate inflammatory signalling pathways and oxidative stress in tumour biology

The TME is bound to the dynamic between malignant and non-transformed cells (Balkwill et al., 2012). Non-malignant cells are reprogrammed to accomplish tumour-

promoting functions during all stages of carcinogenesis (Balkwill et al., 2012). Intercellular interactions modulate the chemical and physical properties of any tissue through a diverse pool of secreted cytokines, chemokines, growth factors, and inflammatory or matrix remodelling enzymes (Fearon, 2017). TGF- β suppresses the differentiation and function of T helper (T_H), CD8⁺ cells, Natural Killer (NK) cells, and tumour-associated neutrophils (TANs), tumour associated macrophages (TAM) and MDSC (Fearon, 2017). Tumour supporting cytokines are released from tumour and stromal cells upon AT1R activation via Ang II including, TGF- β , Interleukin – (IL-1 α , IL-1 β , IL-6, IL-8) and MCP-1 (monocyte chemoattractant protein 1), macrophage colony-stimulating factor (M-CSF), cyclooxygenase 2 (COX-2), C- reactive protein (CRP) (Vinay et al., 2015). Immunomodulatory cytokines may up-regulate immunosuppressive pathways, i.e., COX-2 via prostaglandin E2 synthesis, and impair DC function by reducing their migration (Vinay et al., 2015; Zelenay et al., 2015). Ang II/AT1R signalling induces reactive oxygen species (ROS) generation and related proteins such as inducible nitric oxide synthase in the tumour cells and stroma cells (Jackson et al., 2004). Exposure to ROS in the TME can impair T cell function while enhancing T regs and TAM, as has been previously reported in prostate cancer (Zelenay et al., 2015). Treatment with the ARB candesartan diminishes ROS generation (Uemura et al., 2008).

1.5 Renin-angiotensin inhibitors modulate the function of immune cells within the tumour microenvironment

There are three major immunosuppressive cell types within the tumour mass; MDSC, TAM and CAF. MDSC include both myeloid progenitors cells and immature myeloid cells (Bronte et al., 2016). These cells have been characterized as a population of inhibitory immune cells that lack typical mature myeloid markers, in mouse and human cancers (Bronte et al., 2016). Murine and human MDSC exert their suppressive effects through several mechanisms that inhibit CD8⁺T cell activation (Pyzer et al., 2016). Additionally, they induce the development of the T regs population, the polarization of macrophages to a TAM-like phenotype, the production of ROS, the upregulation of nitric oxide and depletion of nutrients via increased activity of L-arginase (Pyzer et al.,

2016). ACE is required for extramedullary myelopoiesis. A 3-fold increase in the CD11b⁺Gr1⁺ subpopulation with a similar phenotype to MDSC was found in the spleen of the ACE-KO mouse model in response to bone marrow incompetence (Okwan-Duodu et al., 2013). Likewise, ACE may have a role in the accumulation of MDSC in the TME. However, the inhibitory function of MDSC over CD8⁺T cells in the TME requires further investigation (Okwan-Duodu et al., 2013).

Similarly, TAM are usually pro-tumourigenic and have shown that a polarized M2-like phenotype contributes to immunosuppression, whereas M1-like phenotype induces anti-tumour immunity (Chanmee et al., 2014). TAM tend to accumulate in hypoxic and/or necrotic areas of the tumour (Murdoch et al., 2004). Studies with ACE KO and ACE 10/10 mouse model indicated that ACE plays a key role in macrophages to regulate the production of pro-inflammatory cytokines such as IL-12, nitride, and TNF- α in response to lipopolysaccharide (LPS) or chitin (a polymer of *N*-acetylglucosamine). These induce M1 macrophages or activate M2, respectively (Sato et al., 2010). Additionally, M1 induction through granulocyte-macrophage colony stimulating factor (GM-CSF) upregulate ACE expression in human monocytes (Fleetwood et al., 2009; Sato et al., 2010). Recent findings remain inconclusive as to how RAS modulation establishes M2 macrophage induction and polarization. Some studies suggest that there is no difference between ACE KO, ACE 10/10 and WT model (Okwan-Duodu et al., 2013). Others have found an increased or decreased M2 response by RAS blockade (Aki et al., 2010; Kohlstedt et al., 2011). Our laboratory has shown the immunomodulatory role of RAS in influencing TAM. Blockade of ACE with captopril increased Kupffer cells infiltration in the tumour-bearing liver during an early stage of tumour progression in CRC liver metastasis (Wen et al., 2013).

Myofibroblasts are also known as CAF are derived from multiple precursors, such as myoepithelial cells, mesenchymal stem cells, smooth muscle cells and EC (Xing et al., 2010). Their capability to secrete EGF family growth factors hepatocyte and fibroblast growth factors and insulin growth factor confer them the potential to induce malignant transformation of cells (Shiga et al., 2015; Tao et al., 2017). CAF express AT1R through which Ang II stimulates their proliferation and induces the production of various cytokines including TGF- β (Nakamura et al., 2018). TGF- β in addition to its immunosuppressive role as outlined above can stimulate EMT in malignant cells

contributing to an immunosuppressive TME and therapy resistance (Chae et al., 2018; Terry et al., 2017). Our laboratory found that RAS is implicated in the regulation of EMT in CRC via both AT1 and AT2 receptors by Ang II modulated migration (Nguyen et al., 2016). Additionally, CAF tumour promoting effects have been associated with promoting neovascularization and recruitment of immune cells in the TME via NF-kb signalling pathway (Erez et al., 2010). A recent study concluded that local RAS activity can modulate the function MDSC, TAM and CAF, and indeed is a potent inducer of immunosuppression in the TME (Nakamura et al., 2018). Inhibition of local RAS augments the induction and infiltration of tumour antigen-specific T cells, reduces the T cell suppressive activity of tumour-infiltrating CD11b⁺ cells including MDSC and enhances the T-cell stimulatory activity of CAF (Keizman et al., 2011). In addition to CAF, other types of immune cells also express RAS components. These include monocytes, neutrophils, DC and T cells, thus presenting pivotal targets for immune therapy (Pinter & Jain, 2017). For example, the stimulatory and coinhibitory interactions between lymphocytes infiltrating tumour stroma and tumour cells expressing receptor-ligands pairs, like immune checkpoint inhibitors PD-1, and its ligand PD-L1 correlates with T cell dysfunction (Mittal et al., 2014). The inhibition of AT1R enhances the induction of tumour antigen-specific CD8⁺ cytotoxic T lymphocytes via gp70-Tcells (Nakamura et al., 2018). Additionally, a clinical study of non-metastatic pancreatic ductal adenocarcinoma (PDAC) patients reported significant overall survival in the chronic RASi users, while unbiased gene profiling of resected tumours from this group presented enriched gene signatures associated with antigen processing and presentation, and activity of T cells, and reduced gene signatures associated with tumour aggressiveness. These findings may suggest that RAS signalling could modulate the efficacy of immunotherapy on this type of cancer (Liu et al., 2017). Altogether the synergistic use of RASi combined with immune checkpoint inhibitors may improve anti-tumour efficacy by reprogramming the TME towards an immune stimulatory milieu.

1.6 Targeting tumour non-cellular components using Renin-angiotensin inhibitors may enhance antitumour immunity

RAS has been studied under the scope of chronic wound healing and fibrosis (Bernasconi & Nystrom, 2018). During stromagenesis activated fibroblasts, have a critical function in orchestrating ECM remodelling. The effects of tissue renin-angiotensin (tRAS) on chronic wound healing can be divided into two opposite axes: (a) pro-inflammatory / pro-fibrotic and (b) anti-inflammatory / anti-fibrotic (Bernasconi & Nystrom, 2018). RAS signalling modulates ECM remodelling via AT1 by stimulation in dermal fibroblast, which promotes deposition of fibrotic ECM (Min et al., 2004). The contrary effect was observed via AT2 stimulation, which reduced collagen deposition counteracting AT1 signalling through the engagement of tyrosine phosphatase SHP-1 (Cui et al., 2001). In the case of tumour-associated stromagenesis, the pathophysiologic response fails to resolve. RAS activated CAF secrete collagen and other ECM components which leads to a fibrotic reaction so-called tumour desmoplasia (Schnittert et al., 2018). A tumour desmoplastic environment can be either a physical barrier to immune cell infiltration (Watt & Kocher, 2013), or can provide the substratum to their interstitial migration (Mrass et al., 2008). Furthermore, RAS activated CAF and other stromal cells produce immunosuppressive cytokines/ growth factors especially TGF- β in the TME leading to chronic inflammation and effector T- cell dysfunction, apoptosis and failure to infiltrate deep into the tumour (Anderson et al., 2017). RASi targeting of the tumour stroma resulted in collagen I synthesis inhibition by CAF in a dose-dependent manner in different desmoplastic models including human breast, pancreatic, and skin tumours in mice (Diop-Frimpong et al., 2011). A collateral effect of decreasing tumour desmoplasia is the decompression of tumour blood vessels and subsequent reduction of tumour hypoxia by increasing perfusion, which may also enhance antitumour immunity (Chauhan et al., 2013).

1.7 The cross-regulation of the kallikrein-kinin system and Renin-angiotensin system by angiotensin-converting enzyme has pleiotropic effects in the tumour microenvironment

The KKS comprises several peptides that are produced and cleaved in various sites to finally release the vasoactive kinins (da Costa et al., 2014). Kinins are originated from their parental molecules, the kininogens (da Costa et al., 2014). Diverse enzymes known also as kininases such as ACE, neutral endopeptidase (NEP), carboxypeptidase N, carboxypeptidase M, cathepsin X and aminopeptidase metabolize the kinins, (Blaes & Girolami, 2013) (**Fig. 3**). Bradykinin (BK) is the final bioactive peptide produced upon the activation of the KKS, which has pleiotropic functions depending on the pattern signalling triggered through its two different receptors (Leeb-Lundberg et al., 2005). The kinin receptor 1 (B1R) and the kinin receptor 2 (B2R) activate the same signalling pathway while inducing a different duration and intensity of the signal modulated by calcium influx (Moreau et al., 2005). B1R and B2R can be expressed in the same cell type, such as EC, fibroblast, and several tumour cells (Wu et al., 2002). While B1R expression is induced upon inflammatory conditions and tissue injury, B2R is constitutively expressed (Hall, 1997). The overall effect of BK through either B1R, B2R, or both is to promote vascular cell proliferation (Ikeda et al., 2004), influence barrier permeability (Ishihara et al., 2002) and possibly stimulate the release of pro-inflammatory cytokines (Bras et al., 2012). The role of kinin receptors has been investigated in various types of cancer, including melanoma, renal, prostate, lung, breast, and mesothelioma (Chee et al., 2007; Dlamini & Bhoola, 2005; Maria et al., 2016; Molina et al., 2009; Moodley et al., 2005), among others. Recently our laboratory found that both human and mouse CRC cell lines showed a strong positive expression of B1R and B2R, and while the inhibition of both receptors delayed tumour growth, only B1R blockade reduced tumour load and increased tumour apoptosis (Bras et al., 2012). Indistinctively, blockade of B1R or B2R diminished tumour vascularization *in vivo*, and impaired proliferation and migration of CRC cell *in vitro* (da Costa et al., 2018). The effect of RASi -ACEi- leads to accumulation of kinins and subsequent B1R activation (Ignjatovic et al., 2002), which may influence not only the cellular components part of the TME by activating macrophages and DC, but also can up-regulate cytokine production of IL-6 and IFN-g (da Costa et al., 2014). ACEi activate

B2R on DC, with increased production IL-12 (Aliberti et al., 2003). Further studies are required to elucidate the therapeutic opportunities of modulating kinin receptors with ACEi and their influence on the TME.

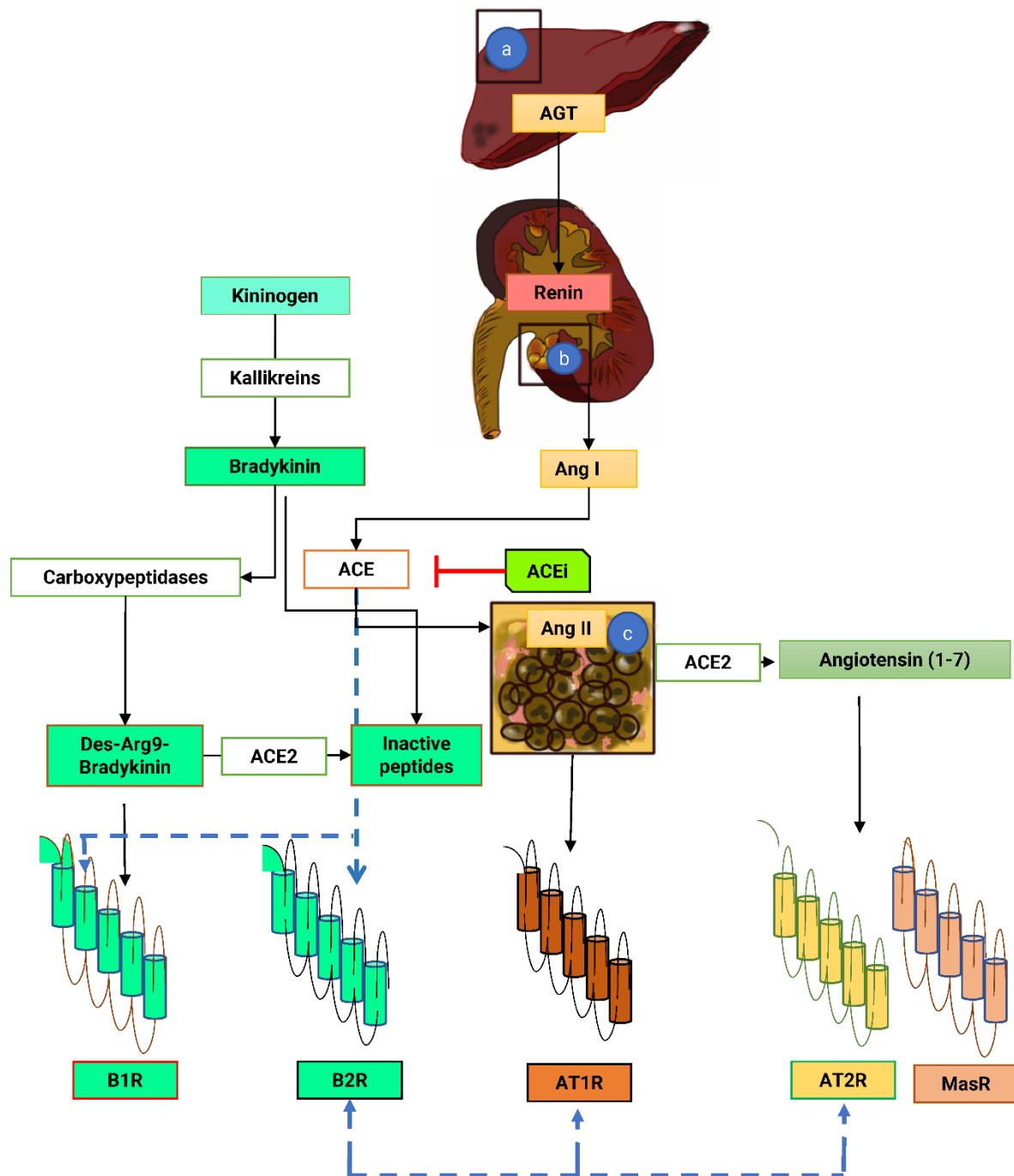


Figure 3. Renin-angiotensin system components intersecting with the kallikrein-kinin system. Angiotensin-converting enzyme (ACE) is a major hub intersecting between the crosstalk of both systems, by regulating the levels of Angiotensin II (Ang II) and kinins. ACE metabolizes bradykinin (BK), and converts Angiotensin I (Ang I) into Ang II. Interactions between ACE and kinin receptor 1 (B1R) and/or kinin receptor 2 (B2R) have been reported, same as interactions between angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) with B2R.

1.8 Perspectives and significance

Over the last decade, many clinical studies have shown the benefits of using RASi in patients at different stages in several types of cancer. The evidence of RASi impairing tumour growth beyond the function of modulating tumour vasculature is rapidly increasing. A major challenge in the field of cancer therapeutics is the increasing rate of resistance to chemotherapy (Zuccala, 2016), and immunotherapy (Jenkins et al., 2018). A better understanding of the complex interaction between non-cellular components, tumour cells, tissue-resident immune cells and infiltrating immune cells within TME is required to develop new cancer treatment strategies. An immunosuppressive TME affects the efficacy of immune checkpoint therapy, which is reflected in two different clinical scenarios, overall survival, and adverse side effects. RASi are able to reprogram the TME, using mechanisms by which they impair hypoxia and acidosis within the tumour stroma, regulate inflammatory signalling pathways and oxidative stress, modulate the function of the non-cellular components and immune cells, and regulate the crosstalk between KKS and RAS. Targeting RAS conventional axis (Ang II/AT1R), KKS and enhancing RAS alternative axis (ACE2/Ang-(1-7)/MasR) seem to be promising strategies to effectively influence TME toward an immunostimulatory milieu, and subsequently, improve immunotherapy outcome for a larger population of cancer patients. Further pre-clinical and clinical studies are necessary to propose that the concomitant use of RASi and immunotherapy could not only improve the overall survival of cancer patients but also could decrease the immunotherapy related side effects. Lastly, additional studies may be required in order to conclude if the effects of RASi on the tumour stroma, not only would depend upon the degree of tumour desmoplasia but also may depend on how they can influence tumour immune infiltration by either impairing or promoting their migration throughout the interstitial space.

1.9 Brief description of the physiology of RAS and therapeutic relevance of targeting the conventional axis

RAS controls hemodynamic stability by regulating blood pressure and maintaining fluid homeostasis and sodium-potassium balance (Gardes et al., 1982; Williams, 2016). The biological activity of RAS can be therapeutically targeted in the management of hypertension (Chen et al., 2018), cardiovascular disease (Martin et al., 2018), chronic kidney disease (Chung et al., 2020) and Coronavirus disease 2019 (Dora Lucía Vallejo Ardila et al., 2020) by hindering the over-activation of the conventional axis, thereby decoupling the hormonal cascade and blocking Ang II signalling or its conversion from Ang I (Arendse et al., 2019). In general, the activation of each axis, conventional and alternative axis, triggers the hormonal cascade in a paracrine or autocrine manner. Therefore, targeting the expression of the AT1R and AT2R receptors at the tissue and cell level can modulate the physiological or pathological response of the blood vessel and haematopoietic cells.

Pharmacological targeting of the AT1R and ACE has been effective and well-tolerated in these pathological conditions because of the potent effects of the inhibitors on vasodilation (Arendse et al., 2019). Nevertheless, the potential immunologic benefits of RASi had never been explored using human CLM collected from patients that have received this treatment as an antihypertensive medication.

Chapter 2

Preface

Material Methods

This chapter aims to describe the experimental design and the information of the material and methods section of regular thesis chapters 4, 5 and 7 and supplement the material and methods section of manuscripts submitted and published.

This chapter contains the methodology section published in the *Journal for ImmunoTherapy of cancer*, as per the following reference:

- Vallejo Ardila, D. L., Walsh, K. A., Fifis, T., Paolini, R., Kastrappis, G., Christophi, C., & Perini, M. V. (2020, May). Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases. *J Immunother Cancer*, 8(1).

Due to copyright obligations, the author accepted the version of this manuscript (Methodology section) in Chapter 2 of this thesis.

The copyedited, typeset published version of this manuscript can be found at:

<https://jitc.bmj.com/content/8/1/e000487.info>

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals and experimental model of colorectal liver metastasis

All animal experiments were conducted in accordance with the guidelines set by the Austin Hospital animal ethics committee (A2018/05549). Male CBA mice (ARC, Perth) of 9 to 11 weeks old with body weight above 20g were used.

Animals were maintained in standard laboratory mouse cages with irradiated food and water supplied *ad libitum*, in rooms with 12 h light/dark cycle, constant temperature and humidity. Environmental enrichment was provided in the form of dry tissue ribbons and cardboard tunnels.

2.2 Primary cancer cell line MoCR maintenance

The primary cancer cell line MoCR was derived from a dimethylhydrazine (DMH)-induced primary colon carcinoma in the CBA mouse and maintained *in vivo* by serial passage in the flanks of CBA (healthy naïve) mice (Kuruppu et al., 1996). Mice used to maintain the MoCR (Mouse Colorectal) cell line are referred to as passage mice. For the initial passage (passage 0), MoCR cells were taken from frozen stocks stored in liquid nitrogen and thawed in a water bath at 37°C. Warm sterile saline was added to (Pfizer Sodium Chloride 0.9% For Injection BP, Australia) the cell suspension and transferred to a 50ml Falcon tube (Falcon, Becton Dickinson, USA) and centrifuged for 5 minutes at 1000 revolution per minutes (rpm) (Beckman coulter, microfuge 18 Centrifuge). Subsequently, the supernatant was discarded, and any traces of the cryopreservation media in the remaining cell pellet were washed with sterile saline. After the sequential washing steps, the pellet was resuspended in 1ml of sterile saline. An aliquot of the cell suspension was transferred into the hemocytometer, and cells were counted. The cell viability of the tumour cells was assessed based on the exclusion of trypan blue. A cell suspension containing approximately 2×10^7 live cells was then injected subcutaneously into bilateral flanks of mice, and tumours were re-passaged once they reached approximately 10mm in diameter or every 14 days. The passage mouse was euthanized by sustained inhalation of 5% isoflurane, and the tumours were excised under aseptic conditions. The resected tumours were rinsed in sterile 0.9% saline (Baxter, Old Toongabbie, NSW, Australia), removing traces of fur, and then finely minced with scissors (Kilner scissor, straight 11.5 cm) and passed

through a stainless-steel mesh. Harvested tumour cells were used to repeat the passage procedure until the passage cell line is discontinued, usually after 15 to 20 cycles of passage and when required for intrasplenic Colorectal Liver Metastases (CLM) induction in CBA mice. Harvested tumour cells were mixed with 10mls of sterile saline and 10mls of 0.01% of EDTA (Biochemical, BDH laboratory supplies, England) in PBS (Apex, Bioresarch products) and stirred in a 37°C water bath for 20 minutes. Cells were centrifuged for 5 minutes at 1000 rpm or 100×g. The supernatant was discarded, and the pellet resuspended in 10mls of Ringer's solution containing 1% glucose. The cell suspension was centrifuged for a further 5 minutes at 100×g to remove red blood cells, ensuring a clean preparation. The supernatant was discarded, and the remaining pellet was resuspended in Ringer's solution containing 1% glucose to adjust the cell concentration to 1×10^6 cells/ml. Viability was determined by trypan blue exclusion. The final volume of cell suspension (1×10^6 cells/ml tumour cell suspension) was then split up into equal number aliquots of 1mls each. Each Eppendorf was kept on ice during the entire tumour induction procedure and gently agitated before the intrasplenic injection.

2.3 Intrasplenic Colorectal Liver Metastases induction in CBA mice

The MoCR-CLM model used in all animal experiments has been well established, characterised and reported on (Kuruppu et al., 1996), using male mice between the ages of 6-12 weeks (Neo et al., 2010; Neo et al., 2007). For the experimental design of this study, male CBA mice (ARC, Perth) of 9 to 11 weeks old with body weight above 20 g were used. CLM were induced by intrasplenic injection of 5×10^4 tumour cells followed by splenectomy. Firstly, mice were anaesthetized with 5% isoflurane for initial induction within 7- 10 minutes (Forthane® isoflurane anaesthetic inhalation, AbbVie Global). To alleviate post-surgical pain, 0.05ml of the analgesic Carprofen (50mg/ml, Rimadyl, Pfizer, West Ryde, NSW, Australia) was given via subcutaneous injection with a 30-gauge needle behind the neck. Eye protection was applied (Lacrilube eye gel, Allergan Australia Pty Ltd, Australia) before the surgery. Before initiating the surgical procedure, the response of animals to stimuli was noted to ensure that the animals were fully anaesthetised before any painful stimuli. The maintenance of dose-

response to inhalation of isoflurane is generally reduced from 5 to 2% during the procedure. This adjustment of the dosage is based on observing the reflexes noted to return or if breathing becomes rapid and shallow during the procedure. The dose was increased to 4 or 5% for a short period of time if pain response is noted. Body temperature was maintained during surgery by placing animals on a heating pad covered with an absorbent pad. To access the retroperitoneal cavity, a 5 mm left subcostal incision was made with surgical scissors (fine cut-straight /pointed & pointed/10.5cm) through the skin and muscle layer. The spleen was exteriorized delicately onto a small piece of distilled water-soaked gauze. Slowly over one minute, 0.05ml of the 1×10^6 cells/ml tumour cell suspension was injected into the spleen body using a 25-gauge needle while holding steady with dissecting forceps (Adsons plain, 11 cm). Distilled water-soaked gauze pads were placed over the injection site, and the needle carefully removed, preventing it from perforating the spleen. The spleen was compressed for 2 minutes allowing for the remaining tumour cells within the spleen to enter via the portal circulation to seed exclusively in the liver. The splenic blood vessels were cauterized, and the spleen was removed by transecting the vessel distal to the cauterized section (Low-Cost Cauteriser Kit; Fine Science Tools, Fisher Scientific). A continuous suture was used to close the abdominal muscle layer, while discontinued suture (3-4 stitches) (Vicryl 4-0, ETHICON) were used to close the skin wound. Mice were closely monitored until full recovery, and their body temperature was maintained after the surgery by placing animals' cages on top of a heating pad.

2.4 Kinetics of the tumour growth in the MoCR mouse model

Colorectal cancer cell seeded in the liver of the immunocompetent mouse model reproduces the Gompertzian growth curve for human colorectal liver metastases (Finlay et al., 1988). Gompertzian curve-based model consists of a lag phase from day 0 to 10 post tumour induction, which macroscopically has been described as an avascular tumour metastasis with a size of less than 2 mm. The exponential growth of the tumour is triggered by the start of angiogenesis at day 10, while the early angiogenic phase encompasses until day 15 post tumour induction. Metastases are fully established by 21-days following tumour induction. The plateau phase is reached

when there is a balance between cell proliferation rate versus cell death (Kuruppu et al., 1996) (**Fig. 4**).

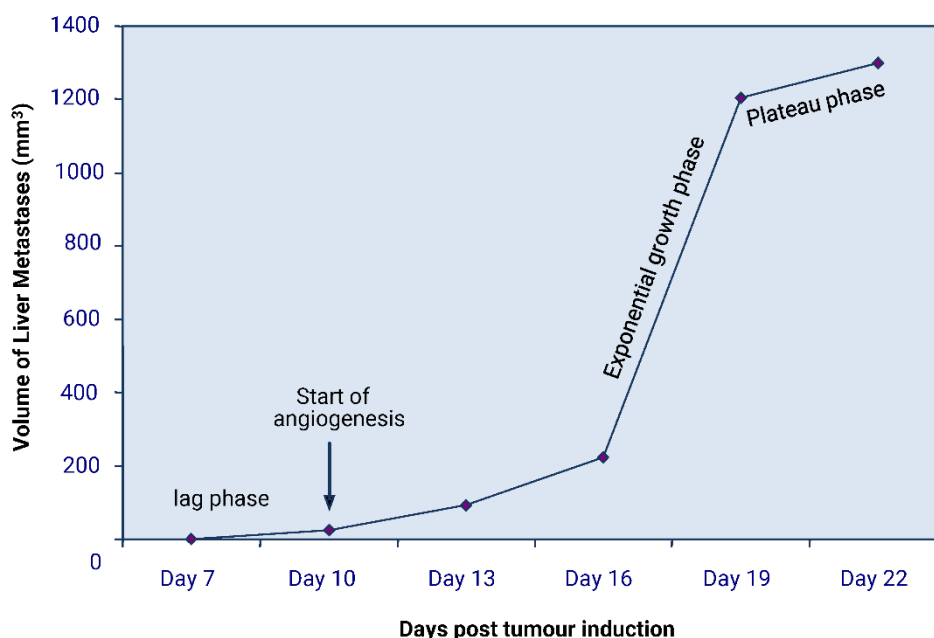


Figure 4. Kinetics of the tumour growth in the MoCR mouse model. Tumour growth kinetics in the MoCR-CLM model follows a Gompertzian curve-based model (Kuruppu et al., 1996).

2.5 Treatment protocol

Mice received tumour induction and were separated into two groups: control (saline) and treatment (captopril). Captopril (D-3-mercapto-2-methyl propanol-L-proline, Sigma-Aldrich, Sydney, NSW, Australia) was obtained in powder form and was made up each day by dissolving it in sterile saline (Pfizer Sodium Chloride 0.9% For Injection BP, Australia) and neutralized with a small amount of sodium hydroxide to adjust the pH to 7.4. Captopril was administered daily via intraperitoneal injection (IP) adjusted to the body weight of each mouse (750mg/kg) from day one post-tumour induction to the endpoint. Control mice received an equal volume of saline. Mice were monitored daily post tumour induction following standard animal experiments guidelines, paying close attention to any change in body weight. The doses dependent effect of the Captopril treatment MoCR-CLM model has been previously characterized (Neo et al.,

2010; Neo et al., 2007). For the present studies, the maximum tolerated dose of 750mg/kg was used.

2.6 MoCR-CLM model Tissue collection

Tissues were collected at days 15 and 21 post tumour induction. At each endpoint, animals were terminally anaesthetized with 5% isoflurane for more than 10 minutes (Forthane® isoflurane anaesthetic inhalation, AbbVie Global) inside a sealed container. An exploratory laparotomy was performed, and the entire abdominal cavity was examined for indications of extrahepatic metastases, including the splenic bed. The liver was carefully perfused through the inferior vena cava with 20 ml saline solution after performing a heart puncture, then excised and harvested. Immediately following excision, liver weights were recorded. To evaluate tumour burden and tumour viability, whole liver tissues were then fixed in formalin (10%) (Sigma Aldrich, Castle Hill, NSW, Australia) for 24 hours and then transferred to 70% ethanol.

Separate experiments were conducted to simultaneously collect tissues for immunohistochemistry (IHC) and multiplex immunohistochemistry (mIHC) using the median, right, and left lobe of the mouse liver while caudate lobes were used either for Fluorescent-activated cell sorting (FACS) or for global proteomics experiment. Day 15 is the earliest time point that enough clean tumour tissue could be isolated for FACS analysis. For the global proteomics experiment, tumours were thoroughly separated from the livers using microsurgery instruments. These tissues were snap-frozen in liquid nitrogen (LN₂) and immediately stored at -80°C (**Fig. 5**).

Lymphadenectomies were performed on day 15 following previously described procedures in mice (Zheng et al., 2013). Inguinal lymph nodes were collected, instead of the portal and celiac lymph nodes (liver-draining lymph nodes) because they showed similar T-cell, DC and macrophage composition, but they do not require fluorescent or other staining procedures for their identification, which may alter the results of subsequent staining protocols (Zheng et al., 2013).

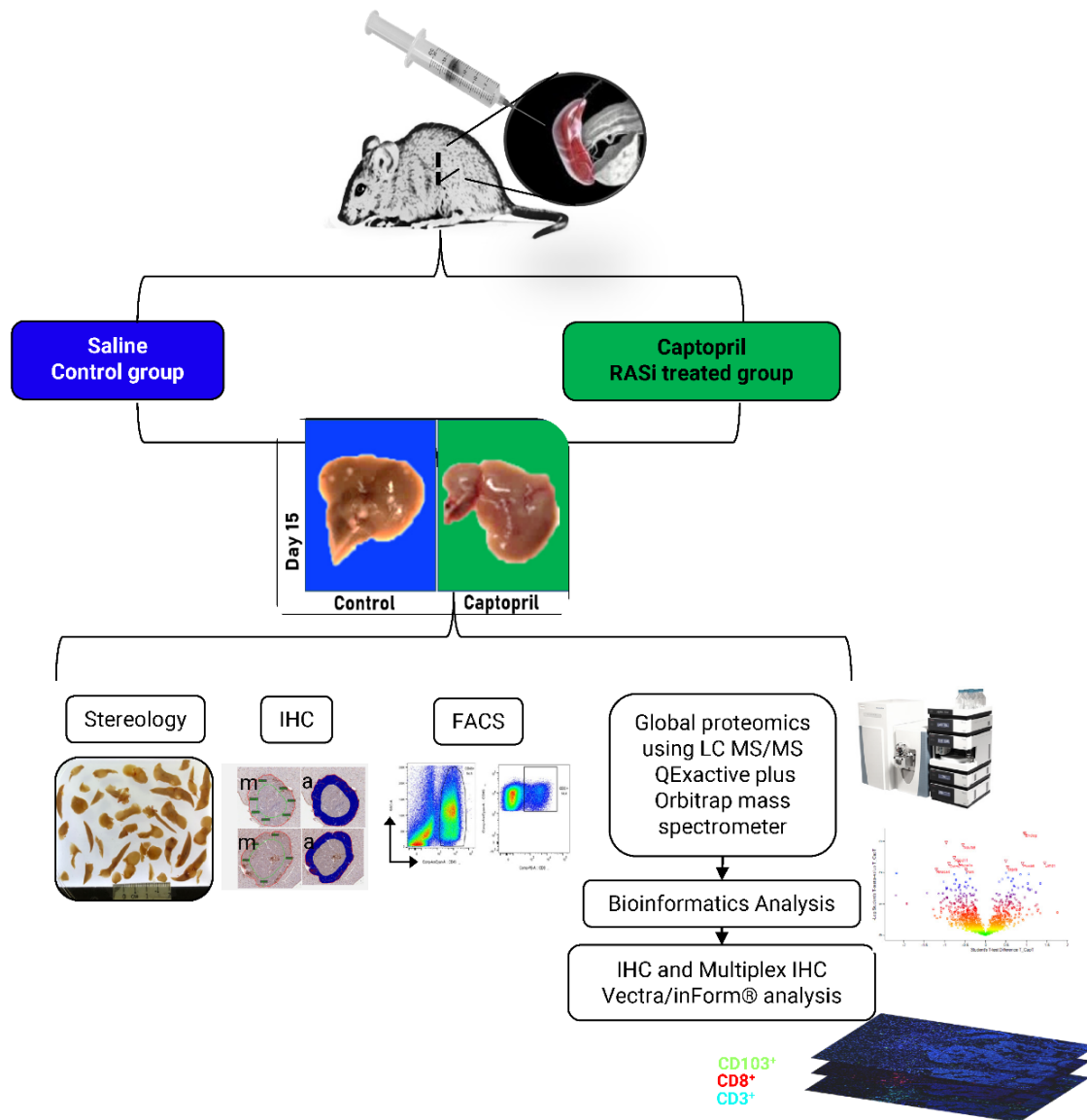


Figure 5. Workflow diagram of experimental design. Liver metastases were induced by intrasplenic tumour induction and allowed to develop for 15 and 21 days. Captopril or saline control treatment was delivered daily via intraperitoneal injection. At each endpoint, mice were terminally anaesthetized, and their livers perfused with saline before removal. Most livers were fixed and prepared for IHC and stereology, while a small proportion was prepared for flow cytometry and global proteomics, including the separation of tumour from the liver tissues using independent experiments for each time point ($n \geq 5$ mice). Adapted from: (D. L. Vallejo Ardila et al., 2020)

2.7 Macroscopic Stereology

Tumour growth was assessed by stereology on each endpoint (day 15 or 21) following tumour induction. Images of the whole liver were taken to examine the tumour distribution, number of tumours per liver, the total tumour area per liver, the total area of the liver, and number of metastatic foci. Each liver was transversely sliced into

sections of 1.5mm thickness using a tissue fractionator. For large livers (saline control), every second slice was sampled for analysis. Every slice was taken for analysis to provide unbiased and quantitative data of the tumour burden and number of metastatic foci in the case of small size livers (captopril treated tumours). Liver slices were placed on a transparent plastic plate; a digital camera (Nikon compact camera) was used to capture the images, and these were analysed using image analysis software (Image Pro-plus, Version 4.5.1, CyberMedia, Perth, WA, Australia). Tumours were visualized as distinctive white/cream-coloured areas against the brown liver tissues. Each tumour outline was annotated following the invasive tumour margin to determine the tumour area and the liver after calibrating the pixel/area (**Fig. 6**).

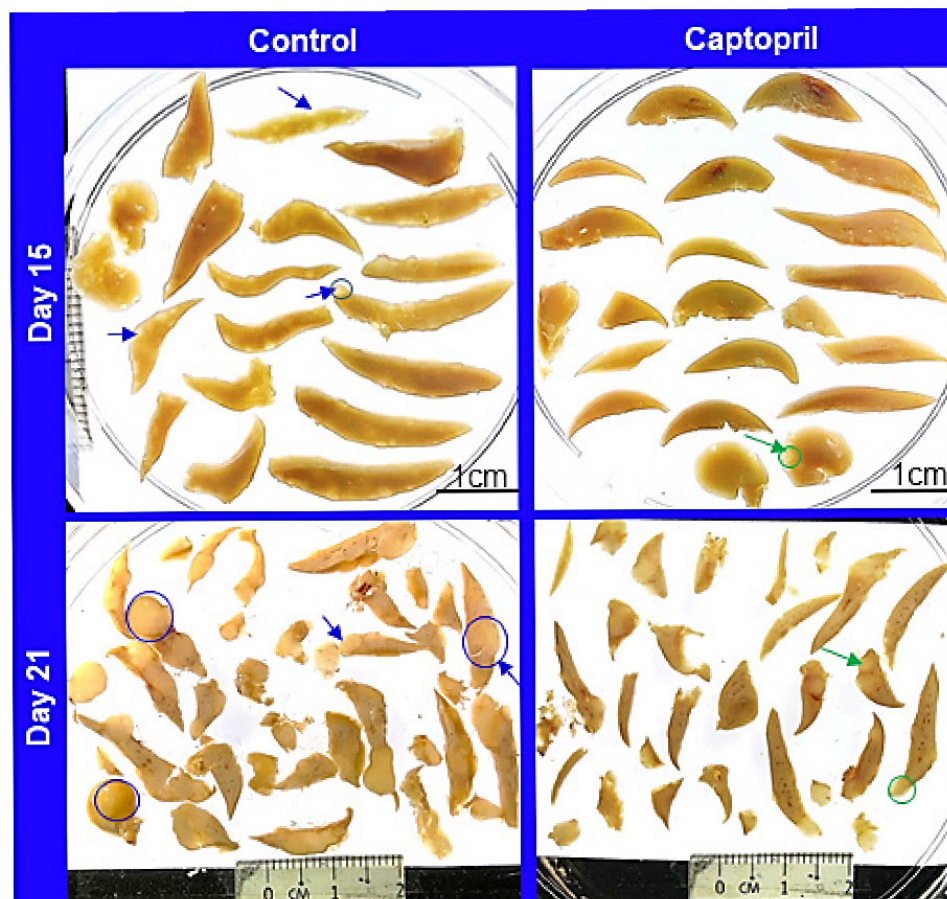


Figure 6. Quantitative stereology for calculating the percentage of tumour burden. Representative livers of each treatment are pictured on day 15 and day 21. Livers were fixed, sectioned, and analysed by stereology for the % tumour burden of the total liver area.

2.8 Hematoxylin and Eosin staining

Hematoxylin and Eosin staining (H&E) was used to delineate tumour masses within the liver tissue, study morphological changes and position of infiltrates, and calculate tumour viability in the mouse liver-tumour tissue (**Fig. 7**). Slides with 4µm sections were de-waxed in Histolene (for 10 min, in two cycles) and rehydrated in histologically graded ethanol concentrations of 100% and 70% for 1 minute each, followed by washing in running tap water for 1 minute. Slides were counterstained in Mayer's hematoxylin for 3 minutes. The hematoxylin was rinsed off in running tap water for 15 seconds and 'blued' in Scott's water for 2 minutes. Slides were then immersed into 1% Eosin Y solution (Sigma, Aldrich, England) for 2 minutes; Slides were dipped three times into water to rinse off excess Eosin and dehydrated in ascending grades of ethanol (70% and two changes of 100%) quickly. Slides were left in Histolene for 10 minutes; cover-slipped, mounted with DPX neutral mounting medium and allowed to dry under the fume hood. The H&E staining uses the principle that hematoxylin is a basic dye with an affinity for the nucleus, while eosin is an acidic dye with an affinity for the cytoplasmic components of the cell.

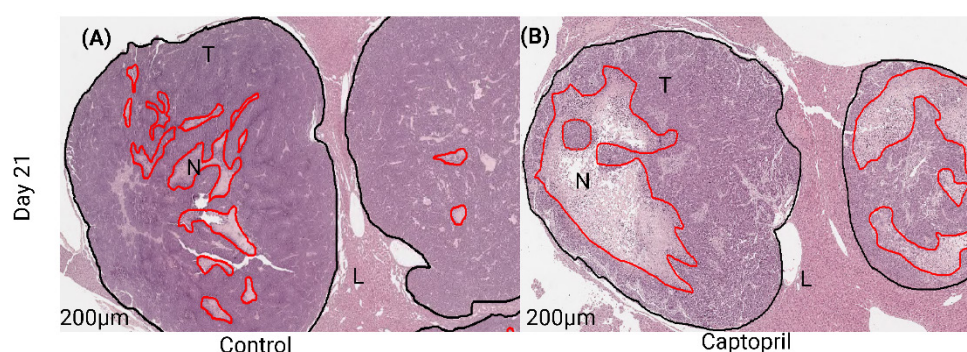


Figure 7. Tumour viability analysis. Stained with H&E for calculating the viable proportion of tumour of the (A) control and (B) captopril treated group. The total area of the tumour (T) annotated in black was calculated, then the necrotic areas (N) annotated in red were subtracted. Adjacent liver parenchyma (L) was not included. *Adapted from: (D. L. Vallejo Ardila et al., 2020)*

2.9 Immunohistochemistry in Mouse liver and tumour tissues

Formalin-fixed paraffin tissue sections (4 µm) were used with an indirect peroxidase labelling technique (Envision Plus, DAKO, Australia). Following sequential steps of deparaffinization, rehydration and washing in Tris Buffered Saline (TBST) with Tween

20 (0.5%) pH 7.5. Endogenous peroxidase activity was blocked with 3% H₂O₂ (Dako REAL Peroxidase-Blocking Solution; Agilent code S2023) for 30 minutes. Afterwards, non-specific binding was inhibited with 10% normal goat serum (01-6201 Thermo Fisher Scientific). Depending on the optimized condition for each antibody, all slides were subjected to either primary heat-induced epitope retrieval (HIER) buffer (pH 6.0 citrate buffer or pH9 buffer [Target retrieval Solution; Dako code S2367]) or IHC SELET® proteinase K (Millipore, concentration 0.2mg/ml). Antigens were visualized by immunohistochemical staining using their respective antibodies diluted as described in **Table 1**, and negative controls were stained without the corresponding primary antibodies. Following primary antibody treatment, sections were incubated with a horseradish peroxidase labelled polymer secondary antibody (peroxidase-conjugated goat anti-rabbit secondary antibody (Envision +®System- HRP kit; Agilent Dako, Australia) for 40 minutes. The antigen-antibody complex was visualized by diaminobenzene DAB chromogen substrate solution (1 drop of DAB (Envision +®System-HRP kit) chromogen added to 1ml of DAB buffer (Envision+®System-HRP kit) was applied to the slides and incubated for 2 minutes depending on the optimized condition to reduce background staining. Slides were then washed in running tap water for 2 minutes to remove any unbound HRP conjugated secondary antibodies. Slides were counterstained with Mayer's hematoxylin (SIGMA, St Louis, Missouri, USA) for 2 minutes, after which they were washed and 'blued' in Scott's tap water for 2 minutes. After a final wash in tap water, the slides were dehydrated in ascending graded ethanol (70% and two changes of 100%) for 2 minutes each. Slides were left in the clearing solution Histolene for 10 minutes before they were coverslipped and mounted with DPX neutral mounting medium (Ajax Finechem Pty Ltd., Taren Point, NSW, Australia) and allowed to dry in the fume hood at room temperature overnight.

Table 1. IHC panel of antibodies for mouse studies.

Target protein	Catalogue No.	Company	Clone	Dilution	Species Cross-Reactivity	Storage	Antigen Retrieval	Detection
PD-L1	13684	Cell Signalling	E1L3N	1:500	Human And Mouse	-20	Citrate	DAB
F4/80*	Sandrin Lab	donated	n/a	1:200	Mouse	-20	Proteinase K Rat linker*	DAB
CD3	M7254	Dako	F7.2.38 A0452	1:100	Human And Mouse	4	Proteinase K	DAB
CD4	14-9766-82	Invitrogen, eBioscience™	4SM95	1:1000	Mouse	4	Citrate	DAB
CD11b	Ab133357	Abcam	EPR1344	1:4000	Human And Mouse	-20	Citrate	DAB
CD11c (Itgax)	97585S	Cell Signalling	D1V9Y	1:100	Mouse	-20	Citrate	DAB
Anti-proteasome 20S LMP7 (Psm8)	Ab3329	Abcam	n/a	1:250	Human and Mouse	-20	Citrate	DAB
PSMB5/ MB1	Ab3330	Abcam	n/a	1:200	Human and Mouse	-20	Citrate	DAB

*** Kindly donated by Professor Mauro Sandrin, The University of Melbourne, Department of Surgery, Austin Hospital, Heidelberg.**

2.10 Image caption and analysis algorithm

Slides were scanned at 20× magnification (Aperio Scanscope AT Turbo; Leica Biosystems). Image analysis software (Aperio ImageScope™) measured the number of positive cells within designated areas. Given the small size of immune cells, the Positive Pixel Count v9 (version 9.1) algorithm was applied as a basis for detecting immune marker positivity, with the intensity thresholds adjusted manually to remove background artefacts as follows: WEAK pixels: 220-175, MEDIUM pixels: 175-100,

STRONG pixels: 100-0, and NEGATIVE pixels: -1. In order to account for variable differences in cell size, and to measure positivity (Number of positive cells/ total number of positive plus negative), and cell density (number of positive cells/per millimetre-square) in the tumour, tumour “periphery” or tumour inner invasive margin, liver, and lymph node (if when collected), thresholds for detection of positivity were manually adjusted as follows: CD3⁺ and CD4⁺ Hue value (centre):0.1; Hue width:0.33 and colour saturation threshold:0.15; CD11b⁺ Hue value (centre):0.1; Hue width:0.33 and colour saturation threshold:0.4; CD11c⁺: Hue value (centre):0.1; Hue width:0.2 and colour saturation threshold:0.03; and F4/80⁺: Hue value (centre):0.1; Hue width:0.33 and colour saturation threshold:0.2.

CD3⁺, CD4⁺, and F4/80⁺ cell distribution between tumour periphery and core was examined at each endpoint post-tumour induction, whereas CD11b⁺ and CD11c⁺ cell distribution between tumour periphery and core were examined only at day 15. The tumour “periphery” was defined to include approximately an equal length of the leading edge of tumour cells and the adjacent stromal interface defined as the invasive inner margin. The “core of the tumour” was defined as the total tumour area excluding the periphery. For each of these regions (periphery/leading edge or core), a total area up to 100 μm per pixel containing the highest density of IHC⁺ cells, were drawn to encompass these areas. When the inner invasive tumour margin or centre of the tumour was smaller than 5 μm per pixel, the whole tumour's positivity and the invasive margin were considered the same. PD-L1 and Anti-proteasome 20S LMP7 expression were counted in the membranous and cytoplasmic tumour region using the same staining intensity thresholds to detect immune marker positivity mentioned above (**Fig. 8**).

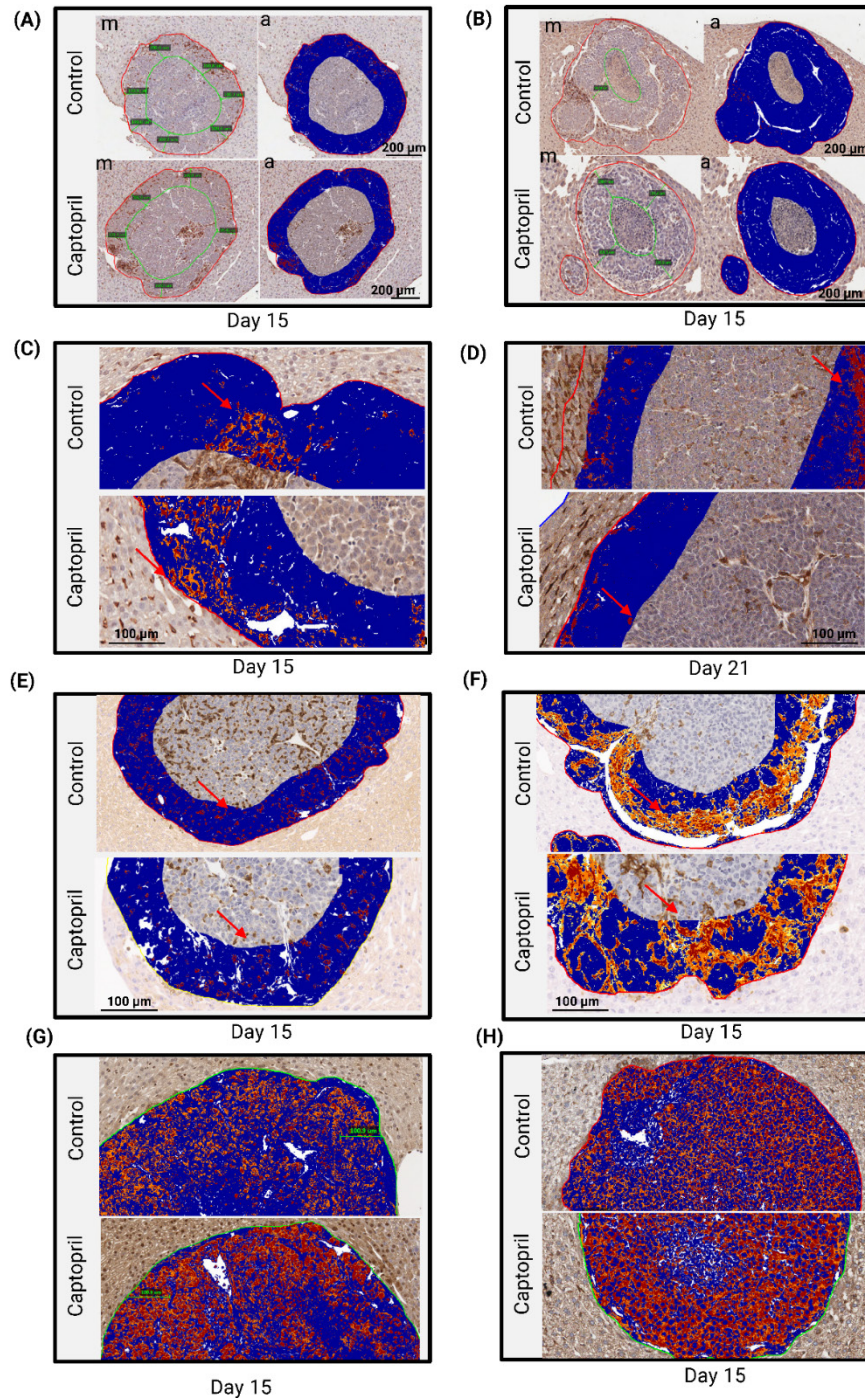


Figure 8. Histological quantitation strategy. (A) representative microphotographs of IHC CD3 staining; (B) representative microphotographs of IHC CD4 staining; marked-up (m) and annotated (a) tumour margin (red line) and intratumoral (green line) distribution at day 15 post-tumour induction (Scale bar = 200 µm). Representative microphotographs of IHC annotated of the tumour margin compared to the total area of the tumour of F4/80⁺ (C) at day 15 and (D) day 21 post-tumour induction (Scale bar = 100 µm measured from the edge of the tumour in direction to the core). Representative microphotographs of IHC annotated of the tumour margin compared to the total area of the tumour of (E) CD11b⁺ and (F) CD11c⁺ at day 15 post-tumour induction (Scale bar = 100 µm measured from the edge of the tumour in direction to the core). Representative microphotographs of IHC (G) LMP7⁺ and (H) PD-L1 annotated intensity of tumour area at day 15 post-tumour induction (Scale bar = 100 µm). *Adapted from: (D. L. Vallejo Ardila et al., 2020)*

2.11 Flow cytometry procedure and dimensionality reduction analysis

At the experimental endpoint, livers were excised as described above, and caudate lobes separated for processing for flow cytometry. Tumours were macroscopically isolated from liver parenchyma, and both tumour and macroscopically tumour-free liver were treated with tissue digest medium (0.25 mg/ml Liberase, Sigma and DNase, Sigma) at 37 °C degrees for 40 minutes. Tissues were then pushed through a stainless-steel mesh, centrifuged at 100g 5 min RT. The cell pellets were treated with red blood cell lysis buffer (10X solution containing ammonium chloride, potassium carbonate, and EDTA, and should be diluted in deionized water) (2 min 37 °C degrees), then centrifuged at 100g 5 min RT. The cell pellets were resuspended in cold FACS wash buffer (10% BSA/5mM EDTA/ 0.01% sodium Azide in PBS PH 7) and stored on ice. 1×10^6 cells were stained 20 min at 4 °C degrees with the optimized diluted antibody cocktails directly conjugated to the following fluorochromes as described in **Table 2**. Each sample was analysed on a FACS CANTO II (BD Biosciences) and data analysed by FlowJo™ (Ashland, Oregon). Lymphocytes were identified as live (DAPI negative), single cells that were negative for autofluorescence, CD45 and CD3 positive. Fluorescence Minus One (FMO) control was used to gate CD4 accurately or CD8 positive T cells and then the expression of PD-1 and CD103 on these T cell subsets, including CD3 positive cells that were expressing neither CD4 nor CD8, termed double-negative T cells (DNT) (**Fig. 9**). The flow-cytometry gating strategy shows CD11b positive myeloid cells gated on CD45 (leukocytes). FMO controls were used to gate CD31 on the CD45+CD11b+ myeloid population accurately. Then F4/80 cells were gated on CD31 myeloid subsets and based on the lack of expression, CD31, F4/80 was assessed. Ly6C expression was gated on F4/80^{low/negative} subset, while PDL1 positive cells were gated on F4/80⁺ (**Figure 10**). Figure 11 illustrates the interpretation of the surface markers on the liver and tumour myeloid-derived cells in mice with CLM. These markers provide a framework for the characterization of MDSC, as they have been previously defined by other studies (Bronte et al., 2000; Connolly et al., 2010; Ling et al., 1997; Y. Wang et al., 2020).

Table 2. FACS panel of antibodies for mouse studies.

Cocktail type	Marker	Fluorophore	Clone	Brand	Catalogue no.
T- cell	CD3e	PE	145-2c11	BD pharmingen™	553064
	CD4	APC cy7	GK1.5	BD pharmingen™	561830
	CD8a	PE cy7	53-67	BD pharmingen™	561097
	CD103	FITC	2 E 7	Miltenyi Biotec	130-118-681
	CD279 (PD1)	APC	REA802	Miltenyi Biotec	130-111-801
	CD45	BV510	30-F11	BD Horizon	563891
	CD16/32	pure	clone93	Miltenyi Biotech	130-092-574
Myeloid- derived populations	CD274/PDL1	PECy7	M1H1	BD pharmingen™	558017
	F4/80	FITC	REA126	Miltenyi Biotech	130-116-499
	CD11b	APC-Cy7	M1/70	BD pharmingen™	561039
	Ly6C	PE	AL-21	BD pharmingen™	560592
	CD31	APC	MEC13.3	BD pharmingen™	561814

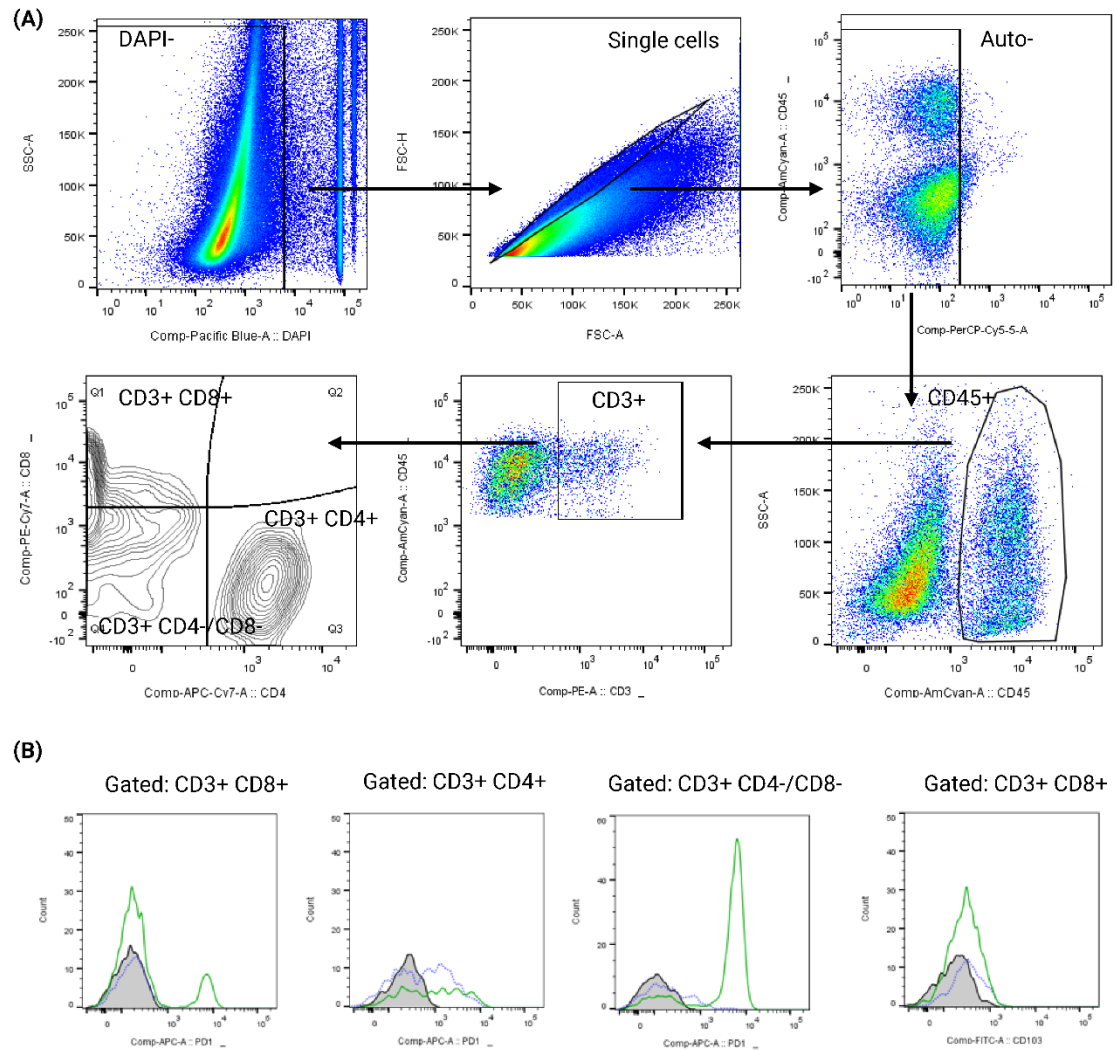


Figure 9. Gating strategy T lymphocytes and DNT. Gating strategy for analysis of lymphocyte subpopulations. (A) Viability, single cells, and autofluorescence were used as optimising strategies to analyse the CD3⁺ lymphocyte subpopulations within the total CD45⁺ leukocyte population in the liver (example given) and tumour. (B) The expression of PD1 and CD103 on gated lymphocyte subpopulations on tissues from captopril treated (green line), saline-treated (blue dash) and unstained for PD1 and CD103 controls (grey fill). (D. L. Vallejo Ardila et al., 2020)

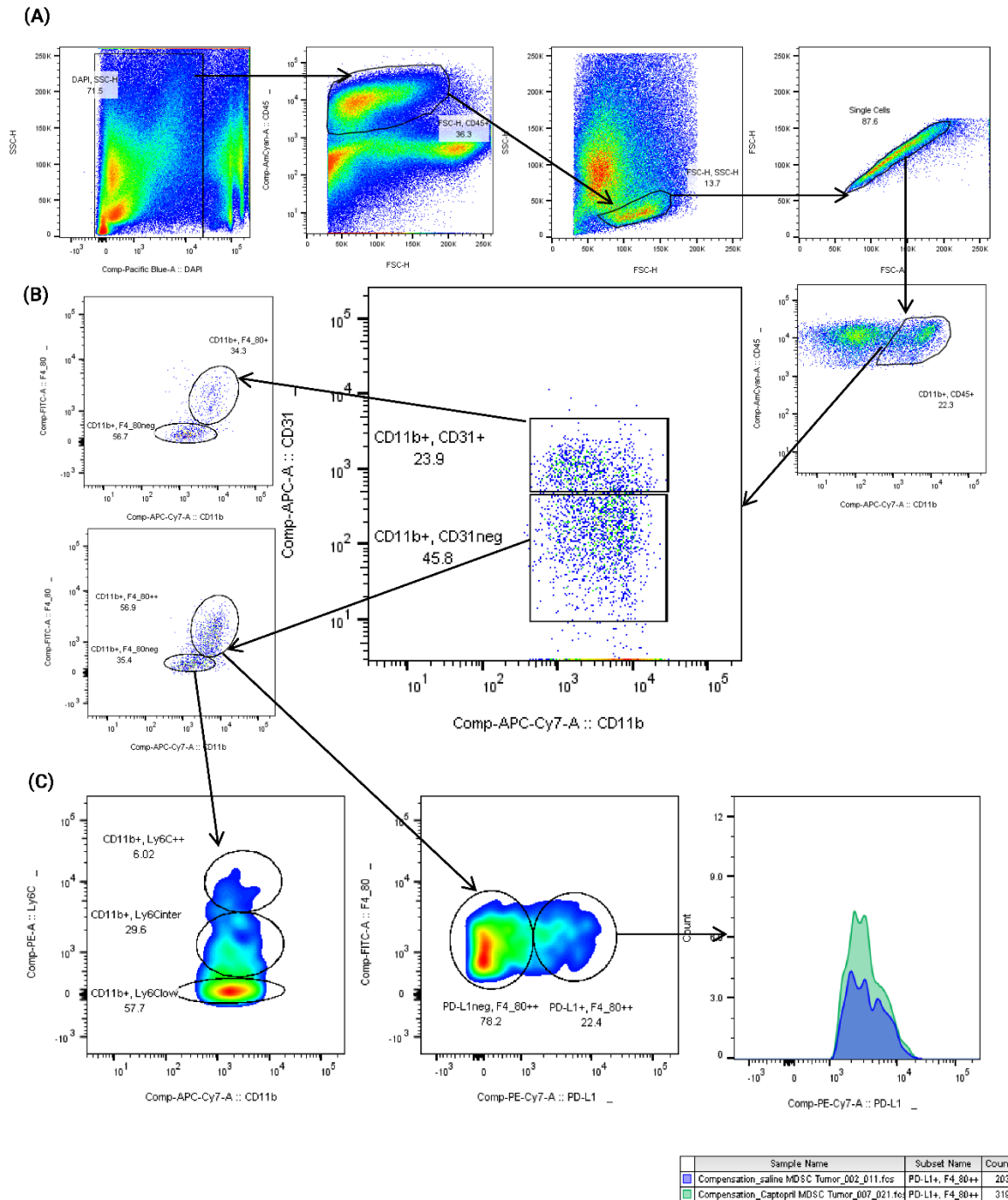


Figure 10. Gating strategy for analysis of Myeloid-derived population. (A) Viability, total leucocytes, size gate, single cells were used as optimising strategies to analyse CD11b⁺ myeloid-derived subpopulations within the total CD45⁺ leukocyte population in the liver and tumour (an example is given). (B) The expression of CD31 was gated to define the presence of a precursor phenotype (CD31⁺) from the mature phenotype (CD31⁻). PDL1 on gated CD11b⁺ CD31⁻ subpopulations expressing F4/80⁺ on tissues from captopril treated, saline-treated comparing their liver and tumour expression (an example is given). (C) Ly6C on gated CD11b⁺ CD31⁻ F4/80⁻ subpopulation on tissues from captopril treated (green line), saline-treated (blue line) comparing their expression in liver and tumour (an example is given).

T- Distributed Stochastic Neighbour Embedding (t-SNE) -guided analysis was performed using flow cytometry processed tumour samples (Toghi Eshghi et al., 2019). Pre-processing steps were conducted the same as in conventional hand-gating flow cytometry to exclude doublets and dead cells from the captopril treated and saline tumour FCS data files. Besides, a subset of events from the parent population was selected and placed in the downsample gate using the downsampling tool FlowJo™ plugin, and only compensated parameters were included for t-SNE analysis. The rationale of the approach compared five samples in the captopril treated group and seven samples in the saline group to downsample and concatenate all samples together by creating categorical populations.

Data were analysed with FlowJo software v.10.7. Captopril treated and saline tumour FCS data files were imported into FlowJo™ t-SNE plugin (Belkina et al., 2019). A subset of more than 10,000 events was selected from each treatment condition at random, their expression matrices containing measured intensities for each marker at a single-cell level were extracted and merged into a single expression matrix using functions from the FlowJo™ t-SNE plugin. A subset of 27,036 cells was concatenated into a merged single expression matrix before t-SNE analysis using keyword-based or categorical gates (sample ID and treatment condition). The categorical populations referred to five samples in the captopril treated condition and seven samples in the saline tumour condition. All compensated parameters except viability channel were selected. t-SNE-guided manual gates were drawn based on observed boundaries of phenotypic marker expression for PD-L1, F4/80, Ly6C, CD45 and CD31. The Barnes-Hut implementation of t-SNE with 1,000 iterations, a perplexity parameter of 30, and a learning rate (eta) 2800 was used for applying the dimensionality reduction algorithm (KNN algorithm: Exact [vantage point tree]). t-SNE maps were generated by plotting each event by its t-SNE dimensions in a dot-plot. Intensities for markers of interest were overlaid on the dot-plot to show the expression of the following markers: CD45, CD11b, CD31, F4/80, Ly6C and PD-L1. The heat map generated to visualize the median expression level of each marker in each cell, which was indicated by colour (**Figure 11A**). Each of these markers was then gated on the t-SNE overlay of the captopril treatment and saline conditions. The overlay displayed the cell islands that are derived

from the captopril treatment condition (contour plots shown in red) and that are derived from the saline condition (contour plots shown in light blue) and present in both treatment conditions (**Figure 11A**). t-SNE-guided cell frequency calculations were mapped into the t-SNE overlay of the captopril treatment and saline conditions according to the conventional gating strategy used to identify and stratify myeloid populations and subpopulations (**Figure 11B**). To further explore cellular islands of the saline condition that did not appear to overlap with the captopril treated cellular tumour islands. A query gate was drawn onto the t-SNE plot shown in the orange contour plot, and each marker expression was shown in the panel of histograms. Computationally defined clusters were qualitatively assessed on Phenograph (Levine et al., 2015).

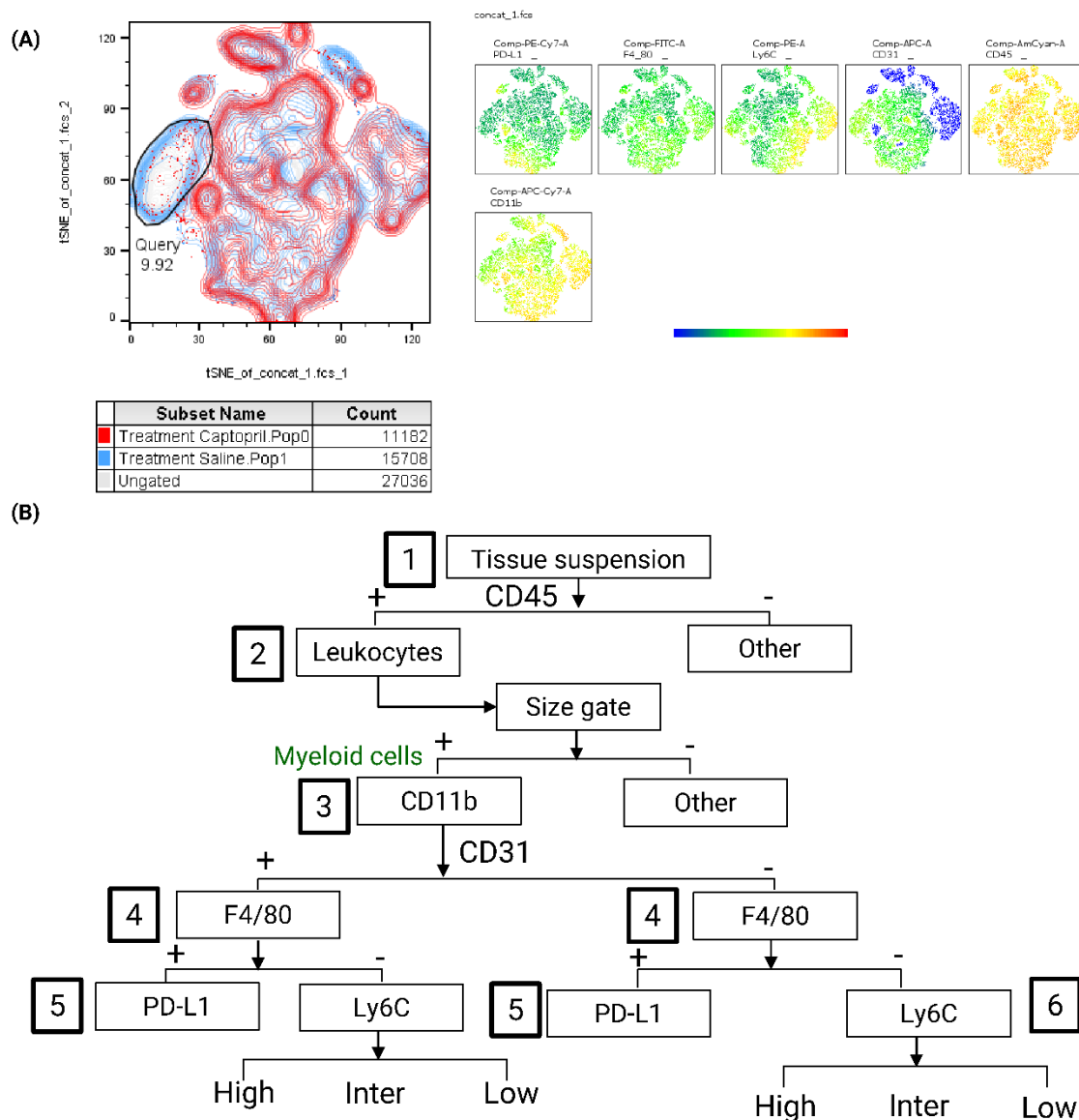


Figure 11. Identification and stratification strategy for t-SNE-guided gating analysis of myeloid population and subpopulations. (A) Heatmap plots were generated using Phenograph for t-SNE visualisation of CD45, CD11b, CD31, F4/80, Ly6C and PD-L1. (B) t-SNE-guided gating analysis of myeloid population indicating (1) cells present in the liver and tumour tissue suspension were divided into two groups. (2) Leukocyte population expressing CD45⁺ in liver and tumour and other (CD45⁻). Size gate and single cells were used to optimise the strategy to analyse CD11b⁺ myeloid subpopulations within the total CD45⁺ leukocyte population. (3) Myeloid subpopulations were distinguished based on the expression of CD31. (4) Further phenotypic analysis of CD31 myeloid cells was based on the expression F4/80. (5) PD-L1 expression was assessed on F4/80⁺. (6) Ly6C monocytes were divided into high, intermediate, and low levels of expression.

2.12 Global proteomics and bioinformatic analysis

At the experimental endpoint of 15 days following tumour induction, livers were excised as described above. Visible tumours (>2mm) were carefully excised from the whole liver and homogenized before freezing. These tissues were immediately snap-frozen in LN₂ and stored at -80°C. For global proteomics, processing liver tissues were separated into two groups: control (saline) and treatment (captopril). Tissue lysates were prepared for each sample included in the control and captopril group. Each group included five biological replicates.

On day 1, the liver and tumour tissues were grinded into fine powder, then protein extraction was carried out using RIPA buffer (25mM Tris HCl, pH 8, 150mM NaCl, 1% Triton x-100, 1% Na deoxycholate, 0.5% SDS, 1mM EGTA for 100mL). In addition, on the day of use, a protease inhibitor cocktail and a phosphatase inhibitor (PhosSTOP™ Sigma-Aldrich, Sydney, NSW, Australia) was added to 10mL RIPA buffer. After removing insolubilized protein, the supernatant was transferred to acetone-compatible tube. Acetone was kept at 20°C, four times the sample volume of cold (20°C) acetone was added to the supernatant.

On day 2, for tissue lysate preparation, the pellet was re-suspended in 500ul of 8M urea in 50mM Tetraethylammonium bromide (TEAB) (SIGMA, St Louis, Missouri, USA) and TCEP/DTT (Tris(2-Carboxyethyl) phosphine hydrochloride) (ThermoFisher) and vortex to solubilize. A protein assay (micro-BCA assay) was run to quantify the same protein amount in each sample (500µg -1mg). Next, iodoacetamide 55mM was added to each sample at room temperature and then diluted to 1M urea. Tissue lysates were digested with trypsin (1µg enzyme:50ug protein) (ThermoFisher) and incubated with shaking overnight at 37°C in the bacterial incubator. On day 3, SPE clean-up with SPE cartridge (Waters, Australia) after setting up the cartridges, so they eluted into the tube below. The cartridge was washed with 1ml of 80% ACN/0.1%TFA after the flow-through was discarded. Then, it was washed with 1.2ml of 0.1% TFA X 2 after the flow-through was discarded. To acidify the sample to 1% (v/v), pure formic acid was added (4µl of formic acid to 4mL sample). The sample is loaded onto the cartridge after the flow-through was discarded. Another wash with 1.2ml of 0.1% TFA X 2 was performed. All samples were eluted with 800µL of 80% ACN/0.1% TFA and collected in 1.5mL

Eppendorf tubes. Finally, aliquots of 20µg equivalent of protein were transferred on ice for speed-vac and freeze-drying.

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) based quantitative proteomics approach allowed quantitatively comparing changes in protein abundance in five biological samples per group. LC-MS/MS was carried out on a QExactive plus Orbitrap mass spectrometer (Thermo Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nano HPLC (Dionex Ultimate 3000). The LC system was equipped with an Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 µm x 50 cm). The tryptic peptides were injected into the enrichment column at an isocratic flow of 5 µL/min of 2% v/v CH₃CN containing 0.1% v/v formic acid for 5 min applied before the enrichment column was switched in line with the analytical column. The eluents were 5% DMSO in 0.1% v/v formic acid (solvent A) and 5% DMSO in 100% v/v CH₃CN and 0.1% v/v formic acid (solvent B). The flow gradient was (i) 0-6min at 3% B, (ii) 6-65 min, 3-22% B (iii) 65-75min 22-40% B (iv) 75-80min, 40-80% B (v) 80-85min, 80-80% B (vi) 85-86min 80-3% and equilibrated at 3% B for 10 minutes before the next sample injection. The QExactive plus mass spectrometer was operated in the data-dependent mode, whereby full MS1 spectra were acquired in positive mode, 70 000 resolution, AGC target of 3e⁶ and maximum IT time of 50ms. Fifteen of the most intense peptide ions with charge states ≥2 and intensity threshold of 5e⁴ were isolated for MSMS. The isolation window was set at 1.2m/z, and precursors fragmented using normalized collision energy of 30, 17 500 resolution, AGC target of 5e⁴ and maximum IT time of 50ms. Dynamic exclusion was set to be 30sec.

The global proteomics data files were analysed using MaxQuant version 1.6.5. (Cox & Mann, 2008; Tyanova et al., 2015), which is open-source software for inspection of raw data, identification of peptide features and quantification results. It is freely available and can be downloaded from www.maxquant.org. Perseus was used for statistical analysis and data visualization (Tyanova et al., 2016). Perseus Software 1.5.1.5 Licence can be downloaded from <http://www.coxdocs.org/doku.php?id=perseus:start>.

Statistical analysis (Welch's t-test) was implemented to compare protein expression differences between saline and captopril treated groups. Statistical significance was defined by p -value ≤ 0.01 and a Log_2 fold change ≥ 2 . Functional enrichment and protein-protein interaction network reconstruction were performed using the Search Tool for Retrieval of interacting Genes version 11.0 (STRING) (<https://string-db.org>) (Szklarczyk et al., 2019) to acquire protein-protein interactions (PPI) networks. Integrative pathway analysis was computed with performed using Functional Enrichment analysis tool (FunRich) version 3.1.3 (<http://www.funrich.org/>) (Pathan et al., 2015). Global proteomics results were validated by IHC as necessary for experimental validation to quantify protein expression.

2.13 Multiplex IHC staining of Mouse liver and tumour tissues

mIHC (OPALTM protocol, PerkinElmer/Akoya Bioscience) was performed to assess the presence and colocalization of T cell markers, immunoproteasome subunits and dendritic cell markers. Formalin-fixed paraffin-embedded (FFPE) tissue sections (4 μm) were dewaxed, rehydrated, and fixed in 10% Neutral-Buffered Formalin for 30 minutes. Then, all slides were subjected to a primary heat-induced epitope retrieval buffer with either pH 6.0 (AR6 buffer; PerkinElmer/Akoya Biosciences cat. No. NEL861001KT) or pH9 buffer (Target retrieval Solution; Dako code S2367). Following antigen retrieval conditions were dependent on the antibody used and performed in the microwave for 2 minutes at 100 % power or until boiling point, then at 20% power for 10 minutes or Proteinase K IHC SELET[®] proteinase K (Millipore, concentration 0.2mg/ml) for 30 minutes at room temperature, which does not require microwave pre-treatment. After washing in Tris Buffered Saline with Tween 20 (0.5%) pH 7.5, endogenous peroxidase was blocked in 0.3% hydrogen peroxide solution (Dako REAL Peroxidase-Blocking Solution; Agilent code S2023) and followed by antibody Diluent/Block (PerkinElmer/Akoya Biosciences cat. N° ARD 1001EA) to block non-specific binding before the incubation with the primary antibody overnight at 4°C. Secondary HRP-conjugated antibody (Opal Polymer HRP Rb; PerkinElmer/Akoya Biosciences) was applied for 40 minutes, and then Tyramide signal amplification (TSA) dye (Opal Polaris 6 colour fluorophore PerkinElmer/Akoya Biosciences cat. No.

NEL861001KT) for 10 min. The slides were washed in TBST/0.5% washing buffer after each application. The procedure was repeated for each antibody, starting with the antigen retrieval step. Following antigen retrieval conditions were dependent on the antibody used and performed in the microwave for 2 minutes at 100 % power or until boiling point, then at 20% power for 10 minutes or Proteinase K for 30 minutes does not require microwave pre-treatment. Antibodies used included CD39/NTPDase 1:200 (E2X6B, Cell Signalling Technology); CD8 α 1:500 (D4W2Z, Cell Signalling Technology); PD-L1 1:1000 (E1L3N, Cell Signalling Technology); F4/80 1:200 (Ascites, kindly donated by Mauro Sandrin's Laboratory); CD11c 1:200 (Clone D1V9Y, Cell Signalling Technology); Anti-proteasome 20S LMP7 1:250 (ab3329, Abcam); and Anti-PSMB5/MB1 1:200 (ab3330, Abcam) (**Table 3**). The immunofluorescent signal was visualized using the OPAL™ 5-colour fIHC kit (Perkin Elmer/Akoya, MA) TSA dyes 520, 540, 570, 620, 650 and 690, counterstained with DAPI (concentration 1mg/ml; dilution 1:4000; ThermoFisher scientific; cat. No. 62248). Image acquisition and quantification were performed using Vectra® 3.0 Automated Quantitative Pathology Imaging System (Perkin Elmer/Akoya, MA) (**Fig. 12**). The procedure described above followed general recommendations to ensure quality data results in mouse FFPE tissue sections (Sorrelle et al., 2019).

The quality of the mIHC staining protocol to evaluate the mouse T-cell phenotypes was markedly limited because the high autofluorescence signal emitted by the mouse liver tissue overlapping with the spectral signal captured by the FITC channel. Different reagents, including TrueBlack Lipofuscin Autofluorescence Quencher (no. 23007; Biotium) and peroxidase-Blocking Solution (Dako REAL; Agilent code S2023), were used. The myeloid cell compartment was characterized in the mouse tissue using CD11c and CD8 makers to measure the ratio of expression of both markers. These markers were paired to dyes in the extreme ends of the spectrum detection in the TRITC and Cy5 channel to unmix the signal of each marker (see chapter 5). More importantly, the selection of highly specific antibodies to reduce background staining was essential for improving image caption on Vectra® imaging and inForm® cell phenotyping (Sorrelle et al., 2019).

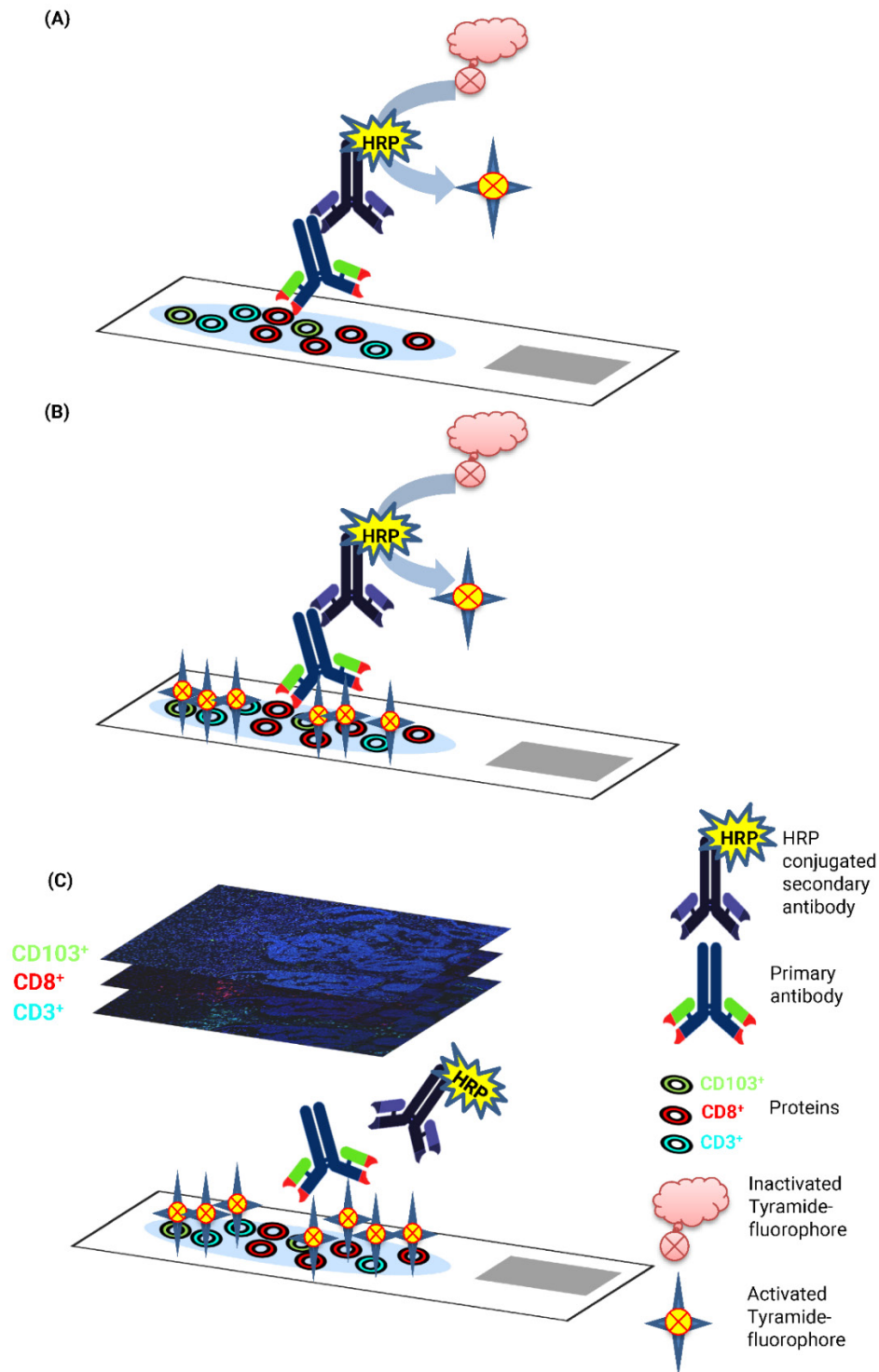


Figure 12. mIHC based on Tyramide signal amplification (TSA) using Opal chemistry. TSA/mIHC protocol depends on the fluorescent signal amplification, in which Tyramide covalently binds to the epitope in a highly specific manner permitting serial staining. In the diagram is shown that (A) after primary antibody incubation (i.e., CD3, CD8 and CD103), (B) a secondary antibody labelled with HRP is applied. (C) A fluorophore-conjugated tyramide molecule serves as the substrate for HRP, resulting in an antigen-associated fluorescence signal or activated tyramide-fluorophore. TSA/mIHC protocol was optimized to demonstrate excellent signal for each primary antibody used for staining the spectral library, mouse (Sorrelle et al., 2019) and human tissue (Halse et al., 2018) to prevent noise (i.e., autofluorescence) through the reduced background. Imaging caption and analysis was performed using Vectra® 3.0 Automated Quantitative Pathology Imaging System (Stack et al., 2014).

Table 3. Multiplex IHC antibody panel for mouse studies.

Target protein	Catalogue N°	Company	Clone	Dilution	Species Cross-Reactivity	Storage	Antigen Retrieval	Detection
PD-L1	13684	Cell Signalling	E1L3N	1:1000	Human And Mouse	-20	AR6	TSA 690
F4/80	Sandrin Lab	donated	Rat linker*	1:200	Mouse	-20	Proteinase K	TSA 520
CD8α (Lyt2)	98941	Cell Signalling	D4W2Z	1:500	Mouse	-20	AR6	TSA570
CD11c (Itgax)	97585S	Cell Signalling	D1V9Y	1:200	Mouse	-20	AR6	TSA540
CD39/ NTPDase 1	14481	Cell Signalling	E2X6B	1:200	Mouse	-20	AR6	TSA650
LMP7 (Psmb8)	Ab3329	Abcam	n/i	1:1000	Human and Mouse	-20	AR6	TSA690
PSMB5/ MB1	Ab3330	Abcam	n/i	1:200	Human and Mouse	-20	AR6	TSA620

2.14 Statistical analysis for Mouse CLM studies

Statistical analysis was conducted using GraphPad Prism® 8.3.0 (GraphPad Software, Inc., San Diego, CA) using both parametric and non-parametric analytical tests as appropriate. All data are presented as the mean value of each group $\pm$ standard error of the mean. The correlation matrix was generated using correlation coefficient r (or r_s) for each pair of variables: length (μm), area (μm^2), tumour margin (TM) positivity, tumour core (TC) positivity and total positivity. A heat map of R^2 calculating P values (two-tail) was generated. All statistical tests were two-sided, and a p -value of 0.05 was considered statistically significant.

2.15 Human Colorectal Liver Metastases immune profiling

2.15.1 Human Tissue collection

Forty-three resected liver specimens (forty metastatic colorectal tumours in the liver, two sclerosed hemangiomas and one benign liver) were taken from patients

undergoing liver resection. Specimens from these tissues were made available to us by the Victorian Cancer Biobank (VCB) after approval from the Austin Health Human Research Ethics Committee HREC reference HREC/54233/Austin-2019.

2.15.2 Inclusion and exclusion criteria

Patients who had undergone liver resection for CLM within Austin Health or other centres and have tissue samples stored within the VCB (with associated appropriate research consents) were included in this study. No exclusion criteria were considered for this study.

2.15.3 Histological investigation of growth patterns

Histological evaluation using H&E stained Aperio scanned slides (x20 Magnification) were used for histological growth pattern classification by an expert pathologist determining the invasive tumour interface, as follows: desmoplastic, replacement and pushing.

2.15.4 Multiplex IHC staining using human CRL

mIHC (OPAL™ protocol, PerkinElmer/Akoya Bioscience) was performed to assess the presence and spatial distribution of T cell markers CD3⁺, CD8⁺, and CD103⁺ and the EMT markers α -SMA and E-cadherin. mIHC was performed as described in the general protocol (see **Multiplex IHC staining of Mouse liver and tumour tissues**), except for the usage of Secondary HRP-conjugated antibody (Opal Polymer HRP Ms + Rb; PerkinElmer/Akoya Biosciences) was applied for 40 minutes. Antibodies used included rabbit monoclonal CD3 (Agilent Dako, clone A0452; code number M7254, proteinase K retrieval, dilution 1:100, dye650), rabbit monoclonal CD8 (ThermoFisher, clone SP16; cat. No MA5-14548, dilution 1:100, high pH retrieval, dye570), rabbit monoclonal CD103 (Abcam, [EPR4166(2)]; cat. No ab129202, 1:1000, high pH retrieval, dye540), rabbit monoclonal alpha-smooth muscle actin (Abcam, clone [E184]; cat. No ab32575; 1:500, low pH retrieval, dye690), mouse monoclonal E-cadherin (Santa Cruz, clone sc-7870, 1: 500, low pH retrieval, dye620), and FoxP3 (Abcam, clone 236A/E7; cat. No ab20034, 1:50, low pH retrieval dye520) (**Table 4**). The immunofluorescent signal was visualized using the OPAL™ 7-colour fIHC kit (Perkin Elmer/Akoya, MA) TSA dyes 520, 540, 570, 620, 650 and 690, counterstained with DAPI (concentration

1mg/ml; dilution 1:4000; ThermoFisher scientific; cat. No. 62248). Image acquisition and quantification were performed using Vectra® 3.0 Automated Quantitative Pathology Imaging System (Perkin Elmer/Akoya, MA). The protocol described above followed the recommendations on best practices for mIHC (Masucci et al., 2016; Taube et al., 2020). Sensitivity in detecting the target signal was also optimized using single spectral libraries of HeLa cells (comparable to the synthetic library, as per manufacturer's recommendation) and CLM whole tissue specimens stained with each primary antibody (see appendix 3). The precise detection of the spectral profiles was optimized by using the appropriate filter with the peak of absorption wavelengths and then unmixing using the spectral intensity of HeLa-stained cells to enable the separation of the signals (Parra et al., 2017). Using optimised exposure times, the fluorescent signal was strong enough to subtract the autofluorescence background using inForm® software v2.4.2 (Stack et al., 2014). This step prevents the “blee-through” of signals that may be a false-positive reading.

Table 4. Multiplex IHC antibody panel for Human CLM studies.

Target protein/Day	Catalogue Number	Company	Clone	Dilution	Species Cross-Reactivity	Storage	Antigen Retrieval	Detection
α SMA Day 1	Ab32575	Abcam	E184	1:500	Human And Mouse	-20	AR6	TSA690
E-cadherin Day 2	Sc-7870	Santa Cruz	H-108	1:500	Human And Mouse	4	AR6	TSA620
Anti-FoxP3 Day 3	Ab20034	Abcam	236A/E7	1:50	Human	4	AR6	TSA520
Anti-CD103 Day 4	Ab129202	Abcam	EPR4166 (2)	1:1000	Human	-20	AR9	TSA540
CD8 Day 5	MA5-14548	Invitrogen	SP16	1:100	Human	-20	AR6	TSA570
CD3 Day 6	M7254	Dako	F7.2.38 A0452	1:100	Human And Mouse	4	Proteinase K	TSA650

2.16 Image analysis using inForm®

2.16.1 Tissue segmentation using Machine learning-based algorithms

Machine learning-based algorithms are divided into supervised and unsupervised learning. The main objective of supervised learning is to map the input images with the appropriate labels inferring on the training data, while unsupervised learning infers on hidden structures from unlabelled images (Komura & Ishikawa, 2018). As part of the mouse and human experimental procedures, both supervised machine-learning algorithms have been performed to analyse mIHC staining results using inForm® software v2.4.2 (Perkin Elmer/Akoya, MA). H&E and OPAL-stained FFPE whole tissue sections were used to characterize the distinct immune profile of CLM. Using the low power image, an average of 10 HPF images were selected from each CLM sample across the liver-tumour interface based on the highest tumour infiltrating lymphocytes (TILs) density to assure an accurate representation of all areas for each whole tissue sample included in the study (Hendry et al., 2017). InForm® Tissue finder algorithm was used for trainable pattern-recognition based on the spectral elements associated with the tissue morphology, and tissue segmentation was determined (95% accuracy) as follows: adjacent liver parenchyma depicted in red (LP), invasive tumour margin in blue (IM) and tumour core in green.

2.16.2 Cell segmentation and cell phenotyping using Machine learning-based algorithms

Cell segmentation and phenotyping algorithm were applied to 386 HPFs in batch analysis using inForm® software v2.4.2 (Perkin Elmer/Akoya, MA). An average of three HPFs per tissue segment was taken for calculating the mean cell count per HPF. Colour separation, tissue and cell segmentation, cell phenotyping and colocalization was performed on inForm® software v2.4.2 (Perkin Elmer/Akoya, MA) to extract image data (**Fig. 13**). Acquired images were examined for the presence of tumour infiltrating of CD3⁺ T Lymphocytes (CD3⁺ CD8⁻ CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺), T_{RM} (CD3⁺CD8⁺ CD103⁺), non-T cells (CD3⁻) expressing CD8⁺ or CD103⁺ and cells expressing α-SMA or E-cadherin. The distribution of positive cells was determined by cell count per HPF and cell density using the previously stated tissue segmentation. A representative HPF illustrating the spectral signature was taken for each fluorophore

and captured using the multispectral fluorescent microscope Vectra® 3.0 Automated Quantitative Pathology Imaging System (Perkin Elmer/Akoya, MA).

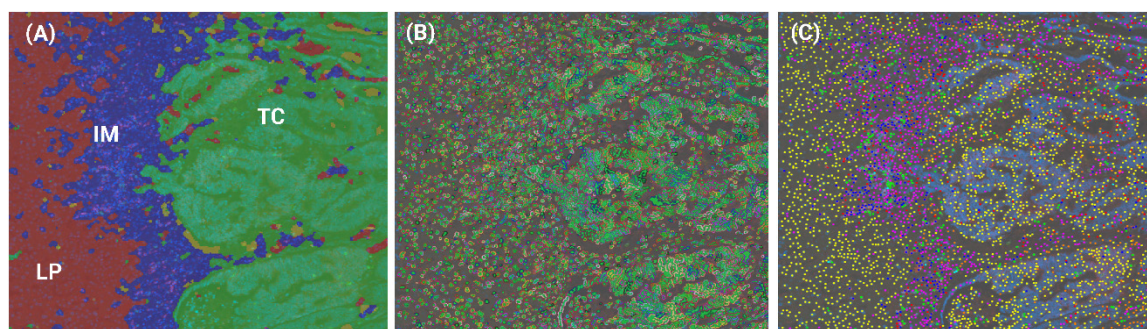


Figure 13. Tissue and Cell segmentation and phenotype scoring algorithm. Representative microphotographs using Vectra® imaging and inForm® cell phenotyping. (A) Tissue segmentation displaying adjacent liver parenchyma (LP) in red, invasive tumour margin (IM) in blue, and tumour core (TC), including tumour stroma and epithelial interface, in green. (B) Nuclear and cell segmentation and (C) Cell phenotype scoring.

2.16.3 Pixel-based colocalization analysis

Pixel-based colocalization analysis was applied to quantitate the fluorescent intensity for the co-expression of the markers of interest using inForm® software v2.4.2 (Stack et al., 2014). First, pixel-count was done after the separation of channels for the detection CD3 and CD8 marker, paired with TSA650 and TSA570 dye, respectively. Then it was compared with an E-cadherin marker paired with TSA620 (**Fig. 14A**). For instance, the co-expression of CD3⁺, CD8⁺ and E-cadherin was assessed on a per-pixel basis. The analysis revealed a co-localization pattern, then utilized to calculate the positive pixel area of the colocalization area of the three markers as indicated using the colour-based colocalization map (**Fig. 14B**). The maximal threshold adjustments for CD3 was 80.00, for CD8 was 119.70, and E-cadherin was 15.

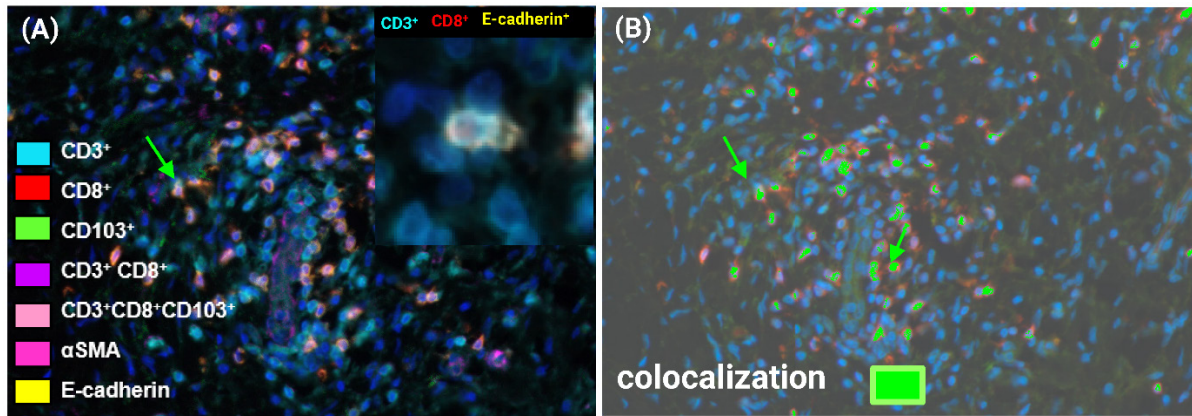


Figure 14. Pixel-based colocalization. (A) Representative image of a multispectral image showing infiltrating cell phenotypes (green arrow indicates CD8⁺ T cell expressing E-cadherin) (B) Colocalization map indicates in bright green the position of the co-expression of all three markers using inForm® to calculate the percentage of co-expression of markers in the whole cohort of CLM.

2.16.4 R analysis of Spatial distribution and spatial engagement

R software (version 3.6.2) was used to evaluate spatial patterns using raw data obtained from inForm® software v2.4.2 (Perkin Elmer/Akoya, MA). Initially, unique cells were identified using cartesian coordinates (x and y) and then labelled cell populations according to the immune markers expressed (i.e., CD3⁺ T Lymphocytes (CD3⁺ CD8⁻ CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺ CD103⁺), non-T cells (CD3⁻) expressing CD8⁺ or CD103⁺ and cells expressing α-SMA or E-cadherin. Each inForm® raw data file was analysed using the R package Inter-cellular Spatial Analysis Tool (ISAT) to evaluate cell population distances (Wang, 2018). This package computes the shortest distance between a cell and a group of cells (*findNN* function), returning summary information about mean distances, median distances and percentage of cells passing a distance threshold (*procDist* function). The percentage of a specific subpopulation is calculated by counting the number of cells of the same phenotype that have the nearest distance within a cut-off ranging from 5 to 30 μm.

2.16.5 Cell density analysis

Cell density (cell/mm²) was calculated by combining cell count from all HPFs normalized according to the area of interest (HPF stamp size 3x3= 2046 x 1530 μm, resolution= 0.5 μm equivalent to 1 pixel, x 20 magnification).

2.16.6 Survival analysis

In chapter 6, the patient's survival post-liver resection was interrogated in two separate univariate analysis. First, we investigated if the median distribution of cell count for each phenotype in areas of interest (LP, IM or TC) had predictive value. Secondly, we investigated if the spatial engagement profile of each phenotype to other phenotypes could be predictive of patient outcome, we stratified it according to the median distribution distance to determine if spatial engagement influences the patient outcome.

In chapter 7, patient's survival post-liver resection was interrogated in two separate univariate analysis to find differences in the immune pattern as follow: intergroup differences (RASi^{high} to Control^{high} and RASi^{low} to Control^{low}) and intragroup differences (RASi^{high} to RASi^{low} and Control^{high} to Control^{low}). The median cell density of each phenotype to stratify patients in whose tumours showed a low density for each group (RASi^{low} and control^{low}), defined as below the median cell density, with those that had high density (RASi^{high} and control^{high}), or above the median cell density in the whole tissue, the LP, IM or the TC. In the first univariate analysis, pairwise comparisons were made between RASi^{high} and control^{high}. Subsequently, RASi^{low} as compared to control^{low}. In the second univariate analysis, pairwise comparisons were performed between RASi^{high} and RASi^{low}. Successively, control^{high} and control^{low} were compared. Log-rank (Mantel-Cox) test comparison was used between the two groups. Statistical analysis was performed using GraphPad Prism Statistics version 8 (GraphPad Software, Inc., San Diego CA). KM plots (for more than two groups) were performed using functions of *ggsurvminer* included in the *ggplot2* package for generating graphics and pairwise statistical comparisons (Wickham, 2016).

2.16.7 Statistical Analysis

The data extracted as cell count per HPF was found not normally distributed (test for normality using Shapiro-Wilk test). Therefore non-parametric analysis was employed. Mann-Whitney U test was used for comparison between two groups (independent group t-test equivalent). Statistical analysis was performed using GraphPad Prism Statistics version 8 (GraphPad Software, Inc., San Diego CA).

2.16.8 *Code availability*

Customized R functions to assess spatial analyses based (on the x, y coordinates) data provided by inForm® software v2.4.2 (Perkin Elmer/Akoya, MA) are available in the supplementary material section. The ISAT (Wang, 2018) package is available online. The *ggplot2* package is available online (Wickham, 2016).

Chapter 3

Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases

This chapter aims to investigate the effects of RASi on liver and tumour T lymphocyte composition and distribution in a mouse model of colorectal liver metastases.

This chapter is published in the *Journal for ImmunoTherapy of cancer*, as per the following reference:

- **Vallejo Ardila, D. L.**, Walsh, K. A., Fifis, T., Paolini, R., Kastrappis, G., Christophi, C., & Perini, M. V. (2020, May). Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases. *J Immunother Cancer*, 8(1).

Due to copyright obligations, the author accepted version of this manuscript is presented in Chapter 3 of this thesis.

The copyedited, typeset published version of this manuscript can be found at:

<https://jitc.bmj.com/content/8/1/e000487.info>

CHAPTER 3

IMMUNOMODULATORY EFFECTS OF RENIN- ANGIOTENSIN SYSTEM INHIBITORS ON T LYMPHOCYTES IN MICE WITH COLORECTAL LIVER METASTASES

Title: Immunomodulatory effects of Renin-angiotensin system inhibitors on T lymphocytes in mice with Colorectal Liver Metastases

Authors:

Dora L Vallejo-Ardila^{1*}, Katrina A Walsh^{1*}, Theodora Fifi¹, Rita Paolini², George Kastrapis¹, Christopher Christofi¹, Marcos Vinicius Perini¹

¹Department of Surgery, Austin Health. The University of Melbourne, Melbourne, VIC 3084, Australia.

² Melbourne Dental School, The University of Melbourne, Melbourne, Vic 3010. Australia

* These authors contributed equally to this work.

*Corresponding authors:

Dr. Dora L Vallejo-Ardila, M.D. and General Surgeon. Ph.D. research scholar. Department of Surgery, Austin Health, The University of Melbourne, Melbourne, VIC 3084, Australia. Phone: (+61) 03 9496 3672; FAX: (+61) 039496 4800; e-mail: dvallejo@student.unimelb.edu.au

Abstract

Background: It is now recognized that many anti-cancer treatments positively modulate an anti-tumor immune response. Clinical and experimental studies have shown that inhibitors of the classical Renin Angiotensin System (RAS) reduce tumor progression and are associated with better outcomes in patients with colorectal cancer. RAS components are expressed by most immune cells and adult hematopoietic cells, thus are potential targets for modulating tumor infiltrating immune cells and can provide a mechanism of tumor control by RAS inhibitors (RASi).

Aim: To investigate the effects of the RASi captopril on tumor T lymphocyte distribution in a mouse model of colorectal liver metastases.

Methods: Liver metastases were established in a mouse model using an autologous colorectal cancer cell line. RAS inhibitor (captopril 750mg/kg) or carrier (saline) was administered to the mice daily via intraperitoneal injection, from day 1 post-tumor induction to endpoint (day 15 or 21 post tumor induction). At the endpoint, tumor growth was determined and lymphocyte infiltration and composition in the tumor and liver tissues were analyzed by flow cytometry and immunohistochemistry.

Results: Captopril significantly decreased tumor viability and impaired metastatic growth. Analysis of infiltrating T cells into liver parenchyma and tumor tissues by IHC and flow cytometry showed that captopril significantly increased the infiltration of CD3⁺ T cells into both tissues at day 15 following tumor induction. Phenotypic analysis of CD45⁺ CD3⁺ T cells indicated that the major contributing phenotype to this influx is a CD4 and CD8 double negative T cell (DNT) subtype, while CD4⁺ T cells decreased and CD8⁺ T cells remained unchanged. Captopril treatment also increased the expression of checkpoint receptor PD-1 on CD8⁺ and DNT subsets analyzed.

Conclusion: Captopril treatment modulates the immune response by increasing the infiltration and altering the phenotypic composition of T lymphocytes and may be a contributing mechanism for tumor control.

Keywords: Immunomodulatory, Colorectal Liver Metastases, Renin Angiotensin System, T-lymphocyte, and Immunotherapy.

3.1 Background

Colorectal cancer (CRC) is the third most common cancer diagnosis, and it is ranked second as cancer-causing death for both sexes combined in the developed world (Keum & Giovannucci, 2019). Over half of the patients with CRC will develop metastatic liver disease. Subsequently, colorectal liver metastases (CRLM) results in death in at least two-thirds of these patients (Tomlinson et al., 2007). Surgical resection of CRLM is the only potentially curative treatment and combined with chemotherapy can achieve 5-year survival rates of 40 to 60% (Tomlinson et al., 2007; Van Cutsem et al., 2014). However, the majority of these patients will develop tumour recurrence, most commonly in the liver (Fong et al., 1999). Current research to improve this clinical outcome is focusing on identifying reliable prognostic indicators of treatment response (Kemeny et al., 2014; B. Y. Zhang et al., 2017), tumours with a high-risk molecular signature for recurrence (Hanahan & Coussens, 2012), and in developing novel strategies to immunomodulate tumour microenvironment (Mlecnik et al., 2016). Established tumours subvert the host anti-tumour immune response through several mechanisms, including the accumulation of immunosuppressive tumour associated macrophages, myeloid-derived suppressive cells and cancer-associated fibroblasts. Also, the production of immunosuppressive factors influences the infiltration and activation of effector immune cells and the upregulation of T cell inhibitory ligands like PD-L1 on the tumour, stromal and immune cells (Xia et al., 2019). During the last few years, many different cancer types have proven responsive to therapeutic targeting of inhibitory T cells receptors by immune checkpoint inhibitors such as ipilimumab (Robert et al., 2011), nivolumab (Borghaei et al., 2015) and pembrolizumab (Garon et al., 2015). In the case of CRLM only a small subset of patients, those with deficient mismatch repair pathways (dMMR), respond to these treatments (Marshall & Djamgoz, 2018). Targeting immunological features that promote metastatic growth such as molecules that are pro-inflammatory, pro-angiogenic, pro-fibrotic, or specific leukocyte subsets that may change in number, phenotype, or function in different types of cancer (Affara et al., 2014; Albini et al., 2018; Smith & Kang, 2013) could tip the balance towards an anti-tumour immune response.

RASi have shown anti-tumour effects that may have a profound impact in cancer therapy (Ager et al., 2008; Neo et al., 2010; Wegman-Ostrosky et al., 2015). The role of the various RASi in modulating inflammation (Pacurari et al., 2014) and fibrosis (G. Kim et al., 2016; Saber et al., 2018) is well-documented. RAS pathway components are expressed by most cells of the innate and adaptive immune system (Haznedaroglu & Beyazit, 2013). *In vitro* studies indicate that RAS signalling modulates the activity of various immune cells, however, *in vivo* studies of cancer immune responses in response to RASi are scarce. One study using mouse renal cancer models reported that captopril increased tumour growth and inhibited the antigen-specific activation of CD8⁺ and CD4⁺ T cells while promoting antigen-specific B cells and their infiltration into tumours (Wysocki et al., 2006). In contrast studies from this laboratory using captopril or angiotensin receptor 1 blockers reported a significant reduction in colorectal liver metastases and modulation of tumour-associated macrophages (Ager et al., 2008; Neo et al., 2010; Neo et al., 2007; Wen et al., 2013). Supporting these findings Nakamura et al. (2017) demonstrated RASi reduced the immunosuppressive activity of TAM, MDSC, and CAF in the TME leading to the induction and tumour infiltration of tumour antigen specific T cells (Nakamura et al., 2018). In this study, the effects of captopril treatment RASi on T-lymphocyte sub-types, and their expression of some activation or inhibitory factors were investigated in a CRC liver metastasis mouse model.

3.2 Materials and Methods

3.2.1 Animals and experimental model of colorectal liver metastasis

All animal experiments were conducted in accordance with the guidelines set by the Austin Hospital animal ethics committee (A2018/05549). Male CBA mice (ARC, Perth) were maintained in standard cages with irradiated food and water supplied *ad libitum*. The primary cancer cell line MoCR was derived from a DMH-induced primary colon carcinoma in the CBA mouse and maintained *in vivo* by serial passage in the flanks of CBA mice (Kuruppu et al., 1996). For passage and experimentation, subcutaneous tumours were teased apart, passed through a filter, treated with EDTA, and washed in PBS to make a single-cell suspension. CRLM were induced by intrasplenic injection of

5x10⁴ tumour cells followed by splenectomy. Metastases are fully established by 21-days following tumour induction (**Fig. 15A, B**).

3.2.2 Experimental design

Tissues were collected at days 15 and 21 post tumour induction and used to evaluate tumour growth and T cell immune infiltration by immunohistochemistry and Fluorescent-activated cell sorting (**Fig. 15 A, B**).

3.2.3 Treatment protocol

Mice received tumour induction and were separated into two groups: control (saline) and treatment (captopril). Captopril (pH adjusted to 7.4) was administered daily via intraperitoneal injection adjusted to the body weight of each mouse (750mg/kg), from day 1 post-tumour induction to the endpoint. Control mice received an equal volume of saline. Tumour growth was assessed by stereology on day 15 or 21 following tumour induction.

At each endpoint, animals were terminally anaesthetized, and laparotomy was performed, and the abdominal cavity examined for indications of extrahepatic metastases, paying close attention to the splenic bed. The liver was carefully perfused with 20 ml saline solution, then excised and harvested. Immediately following excision liver weights were recorded. Livers were then fixed in formalin (10%) (Sigma Aldrich, Castle Hill, NSW, Australia) for 24 hours and then transferred to 70% ethanol.

3.2.4 Stereology

Images of the whole liver were taken to examine the tumour distribution and burden load. The liver was then transversely sliced into sections of 1.5mm thickness using a tissue fractionator. For large livers (saline control) every second slice was sampled for analysis. For smaller livers (captopril treated tumours) every slice was taken for analysis. Liver slices were placed on a clear plastic plate; a digital camera (Nikon Coolpix5000, E500) was used to capture the images, and these were analysed using image analysis software (Image Pro Plus, Perth, Australia).

Tumours were visualized as distinctive white/cream-coloured areas against the red/brown liver tissue. Each tumour outline was traced using image analysis software

to determine the area occupied by the tumour. Stereology technique was used to determine tumour burden,

3.2.5 Immunohistochemistry

Formalin-fixed paraffin tissue sections (4 µm) were used with an indirect peroxidase labelling technique (Envision Plus, DAKO, Australia). Following deparaffinization and rehydration, endogenous peroxidase activity was blocked with 3% H₂O₂ and non-specific binding inhibited with 10% normal goat serum (01-6201 Thermo Fisher Scientific). Heat-induced epitope retrieval was used. Antigens were visualized by immunohistochemical staining using their respective antibodies diluted as follows: CD3 1:100 (Clone A0452 DAKO); CD4 1:1000 (4SM95 e-Bioscience); PD-L1 1:500 (E1L3N, Cell Signalling Technology) and negative controls were stained without the corresponding primary antibodies. Following primary antibody treatment, sections were incubated with a horseradish peroxidase labelled polymer secondary antibody. The antigen-antibody complex was visualized by diaminobenzene (DAB) Peroxidase Substrate Solution (DAKO, Australia). The viable tumour was determined by H&E staining.

3.2.6 Image caption and analysis algorithm

Slides were scanned at 20× magnification (Aperio Scanscope AT Turbo; Leica Biosystems). Image analysis software (Aperio ImageScope™) measured the number of positive cells within designated areas. Given the size of immune cells, the Positive Pixel Count v9 (version 9.1) algorithm was applied as a basis for detecting immune marker positivity, with the intensity thresholds adjusted manually to remove background artefacts and to account for variable differences in cell size (Hue value (centre):0.1; Hue width:0.33 and colour saturation threshold:0.15). CD3⁺ cell distribution between tumour periphery and core was examined at day 15 (**Fig.S1A**, see Supplementary Figures and Tables section, page number 288) and day 21 post-tumour induction. The same conditions were applied to measure CD4⁺ cells at day 15 (**Fig.S1A**, see Supplementary Figures and Tables section, page number 288). The tumour “periphery” was defined to include approximately an equal length of the leading edge of tumour cells and the adjacent stromal interface defined as the inner invasive margin. The “core of the tumour” was defined as the total tumour area

excluding the periphery. For each of these regions (periphery/leading edge or core), a total area up to 100 μm per pixel containing the highest density of IHC+ cells, were drawn to encompass these areas. When the inner invasive tumour margin or centre of the tumour were smaller than 5 μm per pixel, the positivity of the whole tumour and the invasive margin was considered the same. PD-L1 expression was counted in the membranous and cytoplasmic tumour region using the same staining intensity thresholds (Intensity threshold defined as WEAK: 220-175: MEDIUM: 175-100, STRONG: 100-0, and NEGATIVE pixels: -1) (**Fig. 17A, D**) (Parra et al., 2016).

3.2.7 Flow cytometry

At endpoints of 15 days following tumour induction, livers were excised as described above and caudate lobes separated for processing for flow cytometry. Tumours were macroscopically isolated from liver parenchyma and both tumour and macroscopically tumour-free liver were treated with tissue digest medium (0.25 mg/ml Liberase, Sigma and DNase, Sigma) at 37 degrees for 40 minutes. Tissues were then pushed through a stainless-steel mesh, treated with red blood cell lysis buffer, and resuspended in FACS wash buffer (10% BSA/5mM EDTA/ 0.01% sodium Azide in PBS PH 7). 1×10^6 cells were stained with antibody cocktails directly conjugated to fluorochromes (CD45-BV510, CD3-PE, CD8-PECy7, PD-1-APC, CD4-APC-Cy7 (BD Bioscience) and CD103-FITC (Miltenyi biotech) and the viability dye, DAPI (**Table S1**, see Supplementary Figures and Tables section, page number 298). Each sample was analysed on a FACS CANTO II (BD Biosciences) and data analysed by FlowJo™. Lymphocytes were identified as alive (DAPI negative), single cells that were negative for autofluorescence, CD45 and CD3 positive (**Fig.S2A**, see Supplementary Figures and Tables section, page number 289). FMO controls were used to accurately gate CD4 or CD8 positive T cells and then the expression of PD-1 and CD103 on these T cell subsets (**Fig.S2B**, see Supplementary Figures and Tables section, page number 289), including CD3 positive cells that were expressing neither CD4 nor CD8, termed double-negative T cells.

3.2.8 Statistical analysis

All datasets are presented as the mean value of each group $\pm$ standard error of the mean. The correlation matrix was generated using correlation coefficient r (or r_s) for

each pair of variables: length (μm), area (μm^2), tumour margin positivity, tumour core positivity and total positivity. A heat map of R^2 calculating P values (two-tail) was generated. Statistical analysis was conducted using GraphPad Prism® 8.3.0 (GraphPad Software, Inc., San Diego, CA) using both parametric and non-parametric analytical tests as appropriate. All statistical tests were two-sided and a P value of 0.05 was considered statistically significant.

3.3 Results

3.3.1 Captopril inhibits metastatic growth and reduces tumour viability

The stages of metastatic development of CRLM model has been previously characterized (**Fig. 15B**) (Kuruppu et al., 1996). These experiments confirmed previous findings by this laboratory (Connolly et al., 2010), that captopril treatment has profound effects over metastatic growth after the metastases were fully established by 21 days following tumour induction (**Fig. 15C, D**). In this study at day 15 endpoint, there was a trend in the reduction of tumour load by 3.94% difference. In contrast, liver collected at 21 days post-tumour induction from the group treated with captopril, had significantly less tumour burden than the control (28.61% difference), (**Fig. 15D**). In this study, we also examined tumour viability as this is a better indication of treatment efficacy. Quantitation of live tumour area demonstrates that the percentage of viable tumour area in the captopril treatment group was significantly less, compared to the control group at 21 days post-tumour induction (**Fig. 15E, F**). Similar to the findings for tumour burden, while there is a trend, no significant differences in tumour viability could be determined at day 15 (**Fig. 15F**). Captopril treatment reduced the number of metastases although the reduction did not reach significance, and significantly reduced the size of metastatic foci. (**Fig.S3**, see Supplementary Figures and Tables section, page number 290).

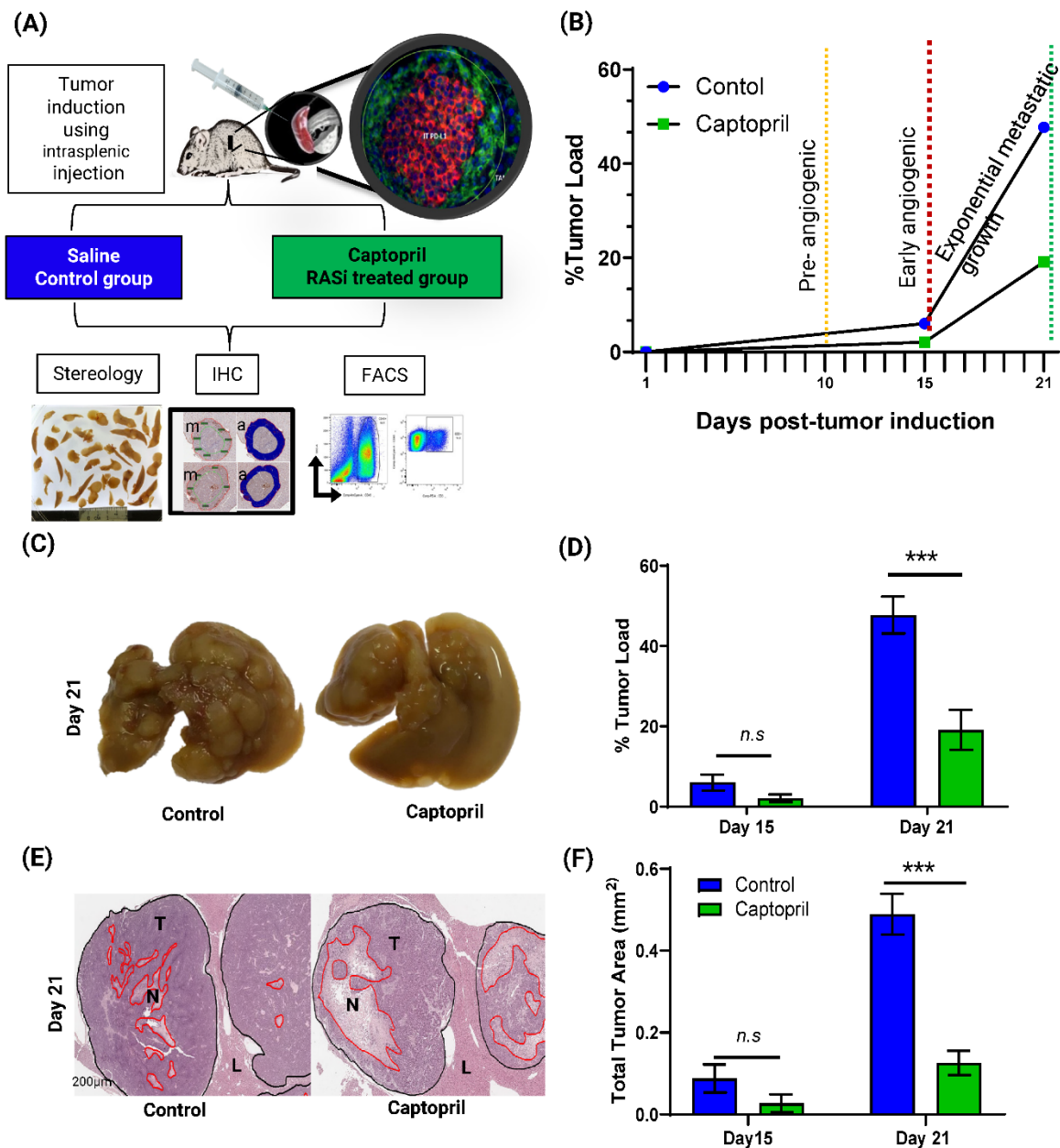


Figure 15. Control of tumour growth and viability by RASi treatment. (A) Liver metastases were induced by intrasplenic tumour induction and allowed to develop for 15 and 21 days. Captopril or saline control treatment was delivered daily via intraperitoneal injection. At each endpoint, mice were terminally anaesthetized, and their livers perfused with saline prior to removal. Most livers were fixed and prepared for IHC and stereology, while a small proportion was prepared for flow cytometry, including the separation of tumour from the liver tissues. (B) Schematic of tumour growth kinetics following tumour induction and daily treatment. (C) Representative livers of each treatment are pictured. (D) Livers were fixed, sectioned, and analysed by stereology for % tumour burden of the total liver area and (E) Stained with H&E for viable tumour analyses. (F) The proportion of tumour viability. Data represents average $\pm$ SEM of $n=5$ per group, significance calculated by Student T-test * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

3.3.2 Captopril treatment modulates spatial and temporal infiltration of tumour Lymphocytes expressing CD3⁺ and CD4⁺

We used immunohistochemistry to examine changes in CD3⁺ and CD4⁺ lymphocytes within the tumour tissues due to captopril treatment. Significant increases were seen in CD3⁺ lymphocytes in the treated group at day 15 (**Fig. 16A, C**). On the other hand, CD4⁺ lymphocytes were significantly decreased at this time point (day15), as seen in **Fig. 16B** and **Fig. 16D**. Visual examination of the staining shows that lymphocyte infiltration tends to accumulate around the intratumoral vessels (**Fig. 16A, B**). A significant increase in the number of CD3⁺ lymphocytes was observed in both tumour margin and intratumoral assessment in the captopril treated group (**Fig. 16A**).

Interestingly, both density and distribution of CD3⁺ tumour infiltrating lymphocytes inversely correlates with the area of liver metastases (**Fig.S4A**, see Supplementary Figures and Tables section, page number 290). While the frequency of CD4⁺ cells in the control group significantly decreased in the same pattern as the CD3⁺ lymphocytes between the two-time points, in the treated group, the CD4⁺ T cells did not change substantially compared to day 15, and in fact, at day 21 the frequency of CD4⁺ in the treated group is relatively higher than day 15, but it is not significantly higher compared to that of the control at day 21 (**Fig. 16C, D**). These results suggest that the increased infiltration of CD3⁺ T cells population at day 15 were clearly not CD4⁺ T cells and that at day 21, these are no longer within the tumour in elevated numbers, giving impetus for a thorough investigation of day 15 T-cell phenotype changes induced by captopril (**Fig. 16**).

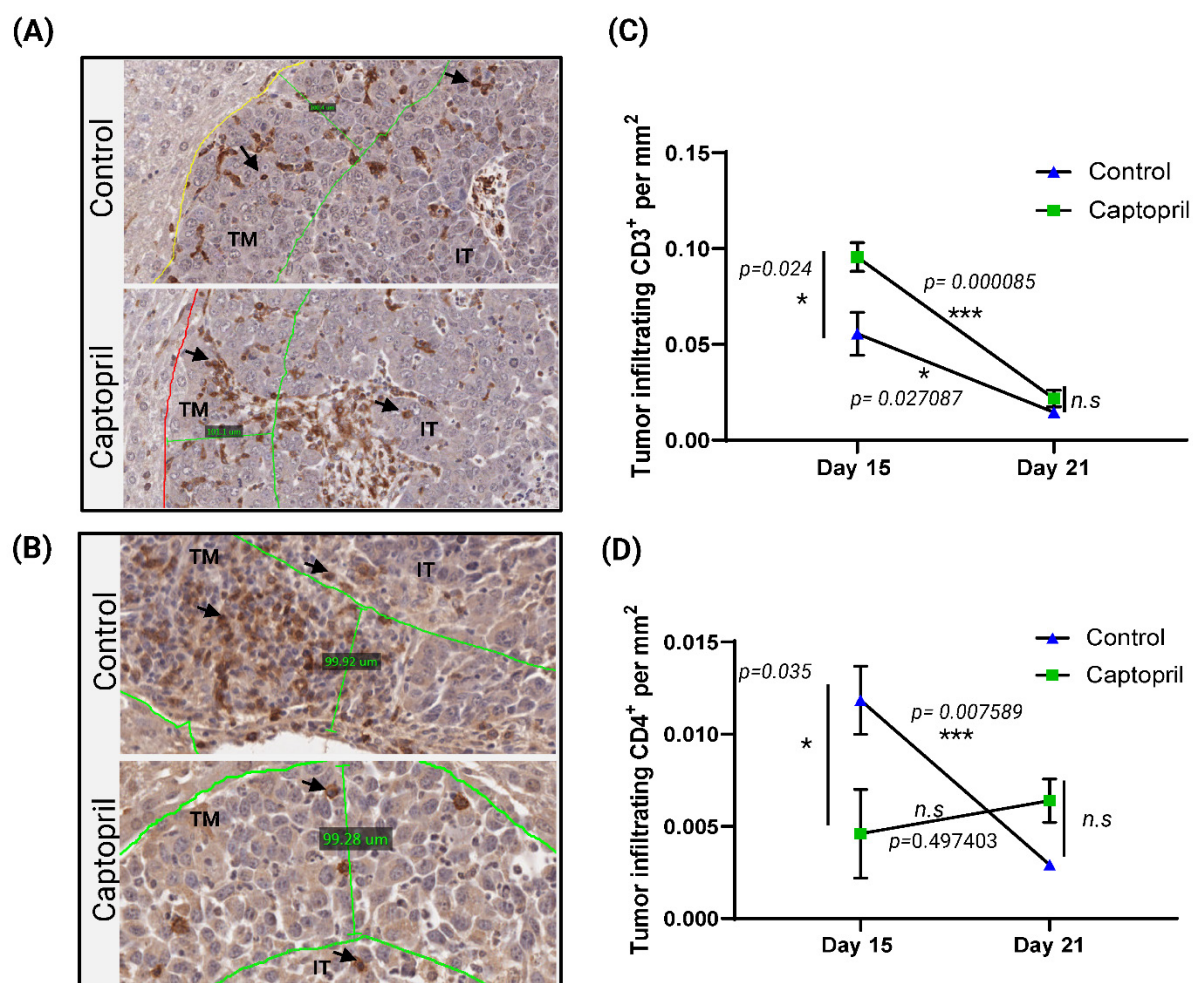


Figure 16. Tumour infiltrating lymphocytes are modulated by RASi. Representative microphotographs of histological quantitation of (A) CD3⁺ lymphocytes and (B) CD4⁺ lymphocytes in tumour tissues at day 15 (scale bar 100 μm), including mark up for tumour margin (TM) and intratumoral (IT) areas. Quantitation of the total area staining positive for (C) CD3⁺ or (D) CD4⁺ as an area per mm² of total tumour area, (excluding necrotic areas) for tissues collected at day 15 and day 21. Data n ≥ 5 presented as mean ± SEM. Significantly different data represented by * p < 0.05, ** p < 0.01, and *** p < 0.001.

3.3.3 Captopril treatment does not alter the tumour PD-L1 expression

Using IHC we also investigated whether the tumour in our model expresses PD-L1 (**Fig. 17A**) and whether captopril treatment alters tumour PD-L1 expression. While there is a strong expression of PD-L1 in the tumour, this expression was not significantly altered at any time point due to the treatment (**Fig. 17B**) and was not tumour size-dependent (**Fig. 17C**). No significant difference was found between different populations of tumour cells expressing PD-L1 at a different intensity between the control group and captopril on day 15 (**Fig. 17D**).

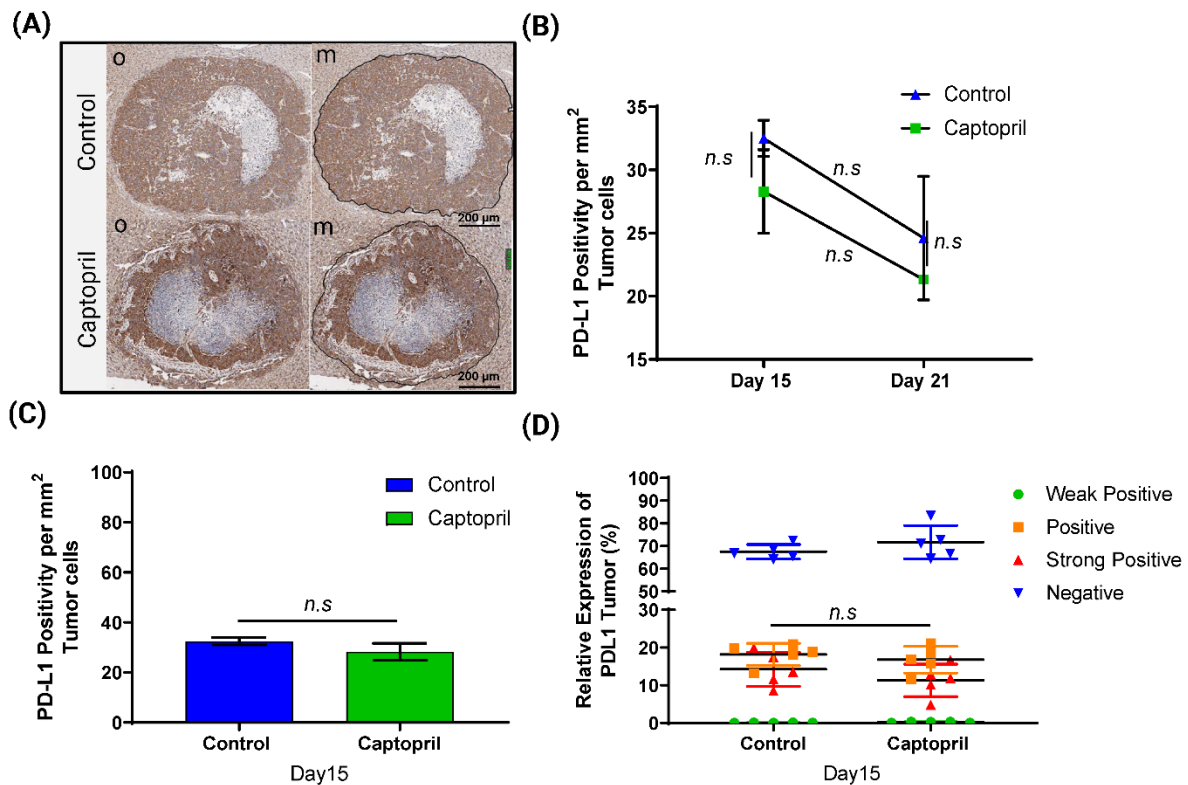


Figure 17. Temporal and spatial PD-L1⁺ expression in tumour cells. (A) Representative microphotographs of IHC PD-L1 staining; original (o) and marked-up (m) at day 15 post-tumour induction (black line) (Scale bar = 200 µm). (B) Histological quantitation of PD-L1 expressions at day 15 and 21 post-tumour induction. (C) Quantitation of the total tumour area staining positive for PD-L1⁺ as an area per mm² of total tumour area, (excluding necrotic areas) for tissues collected at day 15. (D) PD-L1 expression in tumour cells comparison between four levels of staining is shown: weak positive (green), positive (orange), strong positive (red), and negative (blue). Datasets expressed as mean ± SEM with $n \geq 5$ mice.

3.3.4 Captopril treatment differentially modulates T cell subpopulations infiltrating into the tumour and liver tissues

Flow cytometry analysis of dissected tumour tissues was used to identify the phenotype and the subpopulations of CD3⁺ T cells in the tumour that were observed by IHC to be modulated by captopril at day 15 (**Fig. 18A**). We also analysed the T cell subpopulation infiltration into liver tissues that had been macroscopically dissected from tumours. During liver harvesting, livers were perfused with 20ml of saline to remove peripherally circulating lymphocytes from the analysis, thus data represents tissue infiltrating cells.

Flow cytometry analysis confirmed that captopril treatment significantly increased the infiltration of CD3⁺ T cells into the tumour. Interestingly, these changes were also seen within the liver parenchyma (**Fig. 18B**) indicating that CD3⁺ T cells, trafficking from

circulation and/or dividing within the tissue in both liver and tumour, are affected by captopril.

The phenotype of CD3⁺ T cells was examined in terms of the proportion of these cells expressing CD8, CD4 and those expressing neither CD8 nor CD4, termed DNT (**Fig. 18C, D**) and found to be differentially modulated by captopril. Both in the tumour and liver tissues, there is a decrease in the proportion of CD4⁺ and an increase in the proportion of DNT. On the other hand, there is an increased proportion of CD8⁺ T cells in the tumour while in the liver this subpopulation decreases (**Fig. 18C, D**).

When considering these populations as a proportion of total leukocytes (CD45⁺) in these tissues we find that in the liver captopril treatment does not change either CD4⁺ or CD8⁺ subpopulations, whereas the DNT population is significantly increased (**Fig. 18E**). In the tumour, we also see a decrease in the CD4⁺ population while the CD8⁺ and DNT subpopulations significantly increase (**Fig. 18F**). This may indicate that in the liver tissue the increase in CD3⁺ T cells induced by captopril is due to the infiltration of DNT cells. Within the tumour, however, the changes induced by captopril are more complex and may reflect changes in the TME that affects the ability of each subpopulation to exist there.

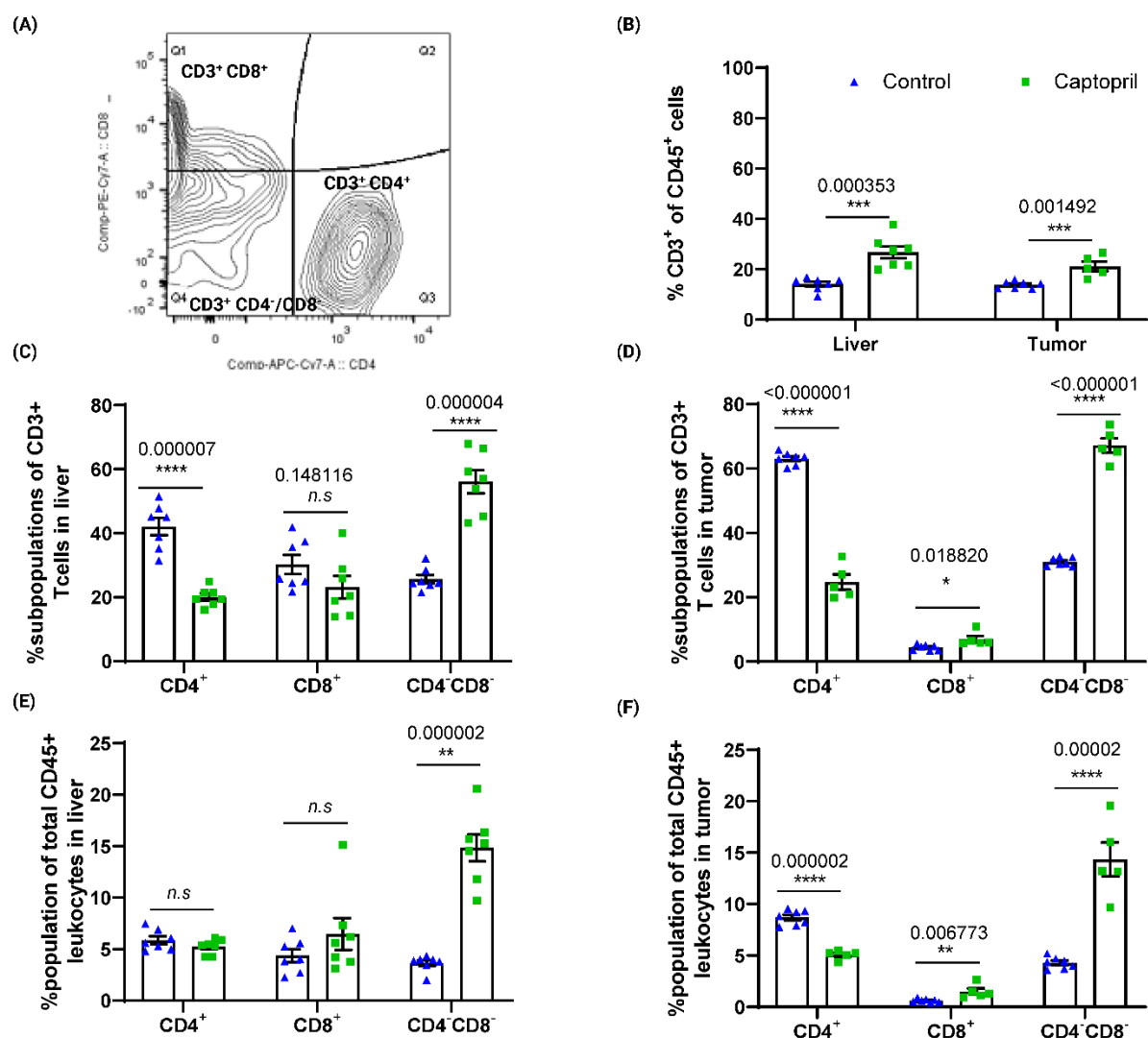


Figure 18. RASi differentially alters the proportions of CD3⁺ subpopulations of lymphocytes in the liver and tumour. (A) Gating strategy for analysis of CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁻CD8⁻. (B) Quantitation of the proportion of CD3⁺ lymphocytes as a proportion of total CD45⁺ leukocytes in the liver and tumour. The proportion (%) of CD3⁺ lymphocytes expressing CD4, CD8 and those negative for both CD4 and CD8 (CD4⁻CD8⁻) in (C) liver and (D) tumour. The proportion of each subpopulation within the total CD45⁺ leukocyte population in (E) liver and (F) tumour. Data $n \geq 5$ presented as mean $\pm$ SEM. Significantly different data represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.5 Captopril treatment differentially modulates the activation of T cell subtypes in the tumour and liver tissues

To investigate changes in the activation state of the CD3⁺ T cell subtypes the cells were also stained for expression of PD-1, an inhibitory receptor upregulated following T cell activation by cognate antigen and is specific for checkpoint ligand PD-L1. Captopril treatment increased the proportion of CD8 and DNT cells subpopulations expressing PD-1 in both liver and tumour (**Fig. 19A, B**).

When considering these activated populations as a proportion of total leukocytes (CD45⁺) in these tissues we find that in the liver and tumour tissues captopril treatment again increases activated CD8 and DNT cells, however in the tumour the proportion of total leukocytes that are activated CD4 significantly decreases. (**Fig. 19C, D**). This may indicate a role for captopril in activation of CD8 and DNT, but also in suppressing the activation of CD4 T cells within the tumour.

We further investigated the proportion of CD8⁺ T cells expressing CD103 to delineate the effect of captopril on the tissue-resident CD8⁺ T cell population. The percentage of CD8⁺ T cells expressing CD103 was significantly reduced in both liver and tumour tissues in the treated group (**Fig. 19E**). However, within the total leukocyte population, the proportion of CD8⁺ CD103⁺ T cells was significantly increased in tumour and was not affected in the liver (**Fig. 19F**).

This suggests a potential mechanism of how captopril may control tumour growth by improving the cytotoxic T cell response.

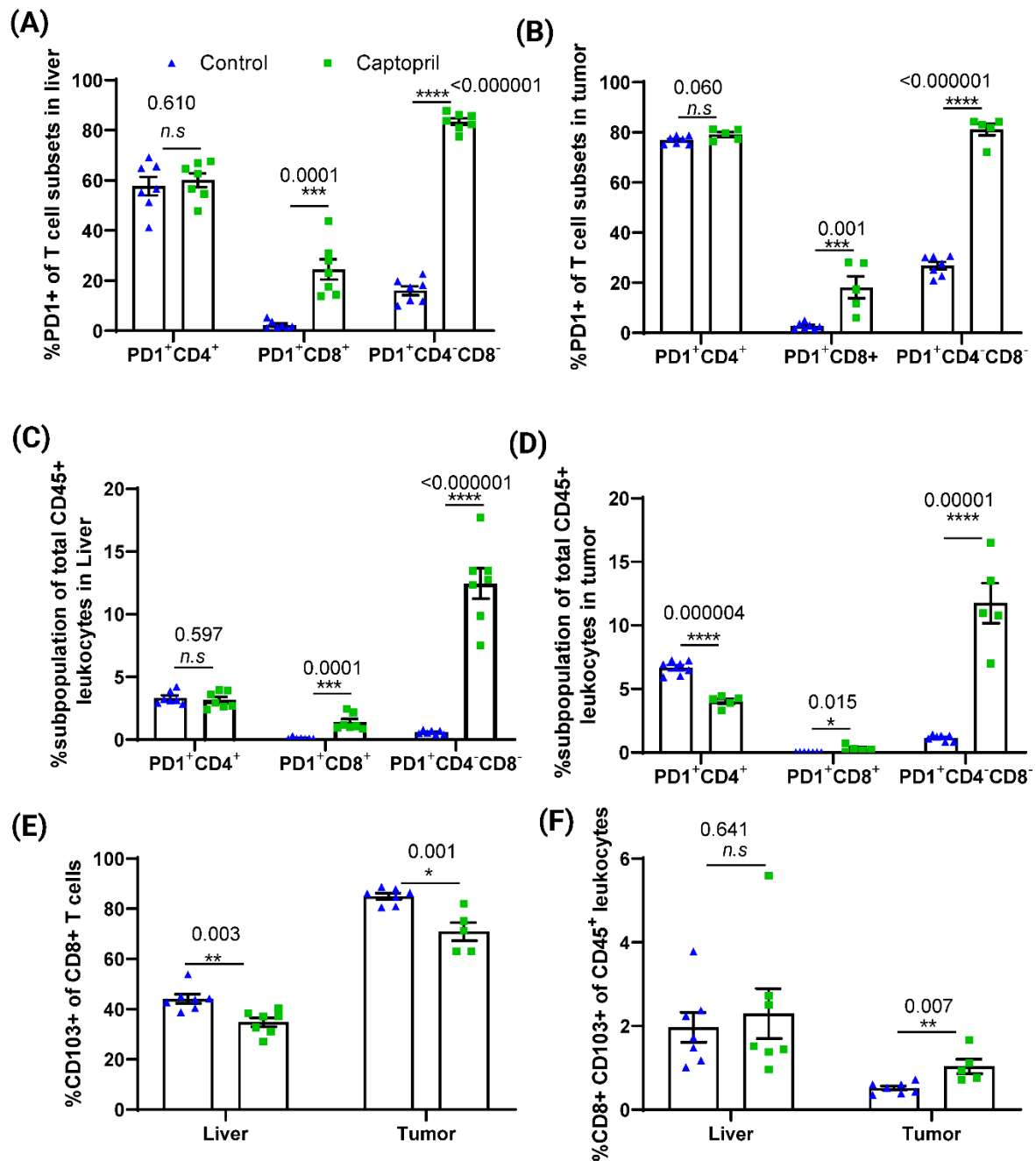


Figure 19. RASi differentially changes the proportions of CD3⁺ subpopulations expressing PD1⁺ and tissue-resident CD8⁺ lymphocytes in the liver and tumour. Quantitation of the proportion of each CD3⁺ subpopulation expressing PD1 in (A) liver and (B) tumour. The percentage of PD1⁺ lymphocyte subpopulations as a proportion of total CD45⁺ leukocytes in (C) liver and (D) tumour. (E) The proportion of CD8⁺ lymphocytes expressing tissue-resident marker CD103 in liver and tumour. (F) The percentage of CD103⁺ CD8⁺ lymphocytes as a proportion of total CD45⁺ leukocytes in the liver and tumour. Data n≥5 presented as mean ± SEM. Significantly different data represented by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

3.4 Discussion

Numerous retrospective clinical studies acknowledge the significant improvement in overall survival, progression-free survival, and disease-free survival in cancer patients using RASi as an antihypertensive medication (Sun et al., 2017). There is a clear upregulation of the RAS component, AT1R within the adenoma-colorectal-liver metastasis axis (Beitia et al., 2019; Zhou et al., 2014). Experimental and clinical studies over the last decade indicate that RASi impair tumour progression through modulation of the tumour vasculature (Carbajo-Lozoya et al., 2012; Shirotake et al., 2011), and tumour desmoplasia (Diop-Frimpong et al., 2011), however, its role in tumour immunoregulation is not yet clear. Almost all immune cells express components of the RAS pathway and several studies implicate a role for RAS in immune dysregulation in several chronic and autoimmune diseases (Capettini et al., 2012). Our previous findings show that the RASi, captopril, reduced tumour inflammation (Neo et al., 2010) and temporally modified the frequency of tumour-associated macrophages within the tumour microenvironment (Wen et al., 2013). Additionally, macrophage depletion experiments show that TAMs are important for RASi control of tumour (Wen et al., 2013).

In this study, we investigated the temporal dynamic changes of specific T-lymphocyte subpopulations, in response to captopril treatment and their trafficking patterns into the liver and tumour tissues. Captopril treatment significantly and temporally increased CD3⁺ T-lymphocyte infiltration both into the tumour and the liver. This finding suggests that increased infiltration of T cells into the tumour may have a role in the observed tumour inhibition as clinical studies report a positive correlation between tumour lymphocyte density and survival outcome (Galon et al., 2006). The greatest observed changes were seen at day 15 post tumour induction, possibly reflecting a timely development of anti-tumour responses and the effect of the captopril treatment. By day 21 the infiltration decreased and the significance of this is not clear as the subpopulation composition was not analysed at this time point. RASi reduces the immunosuppressive TME by altering the activity of TAM, MDSC and CAF (Nakamura et al., 2018) and this may account, at least in part, for the increase in CD3⁺ T-lymphocyte infiltration observed in this study.

In these experiments, the effect of RASi on the infiltration of T cell subtypes appears specific and possibly local to tumour affected tissues. It is important to note that the T cells infiltrating the liver may provide a substantial pool, along with those from the peripheral circulation that would be infiltrating the tumour. Remarkably, we found the inhibition of RAS markedly attenuated tumour infiltrating CD4⁺ T cells. AT1R stimulation has been shown to play a role in the differentiation of CD4⁺ T cells into Th1 cell through the modulation of proteasome function, thus RASi may inhibit this mechanism of CD4 T cell antigen mediated activation (Qin et al., 2018). The proportion of activated circulating CD4⁺ T-cells may have been diminished as a result of reduced Ang II, therefore trafficking into the liver and tumour was profoundly affected, as it was reported during deoxycorticosterone acetate (DOCA)-salt hypertension (Vinh et al., 2010).

RASi appears to not have a selective effect on the infiltration of CD8 T cells into the liver, however the significant increase in CD8⁺ lymphocytes into the tumour, particularly those that are identified as tissue-resident (CD103⁺) and PD-1 positive may indicate a recently activated tumour specific cytotoxic T cell population. Studies reported a positive correlation between CD8⁺ infiltrating lymphocytes and control of tumour growth (Beyrend et al., 2019). Additionally, the intratumoral presence of T cells expressing the integrin CD103 has been strongly correlated with a favourable prognosis for cancer patients (Dumauthioz et al., 2018), independently of the infiltration of CD8⁺ T cells (Topham & Reilly, 2018). Moreover, T_{RM} express immune checkpoint molecules, which may be a potential surrogate marker of response to immunotherapy as they could expand early during anti-PD1 treatment (Webb et al., 2014).

Unexpectedly, the most responsive subpopulation of immune cells to captopril treatment was the DNT cells. CD3⁺ lymphocytes that do not express CD4 and CD8 may include TCR alpha/beta (TcRαβ⁺) and TCR gamma/delta (TcRγδ⁺), however, at this stage, we have not identified what is the type of our CD3⁺ DNT cells. DNT cells represent only a small fraction of the total peripheral lymphocyte composition (1-3%) in healthy subjects (Fischer et al., 2005). However, they are reported to increase in certain diseases with context-specific immune roles such as immune-inflammatory and disease exacerbating activities in autoimmune diseases (Hillhouse & Lesage,

2013) to anti-inflammatory (Martina et al., 2016) or pro-inflammatory roles in tissue injury and ischemia (Meng et al., 2019).

Interestingly, DNT cells have been reported to be present in several solid cancers (melanoma, RCC, and glioblastoma) and to increase upon treatment (Greenplate et al., 2019). The phenotypes of these cells are cancer-specific and distinct from that of resting DNT, but closely resembling DNT in other cancer types and across species (Greenplate et al., 2019). Furthermore, they positively correlate with treatment outcome, in concordance with the findings in this study (Greenplate et al., 2019). DNT cells have been previously reported to contribute to anti-melanoma immunity (De Tullio et al., 2015). In addition, this immunomodulatory effect is congruent with the reduced frequency of CD4⁺ T-cells subpopulation as double-negative T cells have been shown to suppress metabolic activation of CD4⁺ T cell (Haug et al., 2019) and could account for the CD4⁺ T cell reduction in this study. Nevertheless, the contribution of DNTs to antitumour immunity has only begun to be considered.

In the meantime, cancer immunotherapy strategies have mainly focused on modulating the function of infiltrating CD8⁺ and CD4⁺ lymphocytes. The presence and quality of the immune cell infiltrate of the tumour has been shown to be of prognostic potential for the response of different tumours to immunotherapies, regardless of tumour stage (Galon et al., 2006). However, when PD-L1 blockers are used, a selective expansion of tumour infiltrating CD4⁺ and CD8⁺ T cells expressing both activating (ICOS) and inhibitory (LAG-3, PD-1) molecules is seen (Beyrend et al., 2019).

This study showed significant upregulation in PD-1 expression on both the CD8 and DNT tumour infiltrating cells in the captopril treated mice. A recent study reported that DNT cell infusion in combination with anti-PD1 resulted in increased DNT cell-mediated anti-tumour activity during *in vivo* treatment of lung cancer (Fang et al., 2019). Nakamura et al, in the only other *in vivo* study on the RASi immunoregulatory effects, report that RASi in combination with PD-L1 inhibitors improve the effect on tumour inhibition. The increase of lymphocyte populations expressing PD-1 within the RASi treated tumours, as seen in our study, opens up an opportunity to combine RASi with a combination of checkpoint inhibitor treatments that may be able to increase the efficacy and durability of the anti-tumour response (Beyrend et al., 2019).

Humans and outbred animal species display wide variability in the anti-tumour immune responses and drug treatments. In addition to the genetic contribution, numerous studies report a strong sexual dimorphism in immune responses (Capone et al., 2018). Furthermore, studies also indicate that some components of the RAS pathway display a sex-biased differential expression in certain tissues, hence inequality between the sexes response to RASi treatments can be induced (Sullivan, 2008). This study used inbred single strain male mice to overcome genetic and sexual dimorphism variabilities. However immune responses to RASi treatments for CRLM need to be investigated in additional animal models of both genders and clinical studies. Elucidation of genetic and gender differences to RASi response will further define the appropriate patient cohort thus tailoring treatment to those likely to benefit.

3.5 Conclusion

This study described the immunomodulatory effect of RASi using captopril comparing an early and a later stage of tumour development. Treatment significantly increased the infiltration of CD3⁺ T cells into the liver and tumour and altered the phenotypic composition of infiltrating CD4⁺ and CD8⁺ T cells, and double-negative T cells. Additionally, captopril treatment increased the expression of checkpoint receptor PD-1 on the CD8⁺ and DNT cells. The significance of these changes needs to be further characterized, especially the identity and function of the DNT cell population.

Chapter 4

The effects of Angiotensin-converting enzyme inhibitors on myeloid cells in mice with colorectal liver metastases

This chapter aims to investigate the effects of ACEi on liver and tumour myeloid cell composition and distribution in a mouse model of colorectal liver metastases.

This chapter is presented as a regular thesis chapter which includes unpublished results.

CHAPTER 4

THE EFFECTS OF ANGIOTENSIN-CONVERTING ENZYME INHIBITORS ON MYELOID CELLS IN MICE WITH COLORECTAL LIVER METASTASIS

4.1 Introduction

The development of liver metastases (LM) microenvironment encompasses two stages of recruitment of nonparenchymal cells, one that is triggered in the “*premetastatic niche*” by the circulating secreted factors of the primary tumour, and the other during the post metastatic invasion upon entry of tumour cells into the liver (Brodt, 2016). The biological function, phenotype, and development of nonparenchymal cells recruited during the early stages of metastatic growth in LM, including MDSC and KC, remains a current topic of investigation (Milette et al., 2017). Accumulating evidence supports the potential therapeutic benefit of targeting these myeloid subpopulations to impair tumour progression (G. Wang et al., 2016) and to improve response to current cancer immunotherapies (Fleming et al., 2018).

MDSC encompasses a large heterogeneous population of myeloid precursor cells of the macrophages (MΦ), DCs and granulocytes (Laoui et al., 2016). Myeloid-derived populations have been characterized with an immunosuppressive role not only in tumour growth (Fleming et al., 2018) but also in chronic inflammatory pathologies (Sendo et al., 2018), infectious diseases (Dorhoi & Du Plessis, 2017), autoimmune diseases (Boros et al., 2016) and organ transplantation (Ochando et al., 2019). In mice, MDSC have been identified by the co-expression of surface markers such as CD11b and Gr1 (Gabrilovich et al., 2001). Subsequently, they have been subclassified according to the expression level of Ly6C (Youn & Gabrilovich, 2010) into, Ly6C^{high} or monocytic-like (M-MDSCs) and Ly6C^{low} or granulocytic-like morphology (G-MDSCs) (Tcyganov et al., 2018). The phenotype displayed by M-MDSCs is CD11b⁺Ly6G⁻Ly6C^{high}, whereas the phenotype of G-MDSCs is CD11b⁺Ly6G⁺Ly6C^{low} (Fleming et al., 1993; Youn et al., 2008). The suppressing capability of MDSCs over effective T-cell cytotoxic response and T- regulatory development is linked to their origin and maturation status (Medina-Echeverez et al., 2015).

The expression of markers such CD31 (or Platelet Endothelial Cell Adhesion Molecule-1 [PECAM-1]) (Angulo et al., 2000; Bronte et al., 2000) and Major Histocompatibility Complex class II (MHC II) defines MDSCs as immature progenitors and myeloid blast cells (Angulo et al., 2000). In addition, the production of granulocyte colony-stimulating factor in the bone marrow stimulates the production of CD11b⁺Gr1⁺CD31⁺

and the migration of immature CD11b⁺Gr1⁺ cells to secondary lymphoid organs (Kusmartsev & Gabrilovich, 2006). Moreover, CD31 expression has been described in DCs as a coinhibitory receptor that impacts the immunogenic maturation and migration of resident DCs to the draining lymph nodes (Clement et al., 2014). A more recent study found that conventional flow-cytometry gating strategies for identifying monocyte-derived MΦ, particularly KC, have included CD31^{high} endothelial cells causing a bias for cell type and contamination (Lynch et al., 2018). Therefore, the assessment of the expression of CD31 may be a simple method for exclusion for the preparation of hepatic leukocytes for FACS (Lynch et al., 2018).

MDSCs can be phenotypically and functionally distinct depending on whether they are localized at a primary or secondary lymphoid organ (Gabrilovich et al., 2012), within the tumour itself (Zhao et al., 2016) or expanding in the liver (Connolly et al., 2010). For instance, a significant accumulation of CD11b⁺Gr1^{inter} Ly6C^{high} CD31^{high} was described in the liver of mice with intra-abdominal cancer, which also showed a low expression of Ly6G and MHC II (Connolly et al., 2010). Furthermore, the role of these distinct populations as metastases-enabling myeloid cells in the liver of mice harbouring invasive and pre-invasive intra-abdominal tumour has been demonstrated using models of early preinvasive pancreatic neoplasia and advanced CRC (Connolly et al., 2010). That study compared CD11b⁺Gr1⁺ cells from distinct tissue origin, including liver, spleen, bone marrow and tumour, uncovering that hepatic CD11b⁺Gr1⁺ cells expressed high levels of Ly6C, but low Ly6G compared with MDSC populations in other tissues. Likewise, they express low MHC I and MHC II but high CD31, F4/80, and CD195 than the spleen (Connolly et al., 2010). Furthermore, in mice with intraperitoneal MCA38 tumour, hepatic CD11b⁺Gr1⁺ cells comprised a significantly higher portion of CD11b⁺Gr1^{inter} cells than the spleen, bone marrow, or tumour associated CD11b⁺Gr1⁺ cells (Connolly et al., 2010). Another relevant aspect is the retention of tumour-induced MDSC by the liver (Medina-Echeverez et al., 2015). After effectively depleting this population from the tumour and the periphery in tumour bearing models, the liver showed a replenished effect within 24 hours (Ma et al., 2012). These findings were consistent with MDSCs cells being substituted with newly generated precursor cells, either from the BM or peripheral haematopoiesis sites (Ma et al., 2012).

The detrimental influence of MDSCs after disabling KC and natural killer immunity (Li et al., 2009) also corroborates the variety of mechanisms by which they deceive the hepatic immune surveillance mechanism (Ilkovitch & Lopez, 2009). The KC population represents 10% of all liver cells, which replenish from circulating monocytes in a monocyte colony-stimulating factor-dependent manner (Yona et al., 2013). This population's role is context-specific, which means that targeting KC as a potential candidate for cancer immunotherapy relies on its effect to prevent the premetastatic niche formation and enhance its antitumour activity before the establishment of metastases (Milette et al., 2017). Additionally, CD11b^{low}F4/80^{high} MΦ can be recruited to the tumour site and become tumour-associated macrophages (TAMs) (Beltraminelli & De Palma, 2020). Current immunotherapy aims to reprogram M2-type MΦ from a pro-tumour state of activation to an M1-type MΦ or anti-inflammatory (Mantovani et al., 2002) to overcome their immunotolerance activity (Beltraminelli & De Palma, 2020) by exploiting MΦ expression of both ligands for PD-1 (B7H4, PD-L1, PD-L2) and CTLA-4 (B7-1 and B7-2) (Cortese et al., 2019). However, distinct MΦ types are linked to particular prognostic behaviour, which still needs to be better understood (Cortese et al., 2019). Research from our laboratory had previously shown that ACEi effectively altered the frequency of KC in the MoCR-CLM mouse model (Wen et al., 2013). Captopril reduces CLM growth via immunomodulatory effects of KC at the early stage of LM development by increasing KC infiltration in the tumour-bearing liver, which may be associated with distinct functions within the growing tumour (Wen et al., 2013).

ACE activity is known to be involved in the proliferation, differentiation, and maturation of MΦ (Shen et al., 2014) and DCs (Meng et al., 2017). ACE overexpression in myelomonocytic cells promoted differentiation and reduced the expansion of the M-MDSC population in blood and spleen in a mouse model of lung metastasis (Shen et al., 2014). Comparably, Angiotensin II promoted the maturation and migration of DCs, while their ability to proliferate was inhibited in the context of inflammatory disorders (Meng et al., 2017).

In this chapter, the effects of captopril treatment (RASi) on hepatic myeloid cells and tumour infiltrating myeloid subpopulations and their expression of some inhibitory factors were characterized in a CLM mouse model.

4.2 Methods

In this study, tumour metastases were established using the MoCR mouse model, and the captopril treatment protocol was administered as described in Chapter 2 (**Treatment protocol** page number 27-28). At the early angiogenic stage (endpoint day 15), CD11b⁺ and CD11c⁺ cells distribution was examined within the tumour periphery and the total area of the tumour and their distribution in the inguinal lymph nodes using IHC. Besides, F4/80⁺ cell spatial and temporal distribution was assessed at day 15 and day 21 post-tumour induction using IHC. mIHC staining procedures of CD11c⁺ and CD8α⁺ are also fully described in Chapter 2 (**Multiplex IHC staining of Mouse liver and tumour tissues**, page numbers 45-48).

Using the Image analysis software (Aperio ImageScope™), the Positive Pixel Count v9 (version 9.1) algorithm was applied as a basis for detecting immune marker positivity, with the intensity thresholds adjusted manually to remove background artefacts as follows: WEAK pixels (yellow colour): 220-175, MEDIUM pixels (orange colour): 175-100, STRONG pixels (red colour): 100-0, and NEGATIVE pixels (blue colour): -1, as described in Chapter 2 (**Image caption and analysis algorithm**, page numbers 33-34). At day 15 endpoint, livers were excised as described in Chapter 2, and caudate lobes separated for processing for flow cytometry using the optimized diluted antibody cocktails directly conjugated to the following fluorochromes: CD45-BV510, F4/80-FITC, CD11b- APC-Cy7, Ly6C-PE, CD31-APC, CD274/PDL1, and the viability dye, DAPI, as described in **Table 2**. FACS of dissected liver and tumour tissue cell preparations were used to identify the phenotype of KC, MΦ, TAM and M-MDSC, also to investigate changes in their state of activation. The specific antibodies used, the staining procedures, image analysis and quantitation, FACS protocol and dimensionality reduction analysis are also fully described in Chapter 2 (**Flow cytometry procedure and dimensionality reduction analysis**, page numbers 36-42).

4.2.1 Statistical analysis

Statistical analysis was conducted using GraphPad Prism® 8.3.0 (GraphPad Software, Inc., San Diego, CA) using both parametric and non-parametric analytical tests as appropriate. The data was not normally distributed (test for normality using

Shapiro-Wilk test); therefore, non-parametric analysis was employed. Mann-Whitney U test was used for comparison between two groups (independent group t-test equivalent). All datasets are presented as the mean value of each group $\pm$ standard error of the mean. Statistical significance determined using the Holm-Sidak method, with $\alpha = 0.05$. FACS datasets were analysed with FlowJo™ software. The t-SNE analysis was performed using the FlowJo™ v10.7.

4.3 Results

4.3.1 Captopril treatment subtly modifies the liver or tumour expression of myeloid populations identified with CD11b and CD11c markers

We used IHC to examine changes in CD11b⁺ and CD11c⁺ hepatic myeloid populations (**Fig. 20A-D**) within the total area of the tumour (TAT) tissues (**Fig. 20E-H**) and the invasive tumour margin (IM) due to captopril treatment (**Fig. 20I-J**). Captopril treatment reduced the number of CD11b⁺ cells in the liver, but it did not reach significance. The opposite effect was observed for the hepatic CD11c⁺ cell population on day 15 (**Fig. 20C, D**). The results show that both CD11b⁺ (**Fig. 20E, G**) and CD11c⁺ (**Fig. 20F, H**) populations increased in the captopril treated tumours compared to saline, particularly CD11c⁺ cells showed a strong trend of accumulation within TAT (**Fig. 20 H**) and the IM (**Fig. 20I, J**), but no significant difference was reached in the intensity of expression for either of these markers. The findings suggest that after measuring CD11b⁺ (**Fig.S5A, C, E**; see Supplementary Figures and Tables section, page number 291) and CD11c⁺ (**Fig.S5B, D, F**; see Supplementary Figures and Tables section, page number 291) populations, no differences were found between weak positive cells, intermediate positive cells, and strong positive cells in TAT tissues (**Fig.S5C, D**; see Supplementary Figures and Tables section, page number 291) and the IM (**Fig.S5 E, F**; see Supplementary Figures and Tables section, page number 291) between in the captopril treated tumours compared to saline.

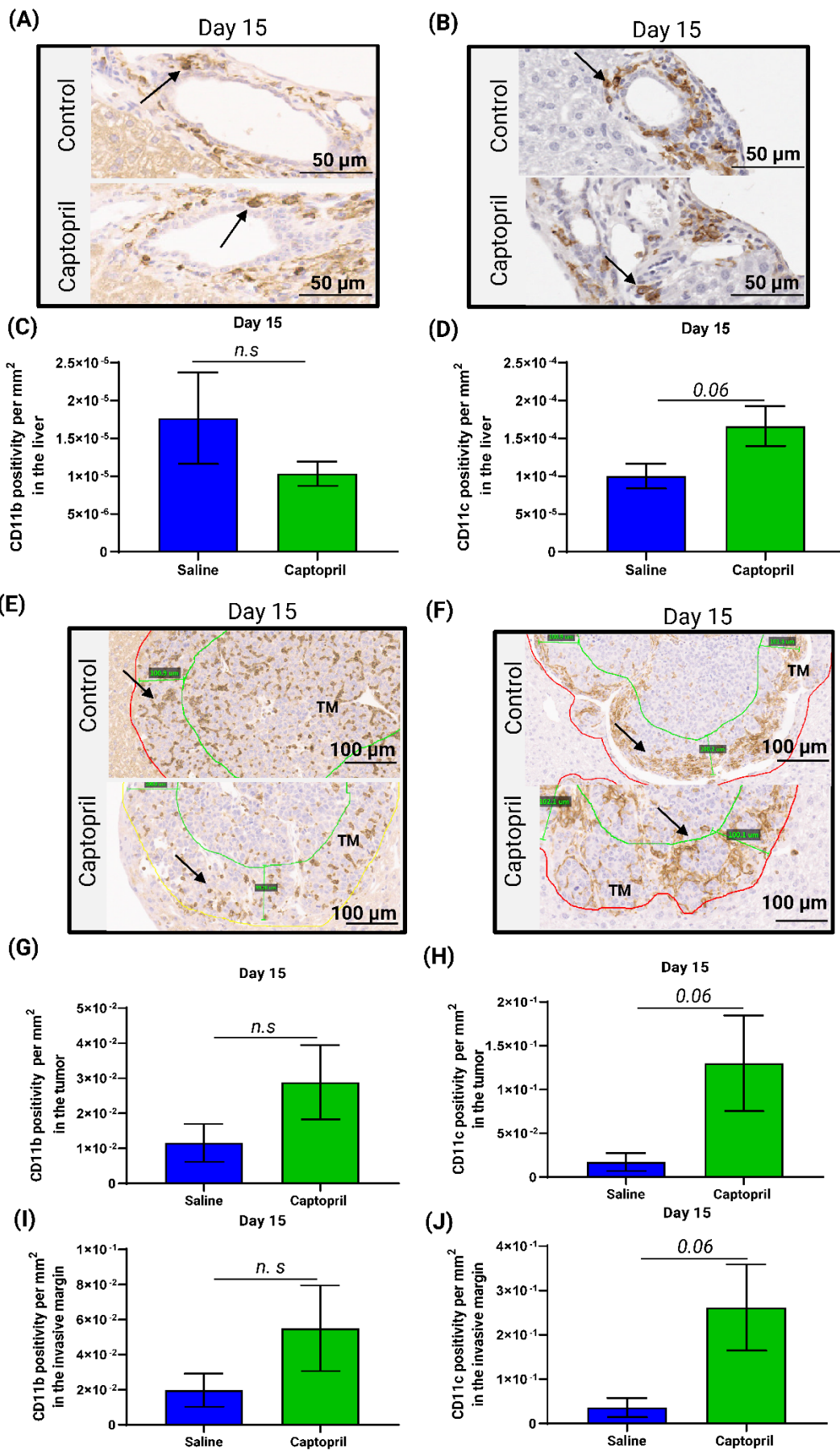


Figure 20. The effect of RASi on CD11b and CD11c populations in the liver and tumour tissues. Representative microphotographs of IHC marked-up of (A) CD11b⁺ and (B) CD11c⁺ in the liver at day 15 post-tumour induction (scale bar 100µm). Quantitation of the total area staining positive for (C) CD11b⁺ and (D) CD11c⁺ as an area per mm² of total liver area. Representative microphotographs of IHC marked-up total tumour area (red line) of (E) CD11b⁺ and (F) CD11c⁺ and invasive tumour margin (TM) (green line) (scale bar = 100 µm measured from the edge of the tumour in direction to the core). Quantitation of the total tumour area staining positive for (G) CD11b⁺ and (H) CD11c⁺ as an area per mm² of the total tumour area and the invasive margin positivity per mm² for (I) CD11b⁺ and (J) CD11c⁺. Datasets expressed as mean ± SEM with n ≥ 5 mice. Significantly different data annotated.

CD11c is typically used to define myeloid DCs. The changes induced by captopril on CD11c⁺ cells are likely to reflect an accumulation of DCs within the tumour, which prompted us to investigate further their phenotype. When developing the DCs Ab panel, we found no cell-specific markers available for DCs; therefore, we used a multimarker such as CD8α expressed in both CD8⁺ T cells and DCs (Sorrelle et al., 2019). The expression of CD8α on DC has been attributed to their maturation status (Traver et al., 2000), their capacity to promote the cytotoxic T cell responses in the tumour (den Haan et al., 2000; Hildner et al., 2008) and to present exogenous antigens on their MHC class I molecules (Schnorrer et al., 2006). The cell densities of CD11c and CD8α were measured using mIHC (**Fig. 21A, B**). A negligible level co-expression of CD8α⁺ on CD11c⁺ was found within the tumour (**Fig. 21C, D**). Also, there was no significant difference in the CD11c⁺ given the wide range of expression in the control group (**Fig. 21 E, F**) and CD8α⁺ (**Fig. 21 G, H**) cell density within the tumour region in the captopril treated group compared to the control at day 15 (**Fig. 21I**).

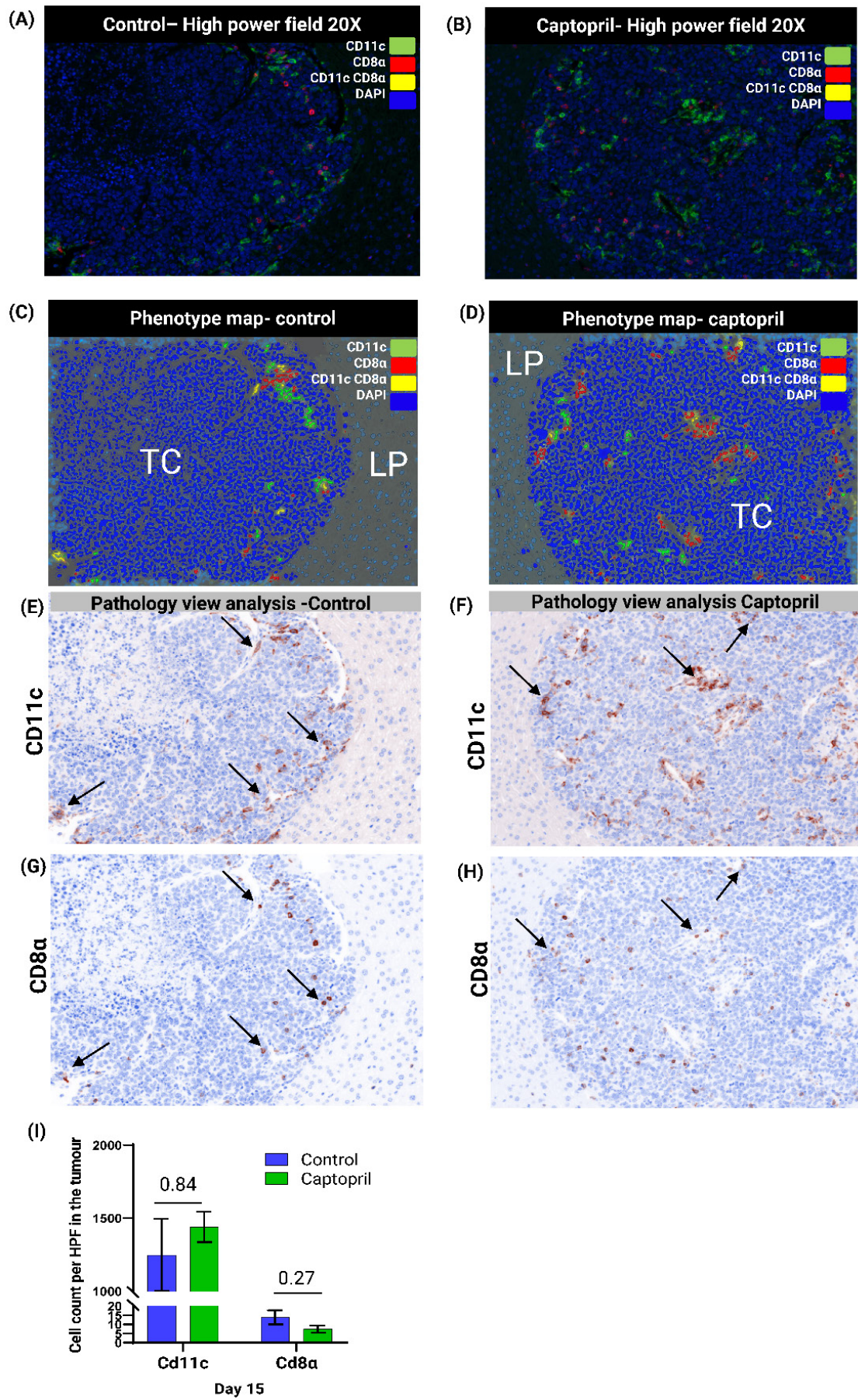


Figure 21. The cell density of CD11c⁺ and CD8α⁺ in the tumour region. Composite images of CD11c in green, CD8α in red and DAPI in blue were captured using Vectra® and analysed on inForm® of (A) the control and (B) captopril treated group. Phenotype scoring of CD11c⁺ in green, CD8α⁺ in red, CD11c⁺CD8α⁺ in yellow and DAPI in blue was performed as shown in the phenotype map of (C) control and (D) captopril. Single staining of CD11c was examined in (E) the control and (F) captopril treated group; CD8α single staining was examined in (G) the control and (H) captopril treated group using pathology view analysis (I) Cell count per high power field (HPF) of CD11c and CD8α. Datasets expressed as mean ± SEM with n ≥ 5 mice for each group. Significantly different data annotated.

We also examined the effects of captopril treatment on the CD11b⁺ and CD11c⁺ cell populations in the lymph nodes localized in the inguinal region, which have been reported to have a similar structure and myeloid cell composition compared to liver-draining lymph nodes such as the portal and celiac lymph node in mice at steady state (Zheng et al., 2013). The size of the inguinal lymph node can vary between a control mouse ($2.32 \pm 0.71\text{mm}^2$) and a tumour bearing mouse model ($3.53 \pm 0.84\text{ mm}^2$) (Zhang et al., 2013). After visual examination of the tumour, inguinal lymph nodes show that the total area of the lymph nodes (**Fig. 22A,B**) and their cellular density was significantly reduced in the captopril treated group (**Fig. 22C-F**). However, the densities of CD11b⁺ (**Fig. 22C**) and CD11c⁺ cells (**Fig. 22D**) were significantly increased at day 15 in the captopril treated group (**Fig. 22E, F**). These results suggest that the effect of captopril on such heterogeneous cell populations requires a thorough investigation of phenotypic changes on myeloid-monocytic subpopulations induced by captopril on day 15, hence the expression of F4/80 to identify MΦ, KC, and TAMs was assessed. In contrast, the expression levels of Ly6C were measured to identify the MDSC subpopulation.

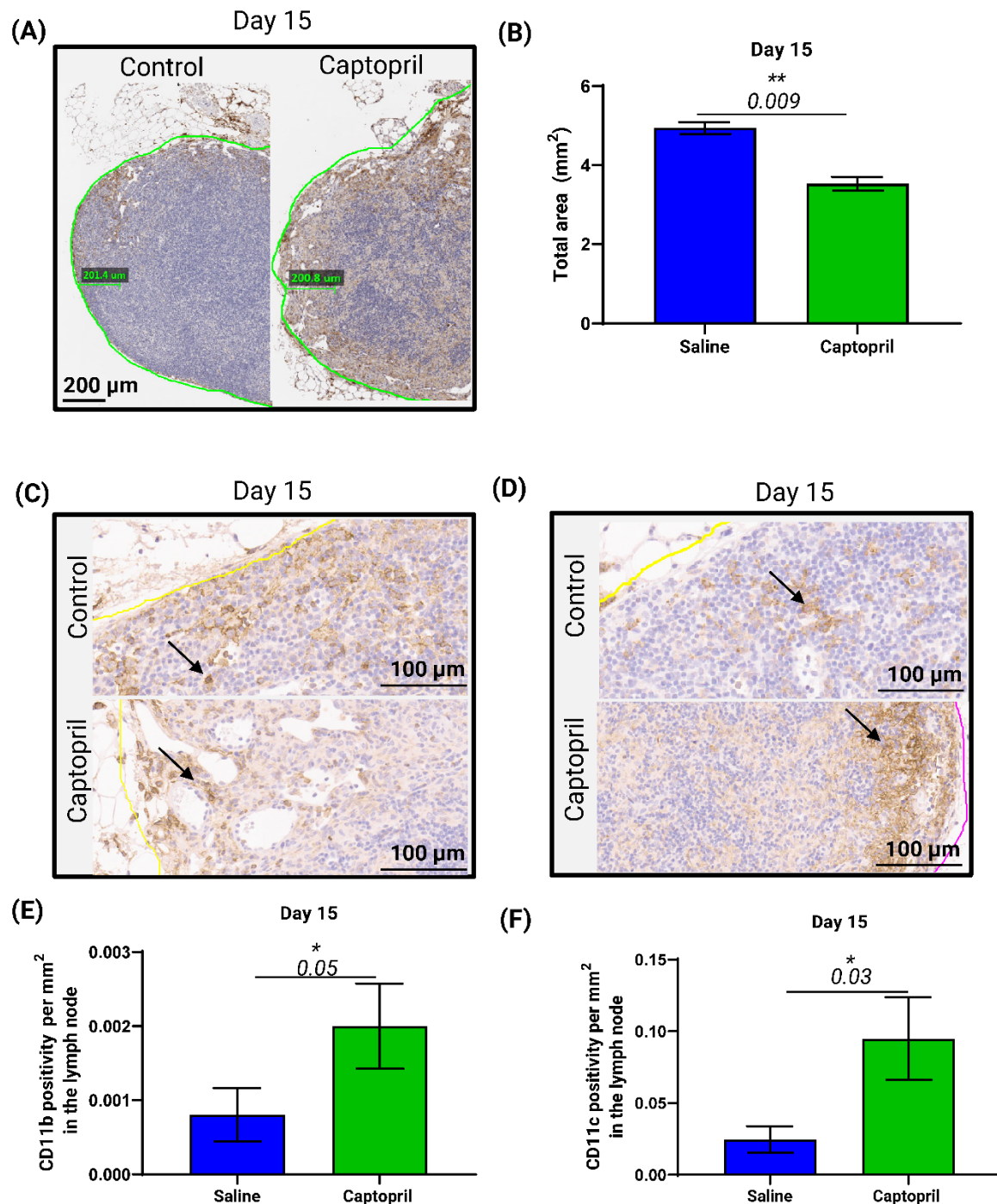


Figure 22. The effect of captopril on inguinal lymph node morphology and CD11b and CD11c populations. Representative microphotographs of whole inguinal lymph node of (A) saline and captopril treated group at day 15. (B) Quantitation of the total area (mm^2) of the whole inguinal lymph node comparing the saline and captopril treated groups. Representative microphotographs of IHC marked-up of (C) CD11b⁺ and (D) CD11c⁺ in the inguinal lymph nodes at day 15 post-tumour induction. Quantitation of the total area staining positive for (E) CD11b⁺ and (F) CD11c⁺ as an area per mm^2 total lymph node area. Data $n \geq 5$ presented as mean $\pm$ SEM. Significantly different data represented by * $p < 0.05$ and ** $p < 0.01$.

4.3.2 Captopril treatment modulates the spatial and temporal distribution of F4/80+ macrophages in the liver and tumour

Using IHC, we investigated whether cell density and distribution of F4/80⁺ including MΦ, KC, and TAMs, would be influenced by captopril during the development of LM by comparing between endpoint day 15 (“early angiogenic stage”) (**Fig. 23A**) and day 21 (“exponential metastatic growth stage”) (**Fig. 23B**). Interestingly, both cell density and temporal changes of F4/80⁺ MΦ and KC were shown in the liver. No significant change was observed in the hepatic F4/80⁺ at day 15 (**Fig. 23C**), whereas F4/80⁺ MΦ and KC were markedly reduced in the captopril treated group compared to saline at day 21 (**Fig. 23D**). An inversed relationship between the density of F4/80⁺ in liver and time indicates that captopril maintains a similar number of F4/80⁺ MΦ and KC over time, whereas in the saline group, a significant accumulation of F4/80⁺ MΦ and KC is shown when comparing endpoint day 15 and day 21 ($p=0.011$) (**Fig. 23E**).

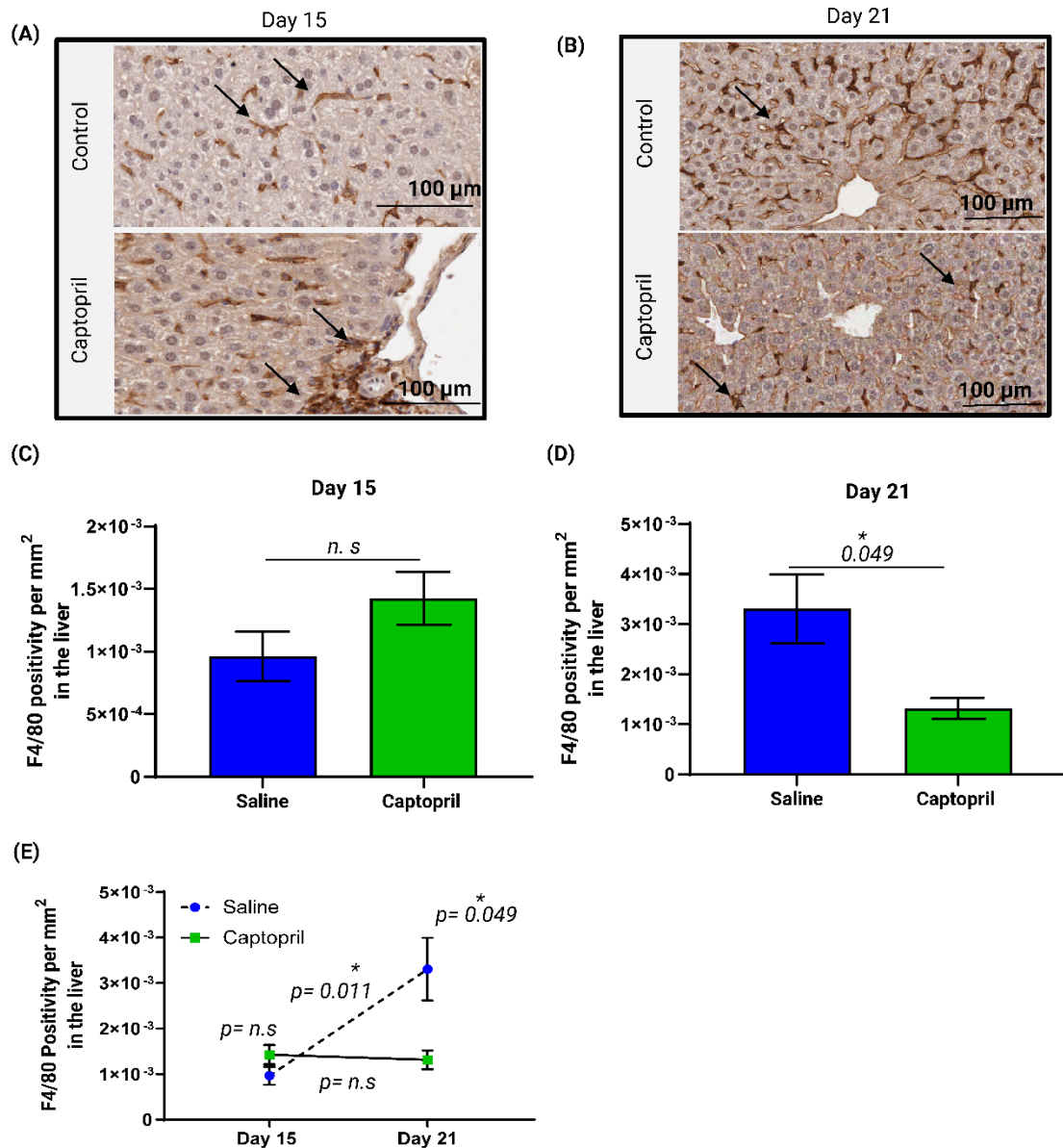


Figure 23. Temporal changes of F4/80⁺ expression in the adjacent liver parenchyma. Representative microphotographs of IHC F4/80 staining (A) at day 15 and (B) day 21 post-tumour induction in the liver area (black arrow showing an F4/80⁺ cell; (Scale bar = 100 μ m). Histological quantitation of F4/80 expression in the liver (C) at day 15 and (D) 21 post-tumour induction. (E) The total area of the liver staining positive for F4/80⁺ as an area per mm² for tissues collected at day 15 and day 21. Datasets expressed as mean $\pm$ SEM with $n \geq 5$ mice. Significantly different data represented by * $p < 0.05$.

At both endpoints, the density of TAMs was increased in the captopril group compared to the saline group (**Fig. 24A-B**). However, F4/80⁺ TAMs distributed in the TAT did not reach significance at day 15 (**Fig. 24C**). Instead, a strong trend of F4/80⁺ TAMs accumulated in the TAT in the captopril treated group at day 21 ($p=0.059$) (**Fig. 24D**). There was no significant difference in the F4/80⁺ TAMs distributed in the IM at day 15

(**Fig. 24E**), whereas they significantly increased in the captopril treated group when compared to saline at day 21 ($p=0.03$) (**Fig. 24F**).

Contrary to the temporal dynamic effect of captopril on MΦ in the liver, TAMs showed a trend of a marked reduction in the captopril group compared to saline. However, it did not reach significance (**Fig. 24G**). The level of intensity of expression of F4/80⁺ was investigated in the TAT, and no significant differences were noticed between weak positive cells, intermediate positive cells, and strong positive cells at day 15 (**Fig.S6A**; see Supplementary Figures and Tables section, page number 292) and day 21 (**Fig.S6B**; see Supplementary Figures and Tables section, page number 292) between captopril treated tumours compared to saline.

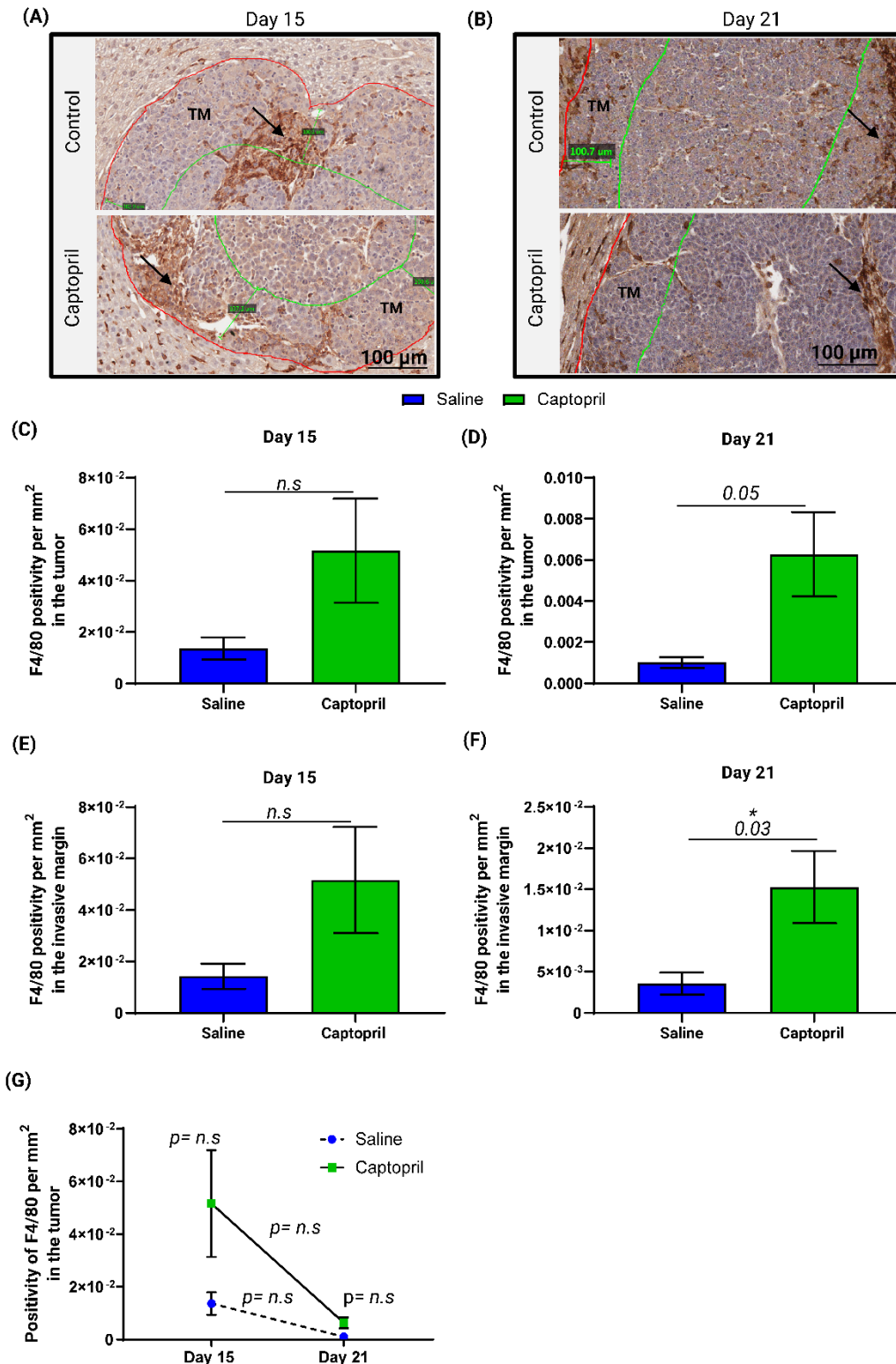


Figure 24. Temporal and spatial changes of F4/80⁺ expression in the tumour. Representative microphotographs of IHC F4/80 staining (A) at day 15 and (B) day 21 post-tumour induction (black arrow showing an F4/80⁺ cell) (Scale bar = 100 μ m measured from the edge of the tumour in direction to the core). (C) Quantitation of the total tumour area staining positive for F4/80 as an area per mm² of total tumour area (red line) for tissues collected at day 15 and (D) day 21. Quantitation of the total tumour area staining positive for F4/80 as an area per mm² of the invasive tumour margin area (green line) for tissues collected at (E) day 15 and (F) day 21. (G) The total area of the tumour staining positive for F4/80⁺ as an area per mm² (excluding necrotic areas) for tissues collected at day 15 and day 21. Datasets expressed as mean $\pm$ SEM with $n \geq 5$ mice. Significantly different data represented by * $p < 0.05$.

4.3.3 Captopril treatment differentially modulates myeloid cell subpopulations expressing CD31 into the tumour and liver tissues

FACS was used to examine further the phenotype of myeloid cell subpopulations in the liver and tumour that were observed to be modulated by captopril on day 15 (**Fig. 25A**). The flow-cytometry gating strategy shows CD11b positive myeloid cells gated on CD45 (leukocytes). FMO controls were used to gate CD31 on the CD45+CD11b+ myeloid population accurately. Then F4/80 cells were gated on CD31 myeloid subsets. Ly6C expression was gated on F4/80⁻ subset, while PDL1 positive cells were gated on F4/80⁺ (**Figure 10**).

FACS confirmed that the percentage of total myeloid cells (CD11b⁺CD45⁺) was significantly decreased in the liver parenchyma due to captopril treatment ($p=0.046$), while no significant change was found in this population within the tumour (**Fig. 25 A, B**). In addition, the distribution of CD31⁺ and CD31⁻ on myeloid cells between the liver and the tumour may provide insights into the influence of captopril over the replenishment of the tumour by myeloid cells present in the liver. Hence, the overall frequency of CD31⁺ myeloid cells was investigated (**Fig. 25C**), which indicated that there was a significant decrease in the captopril treated group ($p=0.002$) when comparing between the frequency of the liver and the tumour, while there is no change in the saline group (**Fig. 25D, upper panel**). In the case of CD31⁻ myeloid cells, captopril does not alter their frequency, whereas there is a marked difference when comparing their frequencies in the liver and the tumour in the saline group ($p=0.00007$) (**Fig. 25D, lower panel**). Overall, captopril diminished the frequency of myeloid cells (CD45⁺CD11b⁺) in the liver, in which the expression of CD31 does not appear to be altered significantly compared to saline (**Fig. 25E**). Furthermore, these findings suggest that even if there was no change in the overall frequency of myeloid cells within the tumour, captopril decreases the number of CD31⁺ myeloid cells within the tumour ($\approx 50\%$ less than the saline group) ($p=0.04$), while the CD31⁻ myeloid subset was significantly increased ($p=0.01$) (**Fig. 25F**).

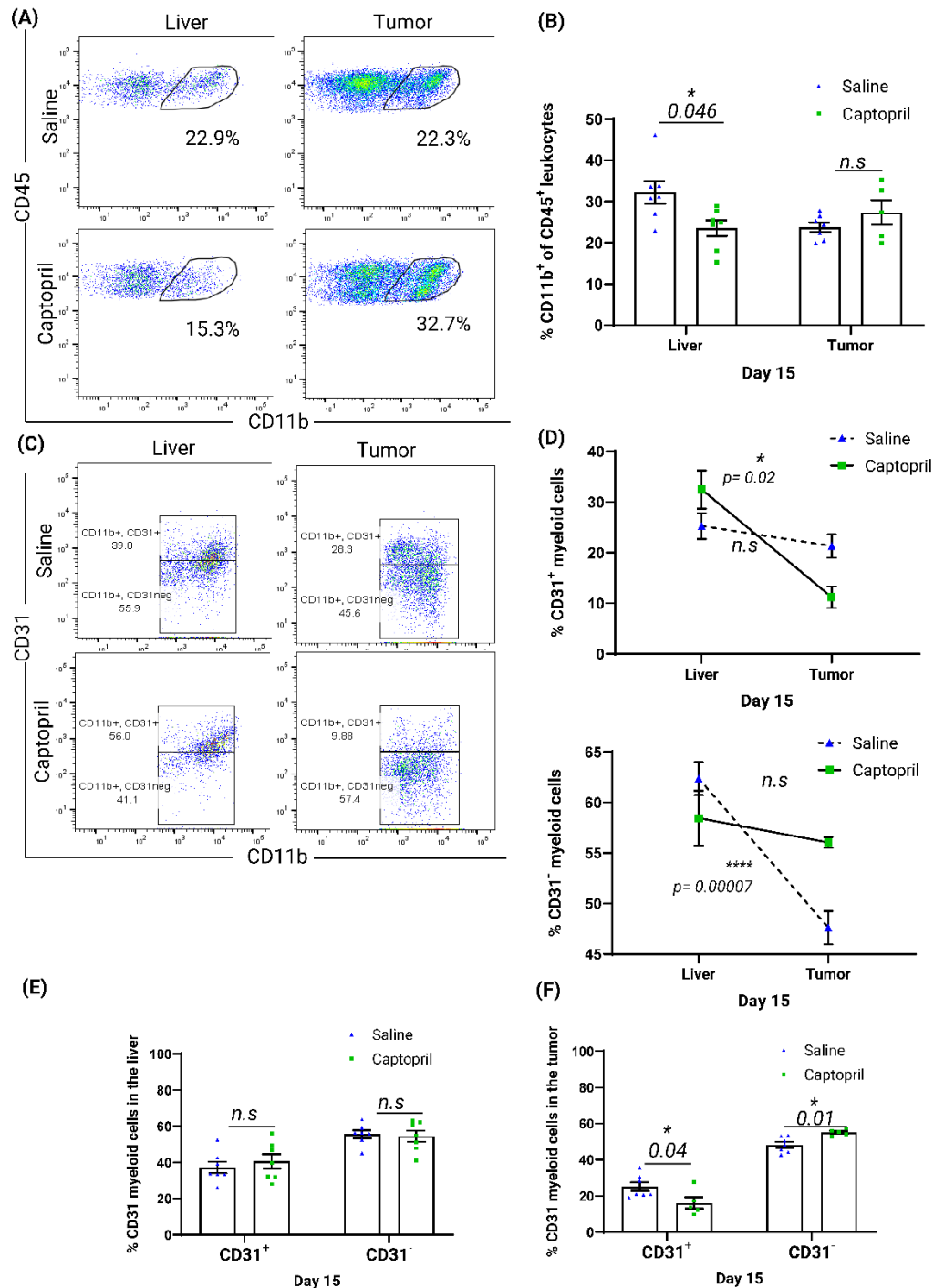


Figure 25. RASi differentially alters the proportions of CD11b⁺ populations in the liver and the tumour. (A) Gating strategy for analysis of myeloid cell population (CD11b⁺CD45⁺), and (B) quantitation of the proportion CD11b⁺ myeloid cells as a proportion of total CD45⁺ leukocytes in the liver and tumour tissues treated with captopril and saline at day 15. (C) Gating strategy for analysis of CD31 expression on myeloid cells (CD11b⁺CD45⁺) and (D) distribution of the proportion of CD31⁺ and CD31^{neg} expression in myeloid populations as a proportion of total myeloid cells in the liver and tumour at day 15. Quantitation of the proportion of CD31⁺ and CD31^{neg} expression in (E) the liver and (F) tumour. Data n≥5 presented as mean ± SEM. Significantly different data represented by * p<0.05 and ****p<0.0001.

4.3.4 Captopril treatment decreases the proportion CD31⁺F4/80⁻ subpopulation within the tumour

The changes in the proportion of CD31⁺ (**Fig. 26A, B**) and CD31⁻ (**Fig. 26C, D**) myeloid cells were further investigated based on the expression levels of F4/80 and their distribution within the liver and the tumour. Captopril does not alter the proportion of F4/80 of CD31⁺ myeloid cells in the liver (**Fig. 26B, upper panel**). Instead, captopril increases the proportion CD31⁺F4/80⁺ myeloid cells ($p= 0.001$), while significantly reducing the proportion CD31⁺F4/80⁻ myeloid cells ($p= 0.00004$) in the tumour (**Fig. 26B, lower panel**). A significant increase in the proportion of CD31⁻F4/80⁻ myeloid cells were found in the liver ($p= 0.002$) (**Fig. 26C, D upper panel**), whereas no significant difference in the proportion of F4/80⁺ or F4/80⁻ was observed CD31⁻ myeloid population in the tumour in the captopril treated group compared to saline (**Fig. 26C, D, lower panel**). In general, the frequency of CD31⁺F4/80⁺ myeloid cells was comparably lower than CD31⁻F4/80⁺ in both tissues in the captopril treated group (**Fig. 26A-D**). However, the most diminished population after captopril treatment was CD31⁺F4/80⁻ myeloid cells.

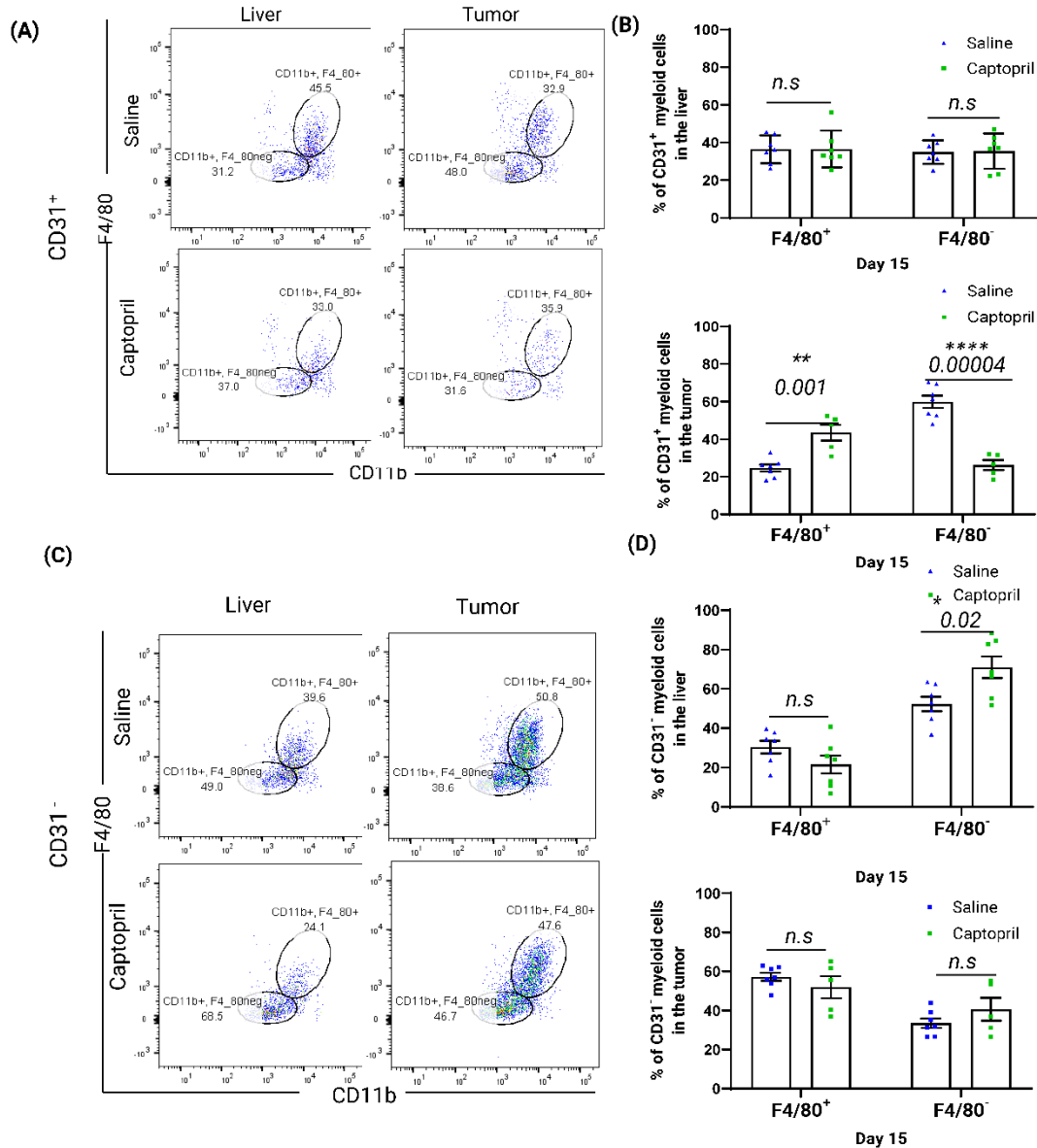


Figure 26. Captopril differentially alters the proportions of F4/80 expression of CD11b⁺CD31⁺ populations in the liver and the tumour. (A) Gating strategy for analysis of F4/80 expression in the CD31⁺ myeloid cells and (B) quantitation of the proportion of F4/80 as a proportion CD31⁺ myeloid populations in the liver and tumour treated with captopril and saline at day 15. (C) Gating strategy for analysis of F4/80 expression in the CD31⁻ myeloid cells and (D) quantitation of the proportion of F4/80 as a proportion of CD31⁻ myeloid cell in the liver and tumour tissues treated with captopril and saline at day 15. Data n≥5 presented as mean ± SEM. Significantly different data represented by * p<0.05, ** p<0.01 and ****p<0.0001.

4.3.5 Captopril treatment reduces PD-L1 expression on CD31⁺F4/80⁺ and CD31⁻F4/80⁺ myeloid subpopulations in the liver

PD-L1 is an inhibitory ligand that binds to PD-1 to suppress T cell activation (Ballbach et al., 2017) and is well-known to be expressed by M2-type MΦ inducing a tumour suppressive microenvironment (Tseng et al., 2018). To determine the immunosuppressive activity of CD31⁺ F4/80⁺ and CD31⁻ F4/80⁺ myeloid cells, we also stained for the expression of the checkpoint ligand PD-L1 (**Fig. 27A-D**). Captopril treatment decreased the expression of PD-L1 in both CD31⁺ F4/80⁺ ($p= 0.0009$) (**Fig. 27A, B upper panel**) and CD31⁻ F4/80⁺ myeloid cells within the liver (**Fig. 27C, D upper panel**) ($p= 0.0009$), whereas PD-L1 expression was not modulated by captopril within the tumour tissues (**Fig. 27B, D lower panel**). These results suggest that RASi influences the immunosuppressive activity of MΦ and KC in the liver.

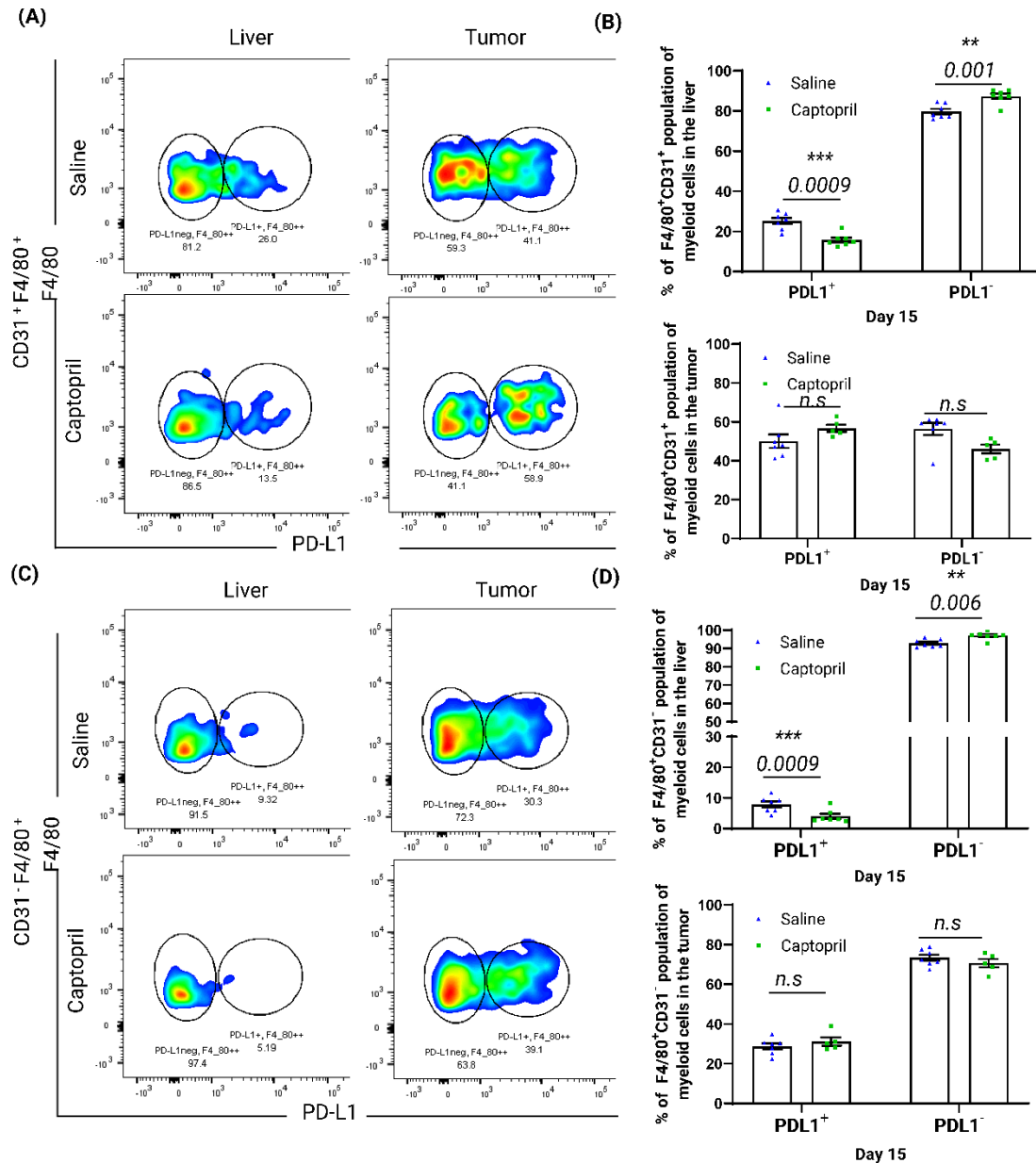


Figure 27. Captopril differentially alters PD-L1 expression on CD31⁺F4/80⁺ and CD31⁻F4/80⁺ myeloid subpopulations. (A) Gating strategy for analysis of PD-L1 expression of CD31⁺F4/80⁺ myeloid subpopulation and (B) quantitation of PD-L1 expression as a proportion of CD31⁺F4/80⁺ myeloid subpopulation in the liver and tumour tissues treated with captopril and saline at day 15. (C) Gating strategy for analysis of PD-L1 expression of CD31⁻F4/80⁺ myeloid subpopulation and (D) quantitation of PD-L1 expression as a proportion of CD31⁻F4/80⁺ myeloid subpopulation in the liver and tumour tissues treated with captopril and saline at day 15. Data n≥5 presented as mean ± SEM. Significantly different data annotated. Significantly different data represented by ** p<0.01 and ***p<0.001.

4.3.5 Captopril reduces the proportion of Ly6C^{high} monocyte-derived subpopulation in the liver and tumour tissues

M-MDSCs are defined as CD11b⁺Ly6G⁺Ly6C^{high} in tumour bearing mouse models (Bronte et al., 2016). The proportion of myeloid cells expressing Ly6C^{high} (CD31⁺F4/80⁺) in both the liver and tumour tissues were significantly diminished (**Fig. 28A, B**), whereas the proportion of myeloid cells expressing Ly6C^{low} (CD31⁺F4/80⁺) increased in the liver at day 15 (**Fig. 28B, upper panel**). These results suggest that RASi affects the development of myeloid-derived immunosuppressive populations in the liver that are later recruited by the tumour (**Fig. 28**). The CD31⁺F4/80⁺ was markedly depleted in the captopril treated group; therefore, accurate quantitation of Ly6C expression comparing saline to captopril treatment was not feasible.

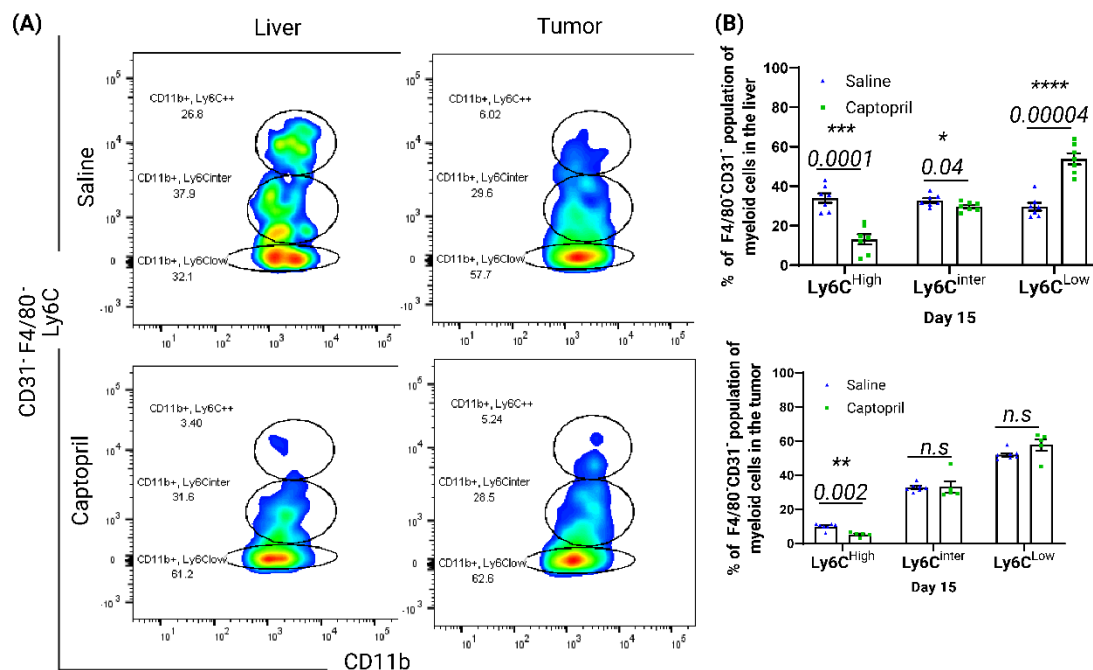


Figure 28. Captopril differentially alters the proportions of myeloid-derived suppressor cells in the liver and the tumour. (A) Gating strategy for analysis of Ly6C expression of CD31⁺F4/80⁺ myeloid cells and (B) quantitation of the proportion Ly6C as a proportion of CD31⁺F4/80⁺ myeloid cells in the liver and tumour treated with captopril and saline at day 15. Data n≥5 presented as mean ± SEM. Significantly different data represented by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

4.3.6 t-SNE analysis identifies and stratifies a discrete monocyte-derived subset targeted by Captopril in the tumour

Dimensionality reduction t-SNE analysis was performed using functions from the FlowJo™ t-SNE plugin (Belkina et al., 2019), explained in detail in chapter 2 (pages 40-42). t-SNE enabled the visualization of the entire sample population in a two-

dimensional density map in the form of cellular islands. The different parameters investigated in the flow cytometry analysis can be visualized by overlaying density plots, enabling specific cell clusters' characterisation. Furthermore, superimposing the 2-dimensional maps or t-SNE plots of the control and treatment data enables us to characterize cells that accurately change the treatment group.

A subset of 11,182 events was selected from captopril treatment, and 15,708 events were selected from the saline condition at random. 27,036 cells were concatenated into merged single expression matrix before t-SNE analysis using keyword-based or categorical gates (sample ID and treatment condition). These populations originated from 5 samples in the captopril treated condition and 7 samples in the saline tumour condition. t-SNE-guided manual gates were drawn based on observed boundaries of phenotypic marker expression for PD-L1, F4/80, Ly6C, CD45 and CD31. The t-SNE analysis plot's overlay displayed cellular islands derived from the captopril treatment shown in the contour plot in red and derived from the saline condition in light blue. The superimposed maps show a cellular island depicted in orange contour in the query gate, which was present only in the saline condition. The levels of expression of CD45, CD11b, CD31, PDL1, Ly6C and F4/80 are shown in the histogram panel comparing the expression of the “query” gate shown in orange colour, while the expression of the t-SNE overlay of the control is shown in light blue and captopril treatment in red (**Fig. 29A**). Using the Barnes-Hut implementation of t-SNE, it was displayed that this cellular island (shown in the orange histogram) referred to as the “query” subpopulation expresses high levels of CD45, intermediate levels of CD11b, CD31 and Ly6C, while shows low levels of F4/80 and PD-L1 when compared to the expression of the cellular islands present in the captopril (red histogram) and saline condition (blue histogram) (**Fig. 29A**).

t-SNE-guided manual gating cell frequency calculations were mapped into the t-SNE overlay of the captopril treatment and saline conditions. The frequency of the total of captopril treatment represented 41.4% (11,182 events), whereas saline condition was 58.1% (15,708 events) of the concatenated single expression matrix (**Fig. 29A**).

Next, using the same t-SNE overlay, we investigated the distributions of the frequencies of the populations within the query gate, representing 9.92% of the saline condition by sequentially using the same hand gating parameters (**Fig. 29**). The

stratification and identification of myeloid cells strategy using t-SNE are explained in **Figure 11**. The “*query*” subpopulation showed that the highest frequency were cells expressing CD11b⁺CD31⁺ (dot plot in light blue), which represented 21.1% of the concatenated events (**Fig. 29B**). CD11b⁺CD31⁻ (dot plot in orange) cells were mainly distributed outside the query gate with a frequency of 51.1%, and the ungated population shown in red did not show any expression of either of these markers (**Fig. 29B**). Subsequently, we gated F4/80 on CD31⁺, the highest frequency population on the query gate. The total frequency of (CD31⁺) CD11b⁺F4/80⁻ was 10.7% (2,889 events), mostly present within the query gate shown in bright green colour dot plot (**Fig. 29C**). (CD31⁺) CD11b⁺F4/80⁺ represented about 6.31% (1,705 events) shown in the orange colour dot plot (**Fig. 29C**). Lastly, the frequencies of the total of Ly6C high (dot plot in purple), intermediate (dot plot in turquoise), and low (dot plot in yellow) were 1.32%, 5.86% and 2.99%, respectively, gated on CD31⁺ myeloid cell island with the CD11b⁺F4/80⁻ (**Fig. 29D**). Altogether, these results showed that the subpopulation impacted by the captopril treatment was expressing CD31⁺ F4/80⁻ (**Fig. 29C**) with a high frequency of Ly6C^{intermediates} (5.86%) followed by Ly6C^{low} and Ly6C^{High} (**Fig. 29D**). The subpopulations expressing F4/80 gated on CD11b⁺CD31⁻ are shown in **Fig. 29E**, while Ly6C was gated on CD11b⁺CD31⁻ F4/80 illustrated in **Fig. 29F**.

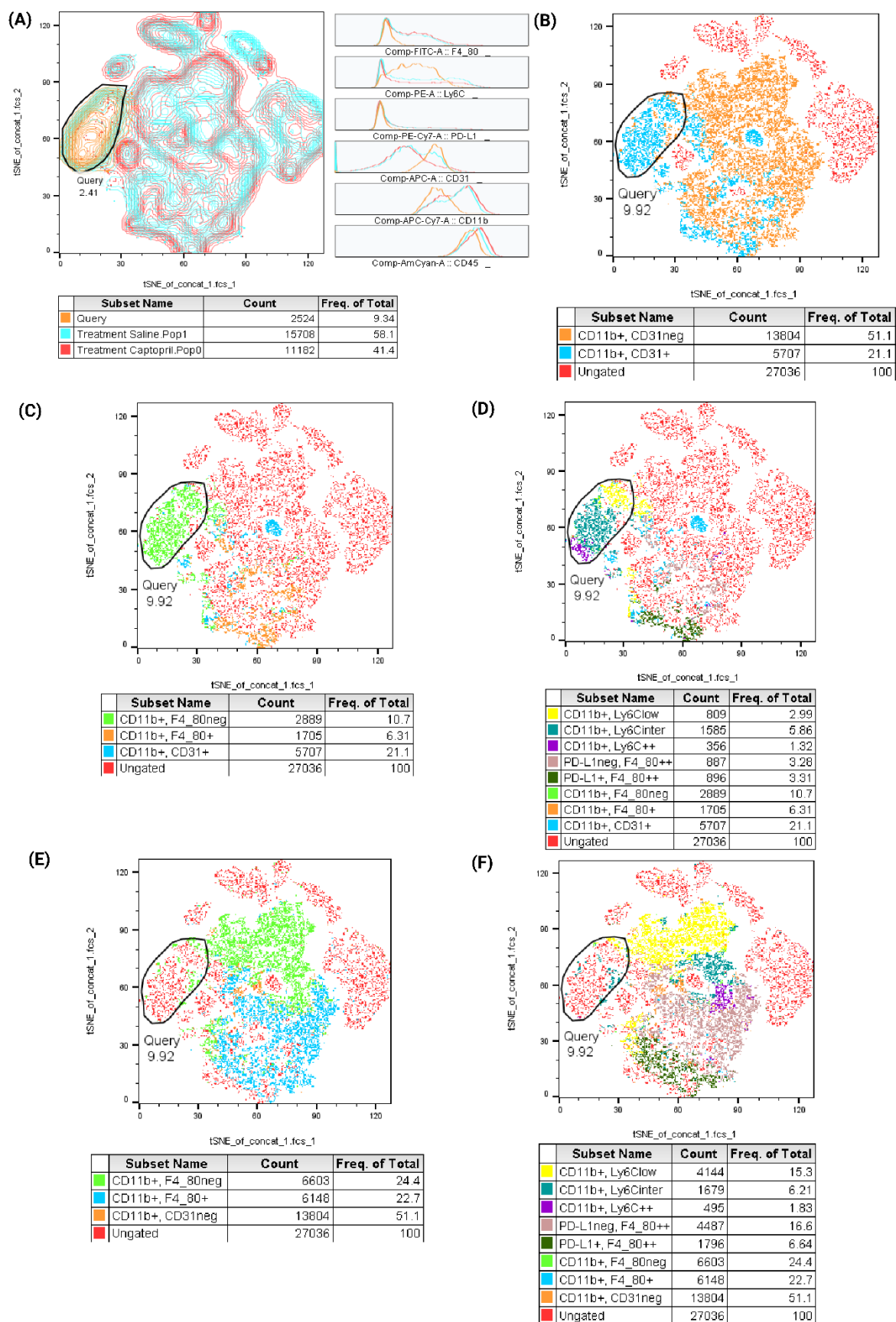


Figure 29. t-SNE analysis and cluster visualization for identifying and stratifying a distinct myeloid-derived suppressor subpopulation impacted by Captopril treatment in the tumour. (A) t-SNE overlay of the captopril (contour plot in red) and saline (contour plot in light blue) treatment conditions and histograms showed surface marker expression for F4/80, Ly6C, PD-L1, CD31, CD11b and CD45. Sequential t-SNE-guided visualization strategy shows (B) the distributions of the frequency of total CD11b⁺CD31⁺ (dot plot in light blue) and CD11b⁺CD31⁻ (dot plot in orange) mapped into the t-SNE overlay of the captopril and saline treatment conditions. (C) Distributions of the frequency of total CD11b⁺F4/80⁻ dot plot in bright green mapped into CD11b⁺ CD31⁺ myeloid cells. (D) Distribution of the frequencies of the total of Ly6C high (dot plot in purple), intermediate (dot plot in turquoise), and low (dot plot in yellow) mapped into CD11b⁺ CD31⁺ myeloid cells. (E) Distributions of the frequency of total CD11b⁺F4/80⁻ dot plot in bright green mapped into CD11b⁺ CD31⁻ myeloid cells. (F) Distribution of the frequencies of the total of Ly6C high (dot plot in purple), intermediate (dot plot in turquoise), and low (dot plot in yellow) mapped into CD11b⁺ CD31⁻ myeloid cells. Datasets are representative of 5 mice in the captopril treated tumour and 7 mice in the control tumour.

4.4 Discussion

In chapter 3, the immunologic effect of RASi on the spatiotemporal dynamics of specific T-lymphocyte subpopulations was demonstrated. Captopril treatment enhances trafficking patterns of T-cells into the surrounding liver and tumour inner margin, which impairs metastatic outgrowth and decreases tumour viability (D. L. Vallejo Ardila et al., 2020). Also, a previous study from our lab had demonstrated the role of TAMs in controlling CLM development (Wen et al., 2013). The current study explains the captopril (ACEi) effect on the tumoral myeloid subpopulations in mice with CLM. Herein, we have investigated the phenotypic composition of the myeloid cell compartment and the changes induced by captopril on their density, spatial and temporal distribution, and expression of co-inhibitory markers such as PD-L1.

The results indicate that the effect of captopril appears to subtly prompt the presence of myeloid cells expressing CD11c⁺ in the liver and tumour. CD11c⁺DC cells may migrate preferentially into the tumour and then also into secondary lymph node organs. ACE is significantly expressed on the surface of DCs (Danilov et al., 2003) when these cells are activated with a pro-inflammatory signal given by INF- γ in the infection context (Shen et al., 2011). Besides, ACE regulates the processing of MHC class I peptides presented by DCs for antigen recognition (Shen et al., 2011). The physiologic implications of ACEi on DCs in the context of tumour development require more investigation to understand the significance of their accumulation in the invasive

tumour margin and the lymph nodes. In contrast, a marked reduction of CD11b⁺ myeloid cells was observed in the liver.

ACE inhibition causes myelosuppression (Chisi et al., 1999) and reduces the differentiation of immature myelomonocytic lineage cells in mice (Cao et al., 2020; Lin et al., 2011). However, CD11b has been used to group various phenotypes of myeloid cells (Bronte et al., 2016), which allows delineating their origin in the liver (Yao et al., 2016; Y.-R. A. Yu et al., 2016) under steady-state condition (Eckert et al., 2015). The lack of specific markers to describe MDSC and to differentiate from other myeloid cells in normal tissues has caused significant confusion in current literature (Bronte et al., 2016; Yao et al., 2016). Hence, myeloid populations were separated according to the expression of CD31, as it has been previously proposed to determine their maturation status (Bronte et al., 2000; Connolly et al., 2010; Yang et al., 2004).

The results showed a significant decline in myeloid cells expressing CD31⁺ induced by captopril, while the relative proportion of CD31⁻ cells was preserved when comparing between the liver and the tumour. This may be attributed to a dual mechanism by which captopril inhibits tumour angiogenesis and reverses TME immunosuppression because CD31 is also expressed by endothelial cell progenitors (Farsaci et al., 2014). CD31 has been regarded to be the most sensitive and specific endothelial marker. However, endothelial cells can be identified based on the expression of CD31 and in the absence of hematopoietic (CD45) MΦ (F4/80) marker expression (Bailey et al., 2006). CD31 is also expressed on the surface of monocytes (Ling et al., 1997). Also, CD31 expressed on monocytes decreases their emigration from the bone marrow to the blood (Ling et al., 1997). The ligation of CD31 drives transendothelial migration and activates antiapoptotic signalling (Ferrero et al., 2003). Previous studies have associated CD31 myeloid cells with pro-angiogenic capacity in the tumour (Rivera & Bergers, 2015; Yang et al., 2004). Further phenotypic characterization of specific lineage markers is required to differentiate between the effect of RASi on endothelial progenitors and immature myeloid cells expressing CD31.

Unexpectedly, the frequency of CD31⁺ TAMs was markedly increased by captopril, which was conflicting criteria to define the maturation of MΦ based on the expression CD31. The surface expression of CD31 on TAMs had been previously described

(McKenney et al., 2001). Furthermore, CD31⁺ TAMs have shown distinct gene signature profiles from tissue or inflammatory MΦ in a melanoma mouse model and human breast cancer patients (Kim et al., 2013), which can potentially be used to predict prognosis or be used as a therapeutic cancer target (Kim et al., 2013). In that same study, CD31⁺ TAMs were reported to infiltrate mainly metastatic lymph nodes (Kim et al., 2013). Further characterization of the effect of captopril on TAMs expressing CD31 and changes to their migration pattern to the hepatic draining lymph nodes (celiac and portal lymph nodes) may offer insights into their role in tumour metastases.

A recent publication reports that the CD45⁺ F4/80^{high}CD31^{high} correspond to endothelial cells attached to the surface membrane of KC and that not even utilizing KC-specific cell markers like Tim4 and CLEC4f (Scott et al., 2016) was possible to differentiate between CD31^{high} and CD31^{low} populations (Lynch et al., 2018). In contrast, several studies described the presence of CD31⁺ not only on TAMs (Kim et al., 2013) but also on different stages of maturation of myeloid cells (Ling et al., 1997) like myeloid progenitor cells (Bronte et al., 2000). Also, CD31 is expressed by MDSC in the context of late sepsis (Brudecki et al., 2012; Dai et al., 2015), chronic hepatitis B virus infection (Chen et al., 2011) and myeloid-populations found in tumour-bearing mouse models (Connolly et al., 2010; Yang et al., 2004).

The expression levels of Ly6C⁺ in the CLM mouse model (High, intermediate, and low) were employed to define their immunosuppressive phenotype. Another study had characterized the frequency of MDSC, which consisted mainly of two distinct subpopulations: Ly6G⁺Ly6C^{low} granulocytic and Ly6G⁻Ly6C^{high} monocytic cells (Bronte et al., 2016), in ten different tumour bearing models of lung, breast, colon cancer, melanoma, and sarcoma (Youn et al., 2008). That study concluded that the immunosuppressive activity of MDSC in cancer depended on the specific phenotypic characteristics of these highly heterogeneous populations on each mouse model (Youn et al., 2008). Furthermore, M-MDSC are known to have a more effective mechanism of suppressing antigen-independent T-cell responses than G-MDSC (Youn & Gabrilovich, 2010). In our CLM mouse model, captopril markedly reduced MDSC expressing Ly6C^{high} (CD11b⁺CD45⁺CD31⁻F4/80⁻) in the liver and tumour, suggesting a mechanism by which RASi decreases TME immunosuppression and hinders tumour

growth. Ly6C^{intermediates} were also decreased in the captopril treated group within the liver, which suggests that RASi also targets the granulocytic myeloid cells. In contrast, Ly6C^{low} was increased exclusively in the liver by captopril, which may indicate their anti-inflammatory nature within the liver, as it has been previously reported that Ly6C^{low} monocytes promoted the resolution of injury and fibrosis (Ramachandran et al., 2012). Still refining phenotypic and functional characterization of Ly6G⁺Ly6C^{low} granulocytic lineage in the context of tumour development is required. Captopril treatment decreased PD-L1 expression in CD31⁺ MΦ and CD31⁻ MΦ, whereas the expression of PD-L1 on TAMs was not modulated by captopril, independently of the expression CD31. These results may suggest that RASi reduces the expression of co-inhibitory molecules on the surface of MΦ and KC, which may contribute to an immunostimulatory TME, since PD-L1 expression on F4/80⁺ MΦ denotes a change in their anti-tumour capability (Gonzalez et al., 2018). Intriguingly, a recent study showed that captopril induces M2-type marker CD206 expression at the cell surface of monocytes engaged in M1 MΦ differentiation *in vitro* (Medjebar et al., 2020). Moreover, the same study reported that ACEi combined with PD-1/PD-L1 inhibitor treatment was associated with poor response and an immunosuppressed tumour state caused by the increased expression of CD206⁺ MΦ in mice (Medjebar et al., 2020). Although captopril did not change the frequency on PD-L1⁺ TAMs, F4/80 positivity was significantly increased in the invasive margin in the CLM mouse model. Defining the polarization status MΦ and the co-expression of CD206 on CD31⁺ and CD31⁻ MΦ may help clarify the benefit of reducing PD-L1 expression.

The t-SNE-guided manual gating put a face and a name on the specific subset of tumour infiltrating myeloid population depleted after RASi treatment. A CD31⁺ monocyte-derived subset expressing F4/80⁻ Ly6C^{intermediate/High} was mapped onto the query gate absent in the captopril treated group. The t-SNE analysis projected the results obtained from the analysis of the 6-parameter flow-cytometry dataset. This workflow of analysis had been previously validated by a different study (Toghi Eshghi et al., 2019). The advantage of using t-SNE map topography in this study was that it enables the visualization and qualitative assessment of the boundaries between cellular island present or absent when overlapping the saline and captopril treated conditions. Further validation is required to quantitatively compare the cell frequency

determined by conventional plot-based and t-SNE-guided manual gating. Lastly, refining phenotypic and functional characterization of Ly6G⁺Ly6C^{low} granulocytic, KC (tissue-resident F4/80^{High}), and tissue infiltrating MΦ would enable a more detailed understanding of the effects of RASi on these subpopulations.

4.5 Conclusion

This chapter described the immunologic effect of RASi using captopril on the phenotypical composition of myeloid-derived populations expressing CD31 within the liver and tumour tissues. In addition, changes in the cell density of F4/80⁺ MΦ were compared between an early and late tumour development stage. RASi significantly reduces a CD31⁺monocyte-derived subset F4/80⁻ Ly6C^{intermediate/High}, which may have a pro-angiogenic and immunosuppressive function. To further determine the mechanisms by which RASi modulates the immunosuppressive activity of MDSC on activated T cells, MDSC and T cells isolated from the tumour can be co-cultured to measure marginal MDSC apoptosis staining with annexin V. Also, captopril treatment decreased the expression of PD-L1 in mature MΦ, which may reprogram them towards an immune stimulatory state. The significance of these findings needs to be further explored, particularly the effect of RASi on other MDSC like Ly6G⁺Ly6C^{low} granulocytic, KC (tissue-resident F4/80^{High}) and tissue infiltrating MΦ.

Chapter 5

Captopril treatment differentially modulates the immunoproteasome in mice with colorectal liver metastases

This chapter aims to assess the effects of captopril (ACEi) on the proteome of liver tissues from a mouse model of colorectal liver metastases using liquid chromatography-tandem mass spectrometry.

This chapter is presented as a regular thesis chapter which includes unpublished results.

CHAPTER 5

CAPTOPRIL TREATMENT DIFFERENTIALLY MODULATES THE IMMUNOPROTEASOME IN MICE WITH COLORECTAL LIVER METASTASIS

5.1 Introduction

In previous studies from our lab, it was shown that captopril (ACEi) significantly impairs tumour growth and tumour viability in the CLM mouse model (Neo et al., 2010; Neo et al., 2007; D. L. Vallejo Ardila et al., 2020). In chapter 3 and 4, the extensive evidence of the potent immunomodulatory function of ACEi during CLM development was provided, showing that T-lymphocytes (D. L. Vallejo Ardila et al., 2020) and myeloid cells infiltrating the TME were significantly modulated. It was hypothesised that additional factors involving both innate and adaptative immune response might contribute to captopril's anti-tumour activity in mice with CLM.

Contradictory findings of the role of captopril promoting tumour outgrowth in an orthotopic renal carcinoma model and its potential immunomodulatory highlight the relevance of using immunocompetent mouse models (Molteni et al., 2003; Wysocki et al., 2006). The anti-tumour properties of captopril were initially examined on immunocompromised *scid/scid* mice receiving a high dosage of the drug (Hii et al., 1998; Small et al., 1999). However, under these particular conditions, the influence of captopril on the mouse host immune response and immunogenicity of the tumour failed to be recognized (Wysocki et al., 2006). Another study later confirmed that captopril impaired the generation of antigen-specific CD8⁺ T cells and enhanced tumour infiltration of activated B-cells (Wysocki et al., 2006).

The most convincing aspect of the findings presented in previous chapters is that the animal experiments were conducted using a well-established immunocompetent orthotopic mouse model (Kuruppu et al., 1996). MoCR-CLM recapitulates the immunological features of CLM (D. L. Vallejo Ardila et al., 2020). Moreover, the dose-dependent effect of captopril to decrease tumour growth had been previously reported by our lab (Neo et al., 2007). In this sense, MoCR-CLM is a powerful immune-oncology model to study RASi modulation of immunosuppressive cells, including CD31⁺TAM expressing PDL-1 and MDSCs, as demonstrated in chapter 4. Other CRC animal models like MC38 have displayed similar immune cell composition of TME (Mosely et al., 2017). The caveat, however, is that the MoCR model uses the carcinogen DMH to induce primary colon carcinoma in CBA animals, which is commonly used to induce mutations primarily CRC that resembles human CRC (Newell & Heddle, 2004), similarly to the origin of CRC cell line MC38, which was isolated from a colon tumour in a

C57BL/6 mouse (Corbett et al., 1975). Chemically induced colon carcinogenesis leads to a high mutational burden (Hegde & Chen, 2020). Emerging evidence suggests that a high tumour mutational burden correlates with the presence of more neoantigens, thereby a better chance of triggering an immunogenic T cell response (Jardim et al., 2020). Another caveat to consider in using the MoCR-model for tumour immunology studies is the effect of splenectomy. The MoCR mice are splenectomised to prevent the local tumour growth after the intrasplenic induction. Previously Christophi's lab had studied the impact of the presence of spleen in the MoCR model would accelerate the kinetics of metastatic development, as observed when compared with the partial splenectomy model (unpublished data).

RAS signalling pathways are associated with stimulation of neoangiogenesis by increasing VEGF (Noble et al., 1993; Yoshiji et al., 2001; Yoshiji et al., 2002), potentiation of EGF-mitogenesis (Norman et al., 1987) and induction cellular oncogenes such as c-myc, c-fos, c-N-ras and TGF- β (Wolf & Neilson, 1990). However, signalling transduction pathways that may influence the efficacy of captopril on immune anti-tumour function have not yet been described.

LC-MS/MS-based proteomic analysis allows the study of transduction pathways with precision due to its high-throughput capability (Edmondson et al., 2002; Herring et al., 2015; H. Zhang et al., 2017). Herein, this method was implemented to investigate and quantitate the changes in the global proteome of liver and tumour tissues collected at the early angiogenic stage of metastases development due to captopril treatment in a systematic and unbiased manner.

5.2 Methods

For global proteomics, processing liver tissues containing micrometastases were separated into two groups: control (saline) and treatment (captopril). Tissue lysates were prepared for each sample included in the control and captopril group. Each group included five biological replicates. State of the art quantitative (LC-MS/MS) proteomic techniques on the QExactive plus Orbitrap mass spectrometer were used to determine relative protein abundance changes (**Figure 5**). Experimental validation was performed using IHC to measure the expression of immunoproteasome subunits on tumour tissues. The specific protein extraction procedures, liver lysates preparation,

quantitation of global proteome protocol and IHC procedures are also fully described in chapter 2 (**Global proteomics and bioinformatic analysis**, page numbers 43-45).

5.2.1 Statistical analysis

The global proteomics pre-processed data was analysed using MaxQuant version 1.6.5.0 to inspect raw data, identify, and quantify peptides features (Cox & Mann, 2008; Tyanova et al., 2015). Perseus version 1.5.1.5 was used for statistical analysis and data visualization of processed proteomics output (Tyanova et al., 2016). Welch's *t*-test was implemented to compare the differences in protein expression between captopril treated samples and saline-treated samples (control group). The cut-off for statistical significance was $p\text{-value} \leq 0.01$ and a Log_2 fold change ≥ 2 . Functional enrichment and protein-protein interaction network reconstruction were performed using STRING v11.0 (Szklarczyk et al., 2019). Integrative pathway analysis was computed with FunRich 3.1.3 and searched against the UniProt rodents database (Pathan et al., 2015).

The correlation matrix was generated using correlation coefficient *r* (or *rs*) for each pair of variables: total tumour positivity of CD11b⁺, CD11c⁺, F4/80⁺, CD3⁺, CD4⁺ and LMP7⁺ for the saline and captopril treated group. A heat map of R^2 calculating *p*-values (two-tail) was generated. Statistical analysis was conducted using GraphPad Prism® 8.3.0 (GraphPad Software, Inc., San Diego, CA) using both parametric and non-parametric analytical tests as appropriate. All statistical tests were two-sided, and a *p*-value of 0.05 was considered statistically significant.

5.3 Results

5.3.1 Impact of captopril treatment on the global proteome of liver tissues at day 15 post-tumour induction

We comprehensively compared the proteomes of captopril treated samples and control samples using LC-MS/MS-based methods and identified 2,964 proteins.

To identify proteins differentially regulated by captopril, the following criteria were utilized: 1) $p\text{-value} \leq 0.01$ and 2) Fold-change values: average increase or decrease of abundance by more than 50% ($\text{Log}_2 > 0.55$ for increase; $\text{Log}_2 < -0.55$ for decrease).

These criteria revealed that when captopril treated liver samples were compared to control liver samples, 13 differentially expressed proteins were found out of 2,964 analysed (p -value ≤ 0.01). Protein and gene names, Welch's t -test (Log_2) difference and cut-off values are described in **Table 5**.

Table 5. Differentially regulated proteins in the captopril liver tissues compared to saline liver tissues

Protein names	Gene names	$-\text{Log}_{10}$ Welch's T-test p-value	Welch's t-test Difference (Log_2)
Proteasome subunit beta type 5	<i>PSMB5</i>	2.20698	-1.82986
Zinc-binding alcohol dehydrogenase domain-containing protein 2	<i>ZADH2</i>	2.78857	-0.963341
ATP-dependent RNA helicase A	<i>DHX9</i>	3.33027	-0.898892
Carboxylesterase 1E	<i>CES1E</i>	2.1796	-0.768901
Proteasome activator complex subunit 1	<i>PSME1</i>	2.25697	0.51452
60S ribosomal protein L17	<i>RPL17</i>	2.08512	0.53331
Endoplasmic reticulum resident protein 44	<i>ERP44</i>	2.3679	0.55593
Actin-related protein 2/3 complex subunit 5	<i>ARPC5</i>	3.16243	0.66923
Proteasome subunit beta type 8	<i>PSMB8</i>	2.00005	0.71876
Histidine-rich glycoprotein	<i>HRG</i>	2.2277	0.80333
Hsp90 co-chaperone Cdc37	<i>CDC37</i>	2.15384	0.91788
Proteasome subunit beta type 10	<i>PSMB10</i>	2.19746	1.40429

Further information for each protein can be retrieved using STRING v11.0 (<https://string-db.org>) (Szkarczyk et al., 2019). The cut-off for statistical significance was p -value ≤ 0.01 and a Log_2 fold change ≥ 2 .

Captopril treatment significantly upregulated the following proteins: PSMB10, CDC37, HRG, PSMB8, ARPC5, ERP44, RPL7, PSME1; while downregulating the expression of CES1E, DHX9, ZADH2 and PSMB5 in the liver. The corresponding volcano plot is shown in **Fig. 30A**. Using STRING v11.0 (<https://string-db.org>) (Szkarczyk et al., 2019) to acquire protein-protein interactions networks, functional clusters were determined using active interaction sources, including text mining, experimental data, databases and co-expression limited to "*mus musculus*" (NCBI taxonomy Id: 10090). UniProt database UniProt (rodents) (<https://www.uniprot.org>) (Consortium, 2020) was used to retrieve functional annotations for each differentially regulated protein as described in **Table 6**. The interaction score of >0.4 was applied to predict functional interactions. K-means clustering algorithm calculated the distance matrix obtained from the STRING global scores determining that PSMB8, PSMB10, PSMB5 and PSME1 were

interacting proteins cluster “Proteasome” (CL: 7204; false discovery rate $1.25e^{-6}$), which had a higher global score part of the same functional cluster in the liver PPI analysis (**Fig. 30B**).

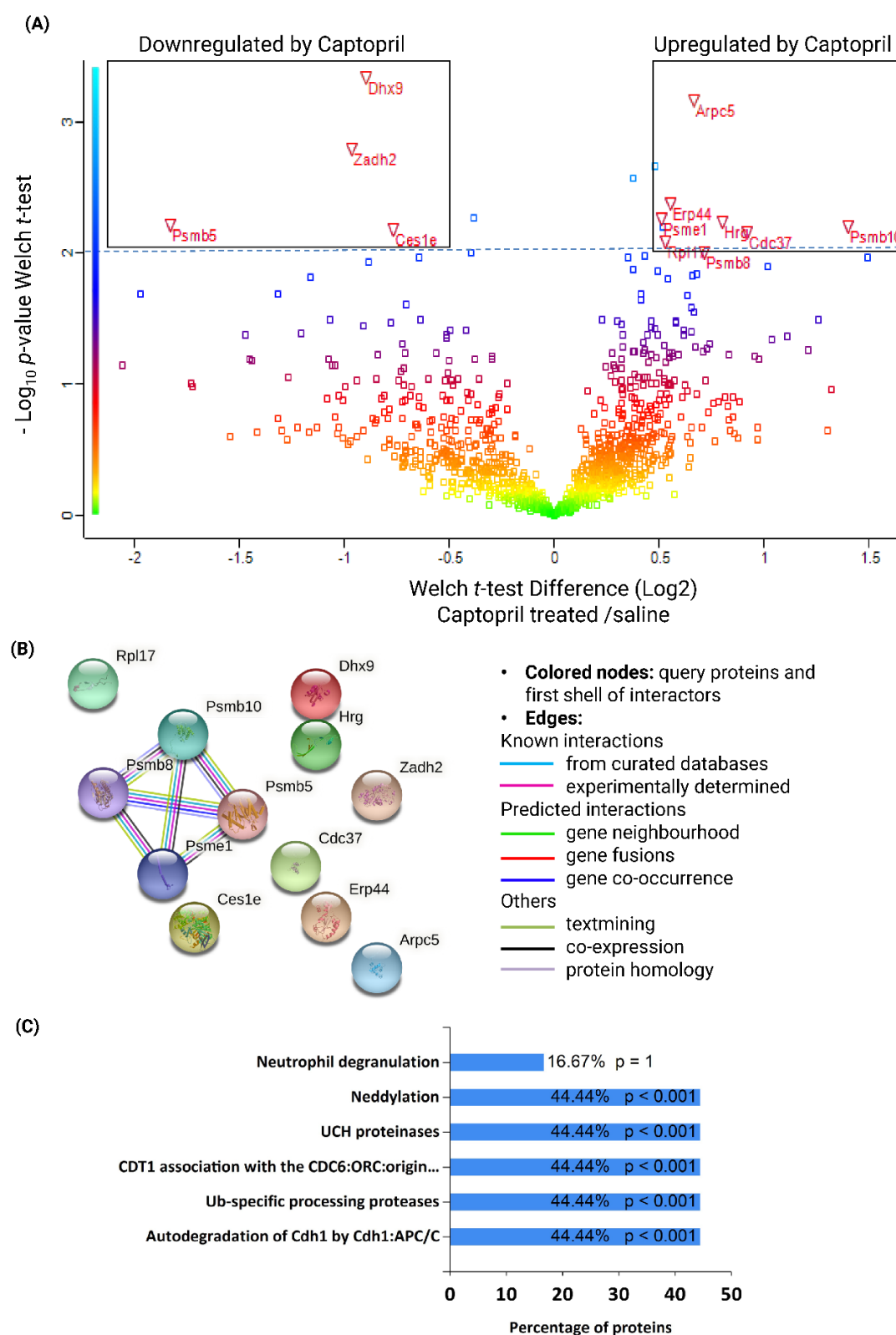


Figure 30. Quantitation of the changes induced by captopril in the global proteome of the liver. (A) Volcano plot showing average fold-change and p -value ≤ 0.01 for the comparison between captopril

treated group and control liver group at day 15 post-tumour induction (n=5). (B) Functional Enrichment using STRING showing protein-protein interaction network and *proteasome* functional cluster. (C) Integrative functional enrichment analysis of significantly regulated proteins in the liver tissues in captopril treated mice using Reactome pathways FunRich v 3.1.3 ®. Statistical significance threshold p -value ≤ 0.001 .

Table 6. Functional enrichments of proteins differentially regulated in captopril treated compared to saline liver tissues.

Protein names	Functional annotation
PSMB5	Component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins. The 26S proteasome plays a key role in the maintenance of protein homeostasis by removing misfolded or damaged proteins that could impair cellular functions.
ZADH2	Functions as 15-oxo-prostaglandin 13-reductase and acts on 15-keto-PGE1, 15-keto-PGE2, 15-keto-PGE1-alpha and 15-keto- PGE2-alpha with the highest efficiency towards 15-keto-PGE2-alpha. Overexpression represses transcriptional activity of PPARG (Peroxisome Proliferator-Activated Receptor Gamma) and inhibits adipocyte differentiation.
DHX9	Multifunctional ATP-dependent nucleic acid helicase that unwinds DNA and RNA in a 3' to 5' direction and that plays essential roles in many processes, such as DNA replication, transcriptional activation, post-transcriptional RNA regulation, mRNA translation and RNA-mediated gene silencing.
CES1E	Involved in the detoxification of xenobiotics and the activation of ester and amide prodrugs (562 aa).
PSME1	Implicated in immunoproteasome assembly and required for efficient antigen processing. The PA28 activator complex enhances the generation of class I binding peptides by altering the cleavage pattern of the proteasome (249 aa).
RPL17	Component of the large ribosomal subunit (184 aa).
ERP44	Mediates thiol-dependent retention in the early secretory pathway, forming mixed disulphides with substrate proteins through its conserved CRFS motif. Inhibits the calcium channel activity of ITPR1. May have a role in the control of oxidative protein folding in the endoplasmic reticulum.
ARPC5	Functions as component of the Arp2/3 complex involved in regulating actin polymerization and an activating nucleation-promoting factor (NPF) mediate the formation of branched actin networks.
PSMB8	The proteasome is a multicatalytic proteinase complex characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. This subunit is involved in antigen processing to generate class I binding peptides. May be involved in the inflammatory response pathway.
HRG	Plasma glycoprotein binds several ligands such as heme, heparin, heparan sulphate, thrombospondin, plasminogen, and divalent metal ions. Binds heparin and heparin/glycosaminoglycans in a zinc-dependent manner. Binds heparan sulphate on the surface of liver, lung, kidney and heart endothelial cells. Binds to N-sulphated polysaccharide chains on the surface of liver endothelial cells. Inhibits rosette formation. Acts as an adapter

	protein and is implicated in regulating many processes such as immune complex and pathogen clearance, cell chemotaxis.
CDC37	Co-chaperone that binds to numerous kinases and promotes their interaction with the Hsp90 complex, resulting in stabilization and promotion of their activity. Inhibits HSP90AA1 ATPase activity (By similarity).
PSMB10	The proteasome is a multicatalytic proteinase complex characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. This subunit is involved in antigen processing to generate class I binding peptides. It plays a role in determining the T-cell repertoire for antiviral T-cell response.

Further information for each protein can be retrieved using STRING v.11 (<https://string-db.org>) (Szklarczyk et al., 2019) and UniProt (rodents) (<https://www.uniprot.org>) (Consortium, 2020).

5.3.2 Functional enrichment analysis of differentially regulated proteins in liver and tumour tissues of captopril treated mice

Functional enrichment and integrative pathway analysis were performed using FunRich v.3.1.3 (<http://www.funrich.org/>) (Pathan et al., 2015). Signalling pathway information was retrieved from the Reactome pathway database (<https://reactome.org/>) (Fabregat et al., 2017). Integrative functional enrichment analysis of differentially regulated proteins identified (**Table 7**) and ranked as the most significant signalling pathways) modulated by captopril in mice (rodents) (p -value <0.001) with their respective stable identifier (SI) are shown in **Fig. 30C**.

Table 7. Reactome pathway analysis of proteins differentially regulated in captopril treated compared to saline liver tissues.

Reactome pathway	Functional annotation	Reference
CDT1 association with the CDC6 origin complex (SI: R-HSA-68827)	Participant of the assembly of the DNA pre-replication complex, in which the function of CDT1 (Cdc10-dependent transcript 1 protein) is regulated during the cell cycle by its tight association with an inhibitory factor, geminin.	(Wohlschlegel et al., 2000)
autodegradation of Cdh1 by Cdh1:APC/C (SI: R-HSA-174084)	Cdh1 (Cadherin-1, also known as CAM 120/80 or epithelial cadherin [E-cadherin]) is degraded by the Anaphase-Promoting Complex/Cyclosome (APC/C) ubiquitin ligase during in G1 and G0 cell cycle phases. This auto-regulation may contribute to reducing the levels of Cdh1 levels during these phases of the cell cycle.	(Listovsky et al., 2004)
UCH proteinases (SI: R-HSA-5689603)	Participant of the deubiquitinating pathway, in which the Ubiquitin C-terminal hydrolase (UCH) family of thiol proteases mediate protein-protein interactions.	(Reyes-Turcu & Wilkinson, 2009)
Ub-specific processing proteases (SI: R-HSA-5689880)	Involved in the detoxification of xenobiotics and the activation of ester and amide prodrugs (562 aa).	(Nijman et al., 2005)
Neddylation (SI: R-HSA-8951664)	Participant of the post-translational protein modification pathway, in which NEDD8 (neuronal precursor cell-expressed developmentally down-regulated protein 8) a ubiquitin-like molecule conjugates to substrate proteins through the ubiquitin ligase E1 to E3 enzyme cascade. The cullin scaffold subunit of cullin-RING E3 ubiquitin ligases (CRLs) is a target of neddylation, while CRLs themselves target numerous cellular proteins for degradation by the proteasome.	(Soucy et al., 2010)

Further information for each protein can be retrieved using Reactome pathway database (<https://reactome.org/>) (Fabregat et al., 2017).

5.3.3 RASi modulates the expression of immunoproteasome subunits

The results obtained indicate that RASi regulates the immunoproteasome functional clustered (PSMB8, PSMB10, PSMB5 and PSME1), which is well-known to specifically degrade proteins to generate antigenic peptides for the presentation to immune cells (Neefjes & Ovaa, 2013). The immunoproteasome encompasses the substitution of three catalytic subunits of the constitutive proteasome, including β 1 (PSMB6), β 2

(PSMB7), and $\beta 5$ (PSMB5), which are constitutively expressed in all cells (Aki et al., 1994). These subunits are also known as inducible subunits, which corresponds to $\beta 1i$ (LMP2, low molecular weight protein 2, PSMB9), $\beta 2i$ (MECL1, multicatalytic endopeptidase complex-like 1, PSMB10), and $\beta 5i$ (LMP7, low molecular weight protein 7, PSMB8), respectively (Aki et al., 1994). Furthermore, the replacement of PSMB5 by PSMB8 enhances the immunoproteasome function to generate proteasomal cleavage products and boosting a peptide-specific CD8⁺ T cell response (Ebstein et al., 2016). To further examine the pattern of expression of PSMB5 and PSMB8, tumour and adjacent liver parenchyma collected at day 15 were assessed using immunofluorescence. The qualitative assessment of expression of these markers indicated that hepatocytes most likely expressed PSMB5 in the liver parenchyma, but it was not expressed in tumour cells (**Fig. 31A**). In contrast, PSMB8 was mainly expressed in the tumour (**Fig. 31B**).

IHC was applied to confirm whether captopril modifies PSMB8 (LMP7) expression within the tumour (**Fig. 31A, B**). It was identified different staining intensity of PSMB8 (LMP7) within the tumour (**Fig. 32A, C**), which indicates a trend of increased expression of PSMB8 (LMP7) in the captopril treated group (**Fig. 32D**). The expression of the PSMB8 can be found on DCs, M Φ , activated T-lymphocytes (Krüger & Kloetzel, 2012) and on solid tumours (Kalaora et al., 2020; Kiuchi et al., 2020; Rouette et al., 2016; Tripathi et al., 2016). To define the specific immune cell type that may be expressing PSMB8, a Pearson's correlation was employed to identify which other cells infiltrating the tumour would indicate a correlation with PSMB8 (LMP7) (**Fig. 32E**). The findings indicate that only CD3⁺ T lymphocytes negatively correlates with LMP7⁺ ($r=-0.15$, p -value = 0.169). In addition, after captopril treatment this inverse correlation becomes significant and stronger ($r=-0.32$, p -value = 0.05), similarly to the relationship between LMP7 and CD11c tumour region positivity (saline $r=0.03$; captopril $r=-0.25$) (**Fig. 32E**). Altogether these findings suggest that the correlation between the expression of PSMB8 (LMP7⁺) and CD3⁺ T cells, CD4⁺ T cells, F4/80⁺ M Φ , CD11b⁺ cells and CD11c⁺ cells is weak; therefore, it is likely that the expression of PSMB8 is not prominent in T cells and myeloid cells such as F4/80⁺ M Φ and CD11c⁺DC within the total area of the tumour as well as it is not significantly modulated by captopril treatment.

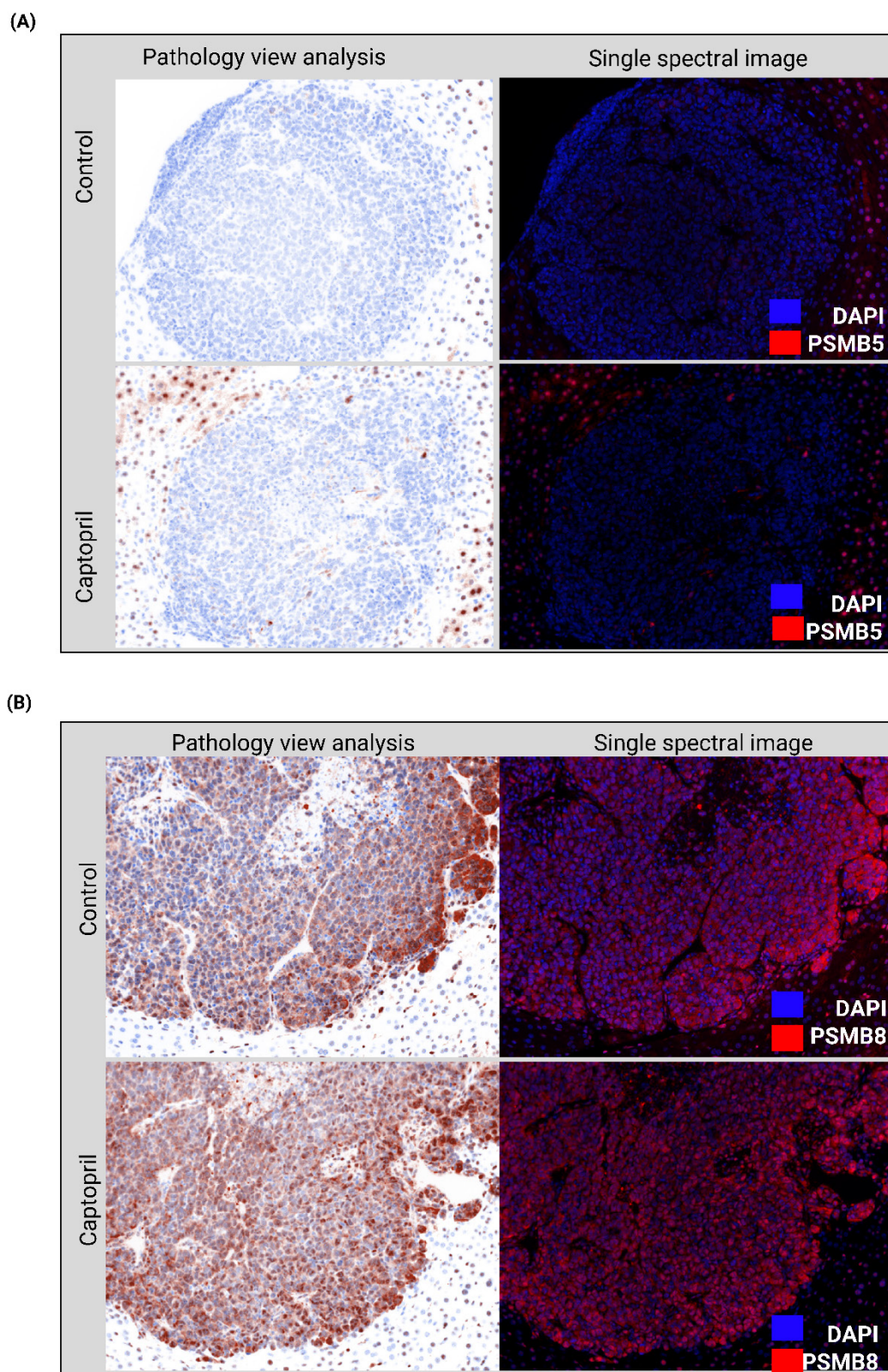


Figure 31. The pattern of expression of PSMB5 and PSMB8 in liver and tumour tissues. Qualitative assessment using mIHC defines the precise location of immunoproteasome subunits PSMB5 and PSMB8 in liver and tumour tissues of control and captopril treated mice. FFPE sections were stained by TSA OPAL 620 (red colour) and DAPI. (a) Nuclear expression of PSMB5 in hepatocytes is shown in red (housekeeping), and (b) cytoplasmic expression PSMB8 (antigenic peptide generation) of tumour cells is shown in red using immunofluorescence.

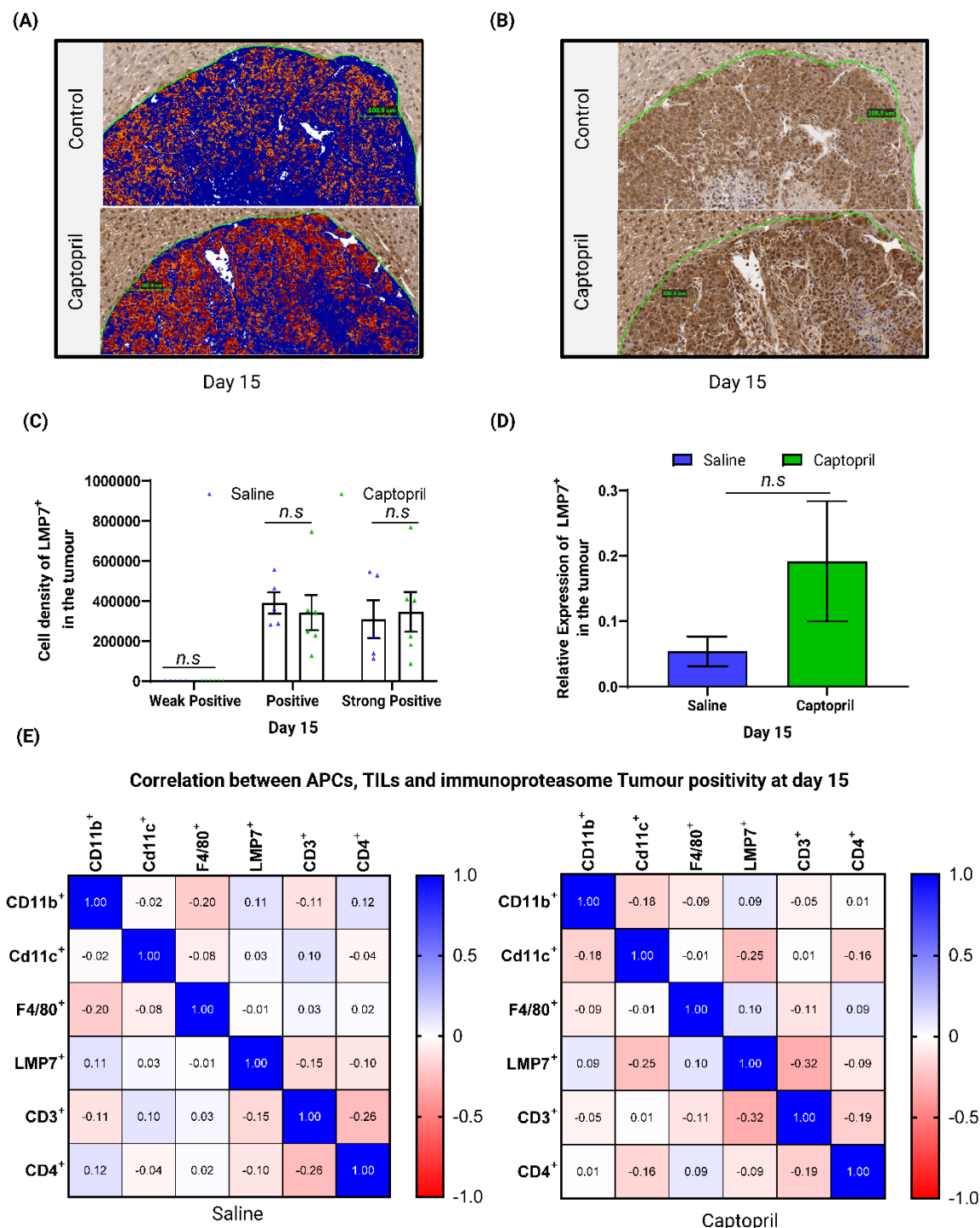


Figure 32. The effect of ACEi in the expression of PSMB8 (LMP7) in the tumour region. Representative microphotographs of IHC LMP7⁺ (a) annotated intensity and (b) marked-up of tumour area at day 15 post-tumour induction (Scale bar = 100 μ m measured from the edge of the tumour in direction to the core). (c) The cell density of LMP7⁺ measured by the intensity of expression as follows: weak positive (in yellow), positive (in orange), and strongly positive (in red) in the total area of the tumour and (d) The effect of captopril treatment against saline on the relative expression of LMP7⁺. (e) Correlation matrix using Pearson r to determine the correlation between tumour expression of CD11b⁺, CD11c⁺, F4/80⁺, LMP7⁺, CD3⁺ T Lymphocytes and CD4⁺ T cells. coefficient r (or rs) are shown for each pair of variables for total tumour positivity. A heat map of R² calculating p-values (two-tail) was generated. Datasets expressed as mean $\pm$ SEM with n $\geq$ 5 mice for each group (equivalent >100 tumour regions in each group) excluding tumour region values outliers ROUT (Q=1%). Significantly different data is annotated.

5.4 Discussion

In this chapter, the effects of RASi on the global proteome of liver tissues at day 15 post tumour induction were assessed. Captopril significantly induced the differential expression of 13 proteins in the liver. Furthermore, clustering and functional enrichment showed that captopril modulates the expression of some of the immunoproteasome subunits, including PSMB8, PSMB10, PSMB5 and PSME1. Several studies have investigated the role of RASi in restoring immune regulation of the TME (Nakamura et al., 2018; D. L. Vallejo Ardila et al., 2020; Wen et al., 2013). T-cell mediated anti-tumour responses require that the immunoproteasome processes MHC-I immunogenic peptides and their presentation by MHC-I on the surface of tumour cells (Lee et al., 2020). The immunoproteasome was thought to be constitutively expressed only by DCs, MΦ and activated T-lymphocytes (Krüger & Kloetzel, 2012). However, recent reports showed that different expression levels could be found on solid tumours and hematologic malignancies (Kalaora et al., 2020; Kiuchi et al., 2020; Rouette et al., 2016; Tripathi et al., 2016).

In the liver tissues treated with captopril, a significant upregulation of PSMB8, PSMB10 and PSME1 were found, while PSMB5 was downregulated. The replacement of constitutive proteasome (PSMB5) for the inducible immunoproteasome (PSMB8, PSME1 and PSMB10) is increased in most cancer types (Rouette et al., 2016). The efficacy of RASi on T-cell anti-tumour function, as was suggested by our previous study (D. L. Vallejo Ardila et al., 2020), may be facilitated by the overexpression of inducible immunoproteasome subunits, particularly PSMB8 and PSMB10. These results indicate that upregulation of the immunoproteasome subunit PSMB8 by captopril treatment may enhance the generation and presentation of antigenic peptides.

On the one hand, immunoproteasome deficiency or low expression of *PSMB8* and *PSMB9* has been associated with recurrence and metastasis in NSCLC patients because this results in a substantially reduced repertoire of HLA-bound peptides and a mesenchymal phenotype (Tripathi et al., 2016). On the other hand, the overexpression of *PSMB8* and *PSMB9* is a strong predictor of better survival and improved response to immune check inhibitors in melanoma patients (Kalaora et al., 2020). Also, it has been correlated with an increased level of CD8⁺ TILs, MΦ and IFN-

γ secretion (Hof et al., 2019). Overall, the weak correlations between PSMB8 (LMP7) tumour expression and CD11b⁺, CD11c⁺, F4/80⁺, CD3⁺ T cells, and CD4⁺ T cells may suggest that any of these immune cells do not predominantly express this immunoproteasome subunit. In the β5i-deficient mice, most of the immunogenic peptides examined were presented by β5i-deficient DCs (Fehling et al., 1994), whereas in triply deficient (β1i, β2i and β5i) mice DCs had notorious defects in presenting several MHC class I epitopes, weakened CD8⁺ T cell responses (Kincaid et al., 2012). In the case of antigen-presenting cells, CD11c⁺ a stronger inverse correlation with PSMB8 (LMP7) was found after captopril treatment, suggesting that the pattern of expression of PSMB8 (LMP7) decreases DC density. Another proposed mechanism impacted by the deficiency or inhibition of PSMB8 is the production of proinflammatory cytokines generated by T helper cells (Th1/Th17), including IL-6, TNF, IL-17 and IL-23, which promote tumour progression in mice with CRC (APC^{Min/+} mouse model) (Koerner et al., 2017). The implications of the upregulation of PSMB8 on antigen presentation, CD8⁺ T cell activation and the functions of T helper cells need to be further elucidated in the CLM mouse model, particularly considering that chemical-induced carcinogenesis associates with a high mutational burden (Hegde & Chen, 2020), which increases the probability of having a larger repertoire of immunogenic tumour antigens (Germano et al., 2017).

Intriguingly, several studies have evaluated the effects of proteasome inhibitors to treat hematologic malignancies and not proteasome activators (Chhabra, 2017; Siegel et al., 2012). Since then, multiple proteasome inhibitors have undergone clinical trials. Nevertheless, immunoproteasome inhibitors have been considered safer since only overexpressing immune cells would be targeted. ONX-0914 has been used on preclinical trials to inhibit β5i (LMP7) and mitigating CRC progression (Koerner et al., 2017). Novel proteasome activators improve the proteasome activity by inducing 20S gate opening and suppressing the proteotoxic effect of the different protein-aggregates (Thibaudeau et al., 2018). The human 20S proteasomes and the immunoproteasome of melanoma cells are characterized by the partial replacement of only one inducible subunit or two at the time of the immunoproteasome assembly (Guillaume et al., 2010). Emerging evidence suggests that the generation of antigenic peptides is organ-specific. It involves cooperative function between the constitutive,

the intermediate, and the immunoproteasome (Guillaume et al., 2010), which needs to be considered when targeting the immunoproteasome in the context of CLM using RASi. In general, there was no detectable expression of PSMB5 (constitutive immunoproteasome) within the tumour, whereas PSMB8 (inducible immune proteasome) was expressed by tumour cells, which may suggest a complete replacement of $\beta 5$ constitutive proteasome. PSMB10 (Larsen et al., 1993) and PSME1 are required for efficient antigen processing (Murata et al., 2001). High expression of genes associated with antigen processing machinery (*B2M*, *CALR*, *NLRC5*, *PSMB9*, *PSME1*, *PSME3*, *RFX5*, and *HSP90AB*) was associated with therapeutic response to checkpoint inhibitors, better progression-free survival and OS in NSCLC and melanoma patients (Thompson et al., 2020). Increased expression of antigen processing genes was also associated with T-cell inflamed status (Thompson et al., 2020). Moreover, cytotoxic CD8⁺ T cell response and tumour recognition are altered by the loss or downregulation of antigen processing machinery genes (Pereira et al., 2017). The changes in the expression of PSMB10 and PSME1 induced by captopril and their implications in the tumour immune response in CLM requires further experimental validation.

The differential regulation induced by captopril was inferred to occur via neddylation, proteasomal ubiquitin-independent and ubiquitin-dependent manner. Reactome pathway analysis is predicted to be related to neddylation, a post-translational modification that adds a ubiquitin-like protein NEDD8 to substrate proteins (Kamitani et al., 1997) such as cullin subunits of CRLs. CRLs regulate cell cycle progression, gene transcription, apoptosis, and DNA replication; thereby, the rationale of targeting CRLs cancer-associated components known as oncogenes (e.g., SKP2 and Cul4A) has been previously explored (Zhou et al., 2018). Additionally, overactivation of the neddylation pathway increases tumour suppressor degradation (Zhou et al., 2018). Neddylation inhibition by MLN4924 (Pevonedistat) reported promising anti-tumour effects via cell cycle arrest, apoptosis, senescence, and autophagy in a context-dependent manner (Zhou et al., 2018). Other signalling pathways related to RASi immunoproteasome activation involve CDT1 association with the CDC6 origin complex (Borlado & Méndez, 2008), proteasomal ubiquitin-independent (Tsvetkov et al., 2010) and proteasome-mediated ubiquitin-dependent protein catabolic process

(Voutsadakis, 2008), which are well-known to contribute to carcinogenesis. The captopril-induced modulation of protein degradation pathways, together with increased immune proteasomal expression, may improve the efficiency of tumour antigen processing, in addition to processing unfolded and possibly dysfunctional proteins that characterise cancer cells, thus relieving their cytotoxic pro-inflammatory side effects.

5.5 Conclusion

This chapter explored the global proteasome changes after captopril treatment at an early stage of tumour development. Captopril differentially regulated the immunoproteasome cluster, specifically upregulating the expression of PSMB8 while downregulating the constitutive expression PSMB5 within the tumour. In addition, functional enrichment inferred that the signalling pathways modulated by captopril were neddylation, proteasomal ubiquitin-independent and proteasome-mediated ubiquitin-dependent protein catabolic processes. Although much remains to be determined about the phosphoproteome and phosphosites that need to be experimentally validated, these findings correlate with the immunomodulatory effect of RASi on T-cell anti-tumour function.

Chapter 6

Spatial Phenotypic Immune Profiling of Colorectal Liver Metastases in the context of Histologic Growth Patterns

This chapter aims to characterize the T cell, α -smooth muscle actin (α -SMA) and E-cadherin, spatial distribution, activation, and spatial engagement within the CLM landscape using multiplex immunohistochemistry to determine whether the tumour growth pattern influences immune cell infiltration.

Justification: In previous chapters using the MoCR-CL mouse model was hypothesized that RASi would have a similar immunomodulatory effect on the TME of human CLM. A prospective human study was not feasible within the term of a PhD candidature. For that reason, archival human CLM samples provided by VCB were utilized. This chapter describes the spatial phenotypic profiling of human CLM using multiplex immunohistochemistry and a machine-learning-based algorithm to determine whether the tumour growth pattern influences immune cell infiltration.

This chapter contains the extended version of the manuscript submitted to, as per the following title:

- **Spatial Phenotypic Immune Profiling of Colorectal Liver Metastases in the context of Histologic Growth Patterns**

Due to copyright obligations, the author submitted a version of this manuscript as appendix 3.

CHAPTER 6

SPATIAL PHENOTYPIC IMMUNE PROFILING OF COLORECTAL LIVER METASTASES IN THE CONTEXT OF HISTOLOGIC GROWTH PATTERNS

6.1 Introduction

Colorectal cancer is the second leading cause of cancer death worldwide, overwhelmingly due to liver metastases (Keum & Giovannucci, 2019). Approximately 25% of CRC patients will be diagnosed with liver metastases during the disease (Engstrand et al., 2019). Surgical resection is considered the only curative treatment option for colorectal liver metastases that offers long-term survival (Kanas et al., 2012). Several prognostic factors have been associated with patient outcome after surgical resection, including tumour size, the number of lesions, tumour progression after chemotherapy, preoperative serum carcinoembryonic antigen (CEA) levels, lymph node status of the primary tumour, disease-free interval from the resection of the primary tumour to the occurrence of liver metastases and the presence of extra-hepatic metastasis (Engstrand et al., 2018; Vera et al., 2019). Clinical risk scoring (CRS) systems employ various combinations of these clinical variables to predict a CLM patient's survival (Fong et al., 1999; Rees et al., 2008). Yet, survival differs between patients with the same CRS score (Wang et al., 2018), which means that a more comprehensive assessment based on better predictive factors is needed.

To this end, several clinical studies have characterized the prognostic value of T-lymphocytes to predict prognosis after liver metastasectomy, with promising results (Halama et al., 2011; Hof et al., 2019; Katz et al., 2013; Wang et al., 2018). In a study of tumour infiltrating CD3⁺ and CD8⁺ T cells in 249 patients with CLM, the immunescore was internally validated for relapse-free survival (RFS) and overall survival (OS) and concluded that CLM patients with high immunescore had significantly longer RFS than those with low immunescore (Rees et al., 2008). Another study on regulatory T cell infiltration found a high proportion of CD4⁺ or CD8⁺ T lymphocytes expressing FoxP3⁺ predicted poor outcome following CLM resection. However, a systematic review on the prognostic value of T lymphocytes (CD3⁺, CD4⁺ and CD8⁺), which analysed high-throughput studies of resected CLM, could not arrive at any conclusions due to the diverse and heterogeneous patient population (Halama et al., 2011). Another systematic review reported no association between CD4⁺ and CD8⁺ T-cell density with OS (Kong et al., 2019). Other studies showed that the intratumoral presence of tissue-resident memory CD8⁺ T cells (T_{RM}), expressing the integrin CD103⁺, has been strongly correlated with favourable prognosis (Dumauthioz

et al., 2018), independently of the infiltration of CD8⁺ T cells in several malignant diseases, including ovarian, lung, endometrial, and breast cancers (Djenidi et al., 2015; Ganesan et al., 2017; Koh et al., 2017; Wang et al., 2015; Webb et al., 2014). Overall, T lymphocytes include several subtypes of T cells at different stages of activation and differentiation; hence the frequencies, spatial distribution and engagement with other cells in their vicinity could be more informative predictors of patient outcome, and it could aid the current stratification of CLM patients for available therapies and particularly immunotherapies (Taube et al., 2020).

Recent reports highlight the importance of integrating spatial and temporal dynamic changes of tumour infiltrating T cells (Bindea et al., 2013), including studies in CLM (Angelova et al., 2018; Berthel et al., 2017; Halama et al., 2011; Wang et al., 2018). Furthermore, the spatial engagement of T cells with neighbour stromal, other immune or tumour cells can provide insights into their state of activation and function (Lazarus et al., 2018). For example, T cells expressing the integrin CD103 were shown to bind on to E-cadherin, an epithelial cell junctional protein, enabling their retention and activation within the infected tissue (Hofmann & Pircher, 2011; Shields et al., 2019).

Patterns of tumour immune cell infiltration may be correlated by the CLM histologic growth pattern (Nielsen et al., 2014). CLM can be categorized into three distinct histologic growths patterns that impact disease progression and patient outcome after surgical resection (Fernández Moro et al., 2018; Nielsen et al., 2014; Pinheiro et al., 2014). Growth patterns in CLM are defined by the liver-tumour interface morphology (van Dam et al., 2017). The most frequent growth pattern reported is the replacement, followed by the desmoplastic and, finally, the pushing pattern (Fernández Moro et al., 2018). Overlaps of these patterns have also been observed (Van den Eynden et al., 2012), and the exact definitions of histopathological criteria varies among different studies (Serrablo et al., 2016; Wiggans et al., 2013). Desmoplastic tumours have been reported to have significantly higher levels of lymphocytic infiltrate compared to the replacement and pushing pattern tumours (Nielsen et al., 2014). However, phenotypic or spatial immune profiles in the different growth patterns have not been determined.

T lymphocytes' spatial phenotypic immune profiling may reveal important insights to CLM development and prognostic treatment outcome. Additionally, a detailed

characterization of how the CLM growth pattern may dictate T cell spatial distribution has never been reported to our knowledge. Thus, in this study, we aimed to unveil the T cell spatial distribution in CLM using multiplex immunohistochemistry and automated image quantitation analysis, which has proven to be a powerful investigative tool to enable spatial and phenotypic characterization of immune and stromal cells in the TME (Blom et al., 2017; Halse et al., 2018; Lazarus et al., 2018; Stack et al., 2014; Taube et al., 2020).

6.2 Methods

A 6-plex OPAL™ protocol was used to assess the composition and spatial distribution of T cell markers CD3, CD8 and CD103 and the EMT markers α -SMA and E-cadherin. The protocol was manually optimized and validated in a cohort of twenty-two CLM whole tissue specimens using well-established antibodies, single spectral library, negative controls, and biological controls for corroborating staining pattern of following the recommendations on best practices for mIHC (Masucci et al., 2016; Taube et al., 2020). The staining patterns of α -smooth muscle actin (α -SMA) were tested on sclerosed hemangiomas, a tissue enriched with blood vessels and desmoplastic fibrotic tissue in the liver, to better differentiated from the expression of smooth fibroblasts and CAF. The pattern of infiltrations was compared between normal liver tissue and CLM. The specific experimental definitions and conditions such as antibodies used, the mIHC staining procedures, image analysis and cell density quantitation, spatial tumour region delineation and distribution of positively stained cells are fully described in Chapter 2 (page numbers 41-46).

6.2.1 Survival analysis and Kaplan-Meier (KM) curves

The patient's survival post-liver resection was interrogated in two separate univariate analysis. First, we investigated if the median distribution of cell count for each phenotype had any predictive value in the IM and TC. Secondly, if the spatial engagement profile of each phenotype: CD3⁺ T Lymphocytes (CD3⁺ CD8⁻ CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺), T_{RM} (CD3⁺CD8⁺ CD103⁺), non-T cells expressing CD8⁺ or

CD103⁺ and cells expressing α -SMA or E-cadherin), to other phenotypes could be predictive of patient outcome according to the median distribution distance.

6.2.2 Statistical Analysis for tissue segmentation distribution (cell count/HPF)

The data was not normally distributed (test for normality using Shapiro-Wilk test); therefore, non-parametric analysis was employed. Mann-Whitney U test was implemented for comparison between two groups (independent group t-test equivalent). Statistical analysis was performed using GraphPad Prism Statistics version 8.3 (GraphPad Software, Inc., San Diego CA).

6.2.3 Code availability

Customized R functions to assess spatial analyses based (on the x, y coordinates) data provided by inForm® software v2.4.2 (Perkin Elmer/Akoya, MA) are available in **Appendix 4**. The ISAT package is available online (Wang, 2018).

6.3 Results

6.3.1 Spatial tumour region delineation and distribution of positively stained cells

Twenty-two CLM specimens were collected from patients (19 males and 3 females, mean age 61 years) who submitted to liver resection at a Tertiary hospital (**Table 8**). FFPE whole tissue sections from the resected specimens from the entire CLM cohort were evaluated by mIHC (OPAL™ protocol) to assess T cell markers' presence and spatial distribution CD3⁺, CD8⁺, and CD103⁺ and the epithelial-mesenchymal transition markers α -SMA and E-cadherin (**Fig.S7**; see Supplementary Figures and Tables section, page number 293). H&E (**Fig. 33A, B**) and OPAL-stained sections (**Fig. 33C**) were used to determine tissue segmentation (**Fig. 33D**). Low-resolution images (10X magnification) of the whole tissue section were scanned on Phenochart® software (Perkin Elmer/Akoya, MA) (**Fig. 33C**). Using inForm® software (Perkin Elmer/Akoya, MA) (Stack et al., 2014) to perform supervised image analysis, the Tissue finder algorithm for trainable pattern recognition was implemented. This algorithm is based on detecting the tissue morphology's spectral elements; therefore, tissue segmentation was determined as follows: adjacent liver parenchyma depicted in red,

invasive tumour margin in blue, and tumour core in green (Huang et al., 2013) (**Fig. 33D**). Regions of interest referred to as high-powered fields (HPFs, 20X magnification) were defined by fixed stamping on the highest tumour infiltrating lymphocyte (TIL) density (**Fig. 33C**) (Halse et al., 2018) of the CLM to accurately represent the whole tumour and adjacent LP as shown in the flow diagram (**Fig.S8**; see Supplementary Figures and Tables section, page number 294). Automated image quantitation analysis of the mIHC stained tissues was used to generate and determine the percentage distribution of cells positive for the markers CD3⁺, CD8⁺, CD103⁺ and the EMT markers α -SMA and E-cadherin within the LP, IM, and TC (**Fig. 33E, K**). Then cell phenotyping was performed on inForm® (Perkin Elmer/Akoya, MA) (Stack et al., 2014), which uses a machine learning-based trainable phenotype scoring of cells that expressed single markers or combined expression as programmed (**Fig. 33F**), thus cells positive for a single marker or a combination of markers are counted only within the population of the assigned phenotype (**Fig. 33G, K**). Based on the markers above the following phenotypes were investigated: CD3⁺ T Lymphocytes (CD3⁺CD8⁻CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺CD103⁺), non-lymphocyte infiltrating cells expressing CD8⁺ or CD103⁺ and cells positive for α -SMA or E-cadherin. A composite image of all these cell phenotypes is shown in **Fig 33L**. Thus, individual immune profiles were created for each CLM patient included in this study and compared with sclerosed hemangioma samples and infiltrate on normal liver tissue (**Fig.S9A-C**; see Supplementary Figures and Tables section, page number 295). The expression was then normalized for exposure confirming that phenotypical characterization was represented within each patient sample (**Fig.S9D**; see Supplementary Figures and Tables section, page number 295). The results demonstrate wide variation for each phenotype within the patient cohort (**Fig. S9D**; see Supplementary Figures and Tables section, page number 295). There was no significant survival difference in the univariate analysis of demographic and surgical variables presented in **Table 8** (data not shown).

Table 8. Clinical, histopathological, and surgical characteristics of the CLM study cohort

Demographic and surgical variables	Percentage in Cohort (%) /(n)
Gender	
Male	86.36 (19)
Female	13.63 (3)
Mean age at liver resection	61.32
Margin Status	
Margin-negative (R0)	77.27 (17)
Margin-positive (R1)	22.72 (5)
Number of tumours	
3 or more tumours	22.72 (5)
Less than 3 tumours	77.27 (17)
Tumour size (mm)	
>50 mm	22.72 (5)
<50 mm	77.27 (17)
Unilobar vs. Bilobar disease	
Unilobar	72.72 (17)
Bilobar	27.27 (5)
Type of Liver rection procedure	
Major	40.90 (9)
Minor	59.09 (13)
Simultaneous colonic resection	
Yes	9.09 (2)
No	90.90 (20)
Histological growth pattern	
Desmoplastic	31.81 (7)
Pushing	31.81 (7)
Replacement	36.36 (8)
Resection of primary tumour	
Right	22.72 (5)
Left	4.54 (1)
Rectum	36.36 (8)
Recto-sigmoidal	4.54 (1)
No resection	9.09 (2)
Unknown	22.72 (5)

Neoadjuvant chemotherapy Regimen	
Pre-colonic resection	4.54 (1)
Pre-hepatic resection	40.90 (9)
Post-colonic resection	9.09 (2)
Post-hepatic resection	13.63 (4)
Pre and Post hepatic resection	4.54 (1)
Unknown	27.27 (5)

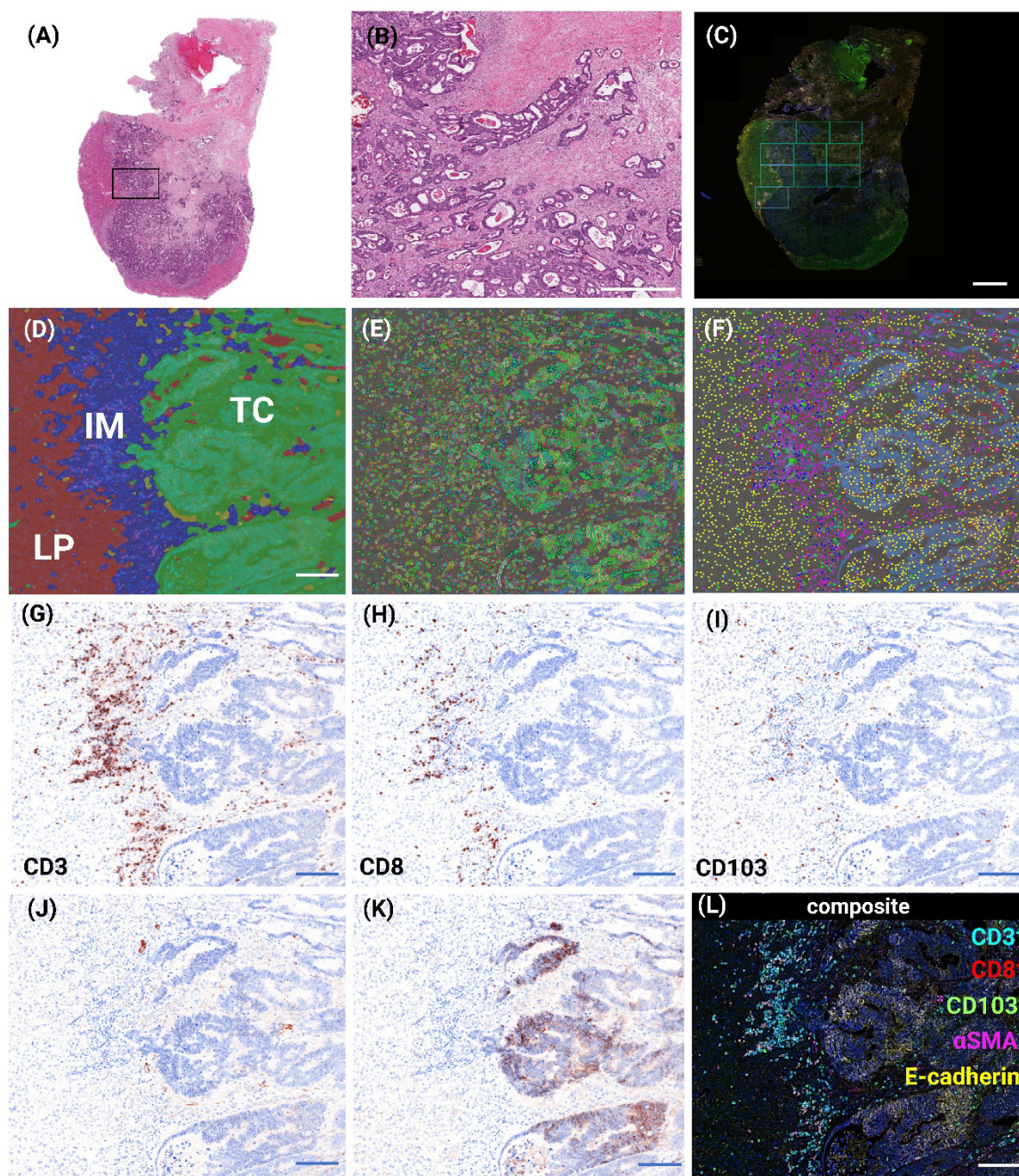


Figure 33. Representative microphotographs of the workflow diagram for analysis using Aperio ImageScope™, Vectra® imaging and inForm® cell phenotyping. (A) Whole tissue scan H&E stained of a CLM. (B) High magnification H&E stained CLM (scale bar: 200µm) (C) Phenochart (Perkin Elmer/Akoya, MA) whole tissue scan reviewer (low-powered field) showing High powered Pre-scan field (scale bar: 2mm) (D) Tissue segmentation displaying adjacent liver parenchyma (LP) in red, invasive tumour margin (IM) in blue, and tumour core (TC) in green (scale bar: 100µm). (E) Nuclear and cell segmentation. (F) Cell phenotyping scoring a composite image representing all biomarkers used. Pathology views analysis of (G) CD3, (H) CD8, (I) CD103, (J) αSMA, and (K) E-cadherin (scale bar: 100µm). (L) Representative composite HPF image displayed all phenotypes, including αSMA and E-cadherin. Key: Cyan: CD3+, Red: CD8+, Bright Green: CD103+, Purple: CD3+CD8+, light pink: CD3+CD8+CD103+, Bright pink: αSMA, and Yellow: E-cadherin (scale bar: 100µm).

Firstly, the distribution of cells positive for the markers CD3⁺, CD8⁺, and CD103⁺ was investigated (**Fig. 34A, C**), and then the spatial distribution of the assigned phenotypes (**Fig. 34 D-H**). The spatial distribution of CD3⁺ T cells (CD3⁺ CD8⁻ CD103⁻) and overall, was found significantly higher in the IM when compared with LP or the TC (**Fig. 34D**). Subsequently, the staining for CD8⁺(CD3⁻ CD103⁻) non-T cells (**Fig. 34E**), which showed significantly higher levels in the IM when compared with LP, was investigated (**Fig. 34E**); whereas the spatial distribution of CD103⁺(CD3⁻ CD8⁻) non-T cells staining showed no significant difference across the liver-tumour interface (**Fig. 34F**). These populations did not co-express CD3, but CD8⁺ expression, which has also been reported on natural killer cells (Ahmad et al., 2014), while CD103⁺ is known to expressed on dendritic cells (Williford et al., 2019) and tissue-resident NK cells in humans (Marquardt et al., 2019). After defining T cell subpopulations expressing CD3⁺CD8⁺ (CD8⁺ T cells) (**Fig. 34G**) and CD3⁺CD8⁺CD103⁺ (resident memory CD8⁺ T cells or T_{RM}) (**Fig. 34H**). These T cell subpopulations like CD3⁺ T cells were found in a significantly higher percentage located in IM when compared with the TC and LP. There was a distinct spatial distribution pattern between T cell populations compared to non-T cell populations, which may be related to the specific relationships and functions they execute within the liver-tumour interface.

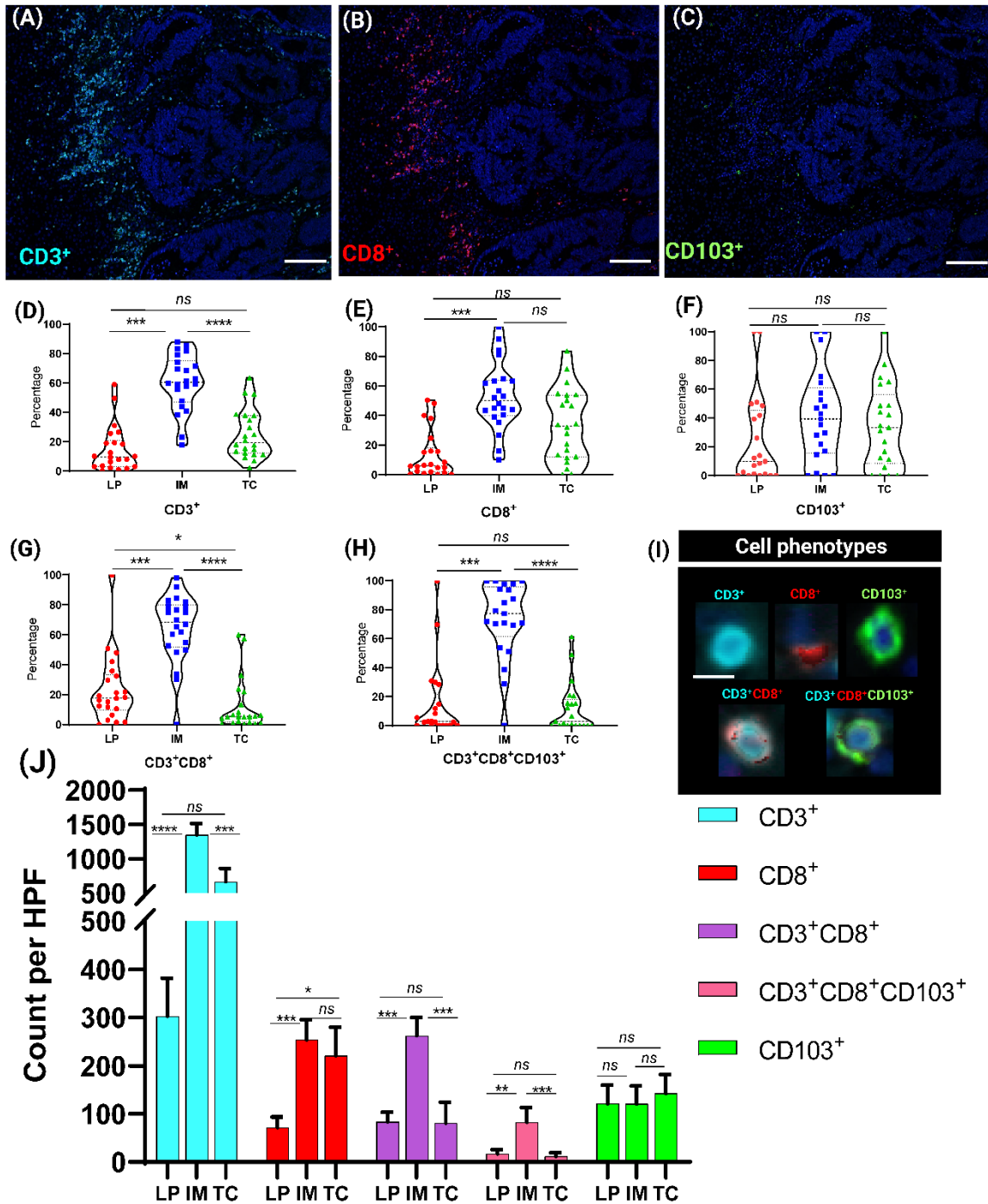


Figure 34. Spatial distribution of T Lymphocytes (CD3⁺CD8⁻CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), TRM (CD3⁺CD8⁺CD103⁺), and non-lymphocytes expressing CD8⁺ or CD103⁺ in the CLM cohort. Representative opal-stained images of (A) CD3⁺, (B) CD8⁺, (C) CD103⁺, expressing cells (scale bar: 100µm). Spatial quantification of immune cell phenotypes in the LP, IM, and TC of (D) CD3⁺, (E) CD8⁺, (F) CD103⁺, (G) CD3⁺CD8⁺, (H) CD3⁺CD8⁺CD103⁺ as percentage of total cells per HPF (n=22). (I) Representative cell phenotypes stained by OPAL (scale bar: 10µm). (J) CLM immune profile plotted as cell count per HPF (20X). Statistical analysis was performed comparing tissue segmentation using Mann Whitney U test, Data presented as mean ± SEM. Significantly different data represented by * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.; not significant (n.s).

6.3.2 Spatial and phenotypical characterization of CLM infiltrating immune cells within the tumour and adjacent liver parenchyma

The initial intention was to explore the T cell phenotype distribution; however, there was a high frequency of non-T cells expressing CD8 or CD103; thereby, those phenotypes were also included in the analysis (**Fig. 34I, J**). Firstly, the spatial distribution of T lymphocytes (CD3⁺ CD8⁻ CD103⁻) was investigated (**Fig. 34J**; [bar colour: cyan]), and overall, it was found significantly higher in the IM when compared with LP or the TC. Similarly, subpopulations of CD8⁺ T cells (CD3⁺CD8⁺CD103⁻) (**Fig. 34J**; [bar colour: purple]) and the tissue-resident memory CD8⁺ T cells or T_{RM} (CD3⁺CD8⁺CD103⁺) (**Fig. 34J**; [bar colour: pink]), had significantly higher percentage of cells located in IM when compared with the TC and LP. In general, there were significant differences in cell count according to the spatial distribution of T lymphocytes and T cell subtypes, which were highly concentrated at the IM, rather than in the TC or LP among the CLM patient cohort (**Fig. 34J**). In contrast, non-T cell populations expressing CD8⁺ (**Fig. 34J**; [bar colour: red]) or CD103⁺ (**Fig. 34J**; [bar colour: bright green]) accumulate equally in the IM and TC (**Fig. 34J**). The CD8⁺ T cell count (mean 261.9, Std. Error of Mean [SEM] 37.99; *p-value* =0.0001) and T_{RM} cell (mean 82.82 SEM 30.04, *p-value* =0.0001) was significantly lower in IM, when compared with CD3⁺ (CD8⁻CD103⁻) cell count (mean 1348, 169) (**Fig. 34J**). Interestingly, CD8⁺ (CD3⁻ CD103⁻) cell count reached a significantly higher level (mean 221.3, SEM 58.01) when compared with CD8⁺ T cells (80.70, SEM 43.66) in the TC (*p-value* =0.0036). Moreover, a higher cell count was found for CD103⁺ (CD3⁻ CD8⁻) (mean 120.4 SEM 37.48) than for T_{RM} cell account in the TC (**Fig. 34J**) (*p-value* = 0.0019). Lastly, the percentage of CD8⁺ T cells (CD3⁺ CD8⁺ CD103⁻) and T_{RM} (CD3⁺CD8⁺CD103⁺) was calculated relative to the total T lymphocytes expressing CD3⁺ using the median cell count per HPF, and it was found that CD8⁺ T cells and T_{RM} only accounted for 17.09% and 1.46% respectively in the IM out of the total number of CD3; whereas in the TC, CD8⁺ T cell represented 3.83% and T_{RM} was only 0.25 % (**Table S2**).

6.3.3 Marker predictive value for CLM patient survival integrating frequency of tumour immune infiltrates and spatial distribution

To determine if these markers could predict OS post liver resection, OS in patients whose tumours showed a low marker frequency, defined as below the median cell count distribution, as compared with those that had high frequency or above the median cell count distribution for tumour infiltrating cell populations in the IM or the TC. Using annotated patient data, the effect of the spatial distribution of immune infiltration on OS was calculated. In a univariate analysis, the parameters taken into consideration were overall frequency and spatial distribution. The median follow-up was 5.035 years (range of 1.08-13.41 years) (**Table S3**). Thus, high levels of CD3⁺ T lymphocytes in the IM showed no significant effect on patient outcome (**Fig. 35A**), while in the TC had a significant effect (Log-rank $p=0.0054$) (**Fig. 35B**) (CD3⁺ median cell count in the IM was 1149 and in the TC was 379.8).

In contrast, high levels of CD8⁺ T cells in the IM had a significant impact on patient OS (Log-rank $p=0.0486$) (**Fig. 34C**), while no significant difference was found in the TC (**Fig. 35D**) (CD8⁺ T median cell count in the IM was 241.2 and in the TC was 15.17). There was no survival difference in patients with high levels of T_{RM} expressing CD3⁺CD8⁺CD103⁺ compared to those with low levels at either the IM (**Fig. 35E**) or the TC segments (**Fig. 35F**), (CD3⁺CD8⁺CD103⁺ median cell count in the IM was 20.67 and in the TC was 1). Upon interpretation of the inForm® (Perkin Elmer/Akoya, MA) cell phenotyping analysis, it was revealed that cells other than lymphocytes expressed these markers, and therefore we followed up analysing them. Any prognostic value of these markers towards predicting patient outcome was investigated. There were no survival differences associated with the frequency of the non-lymphocyte immune infiltrates expressing only CD8⁺ (CD8⁺CD3⁻CD103⁻) (median cell count in the IM was 176.8 and in the TC was 143.5) (**Fig. 36A, B**) or CD103⁺ (CD103⁺CD8⁻CD3⁻) (median cell count in the IM was 44 and in the TC was 76.67) regardless of their spatial location (**Fig. 36C, D**) (**Table S3**). In summary, these results indicate that the frequency of the particular lymphocyte subpopulation and their spatial location within the tumour impacts the patient's survival outcome.

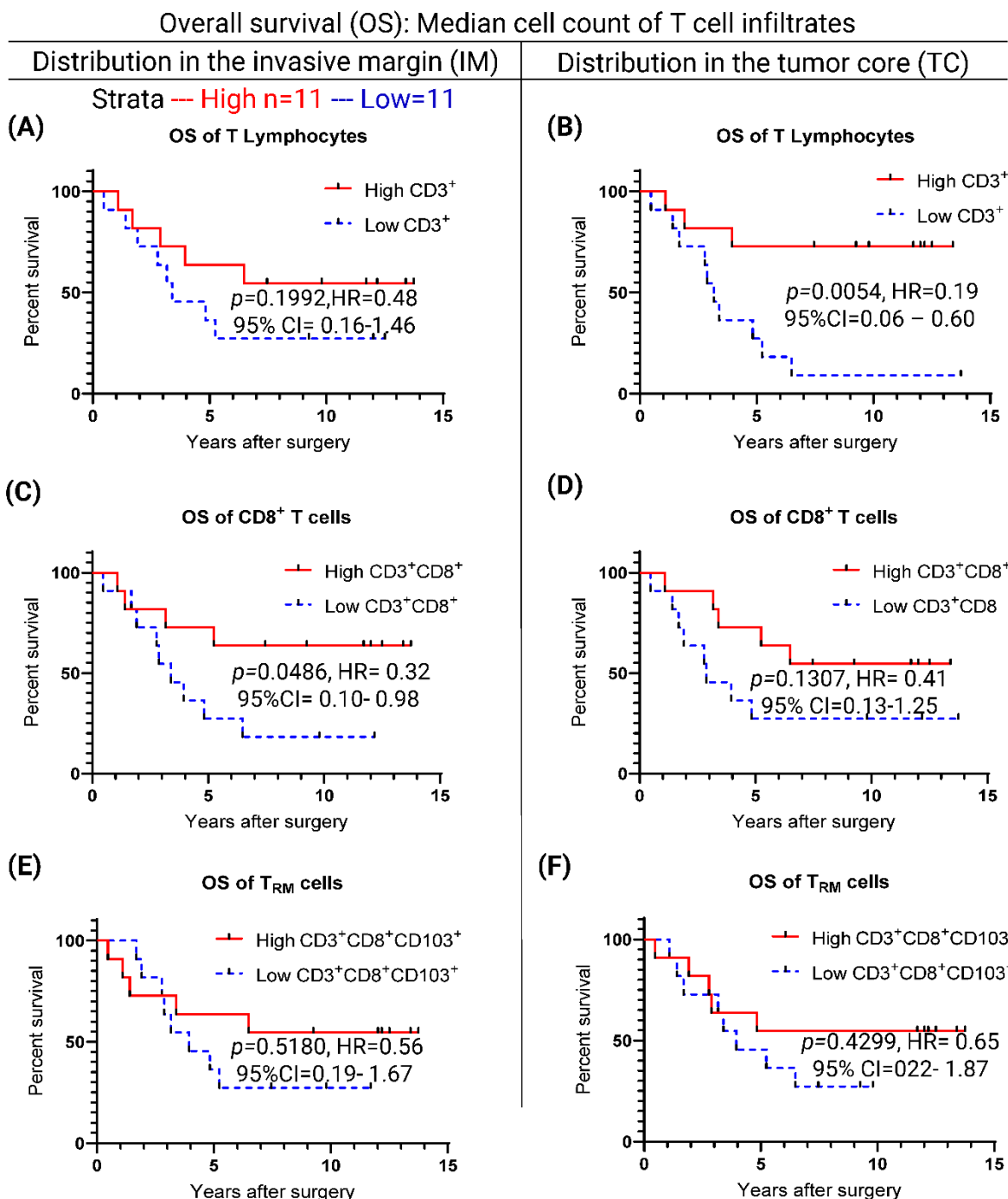


Figure 35. CLM patient survival post-liver resection univariate analysis using the median distribution of cell count per HPF and T cell subpopulations' spatial distribution. Patient's overall survival (OS) classified by the median distribution of cell count per HPF of (A-B) T Lymphocytes (CD3⁺), (C-D) CD8⁺ T cells (CD3⁺CD8⁺), (E-F) T_{RM} (CD3⁺CD8⁺ CD103⁺) according to the tissue segmentation. High cell count: red line (n=11), Low cell count: blue dash line (n=11). Log-rank (Mantel-Cox) test *p*-value is annotated.

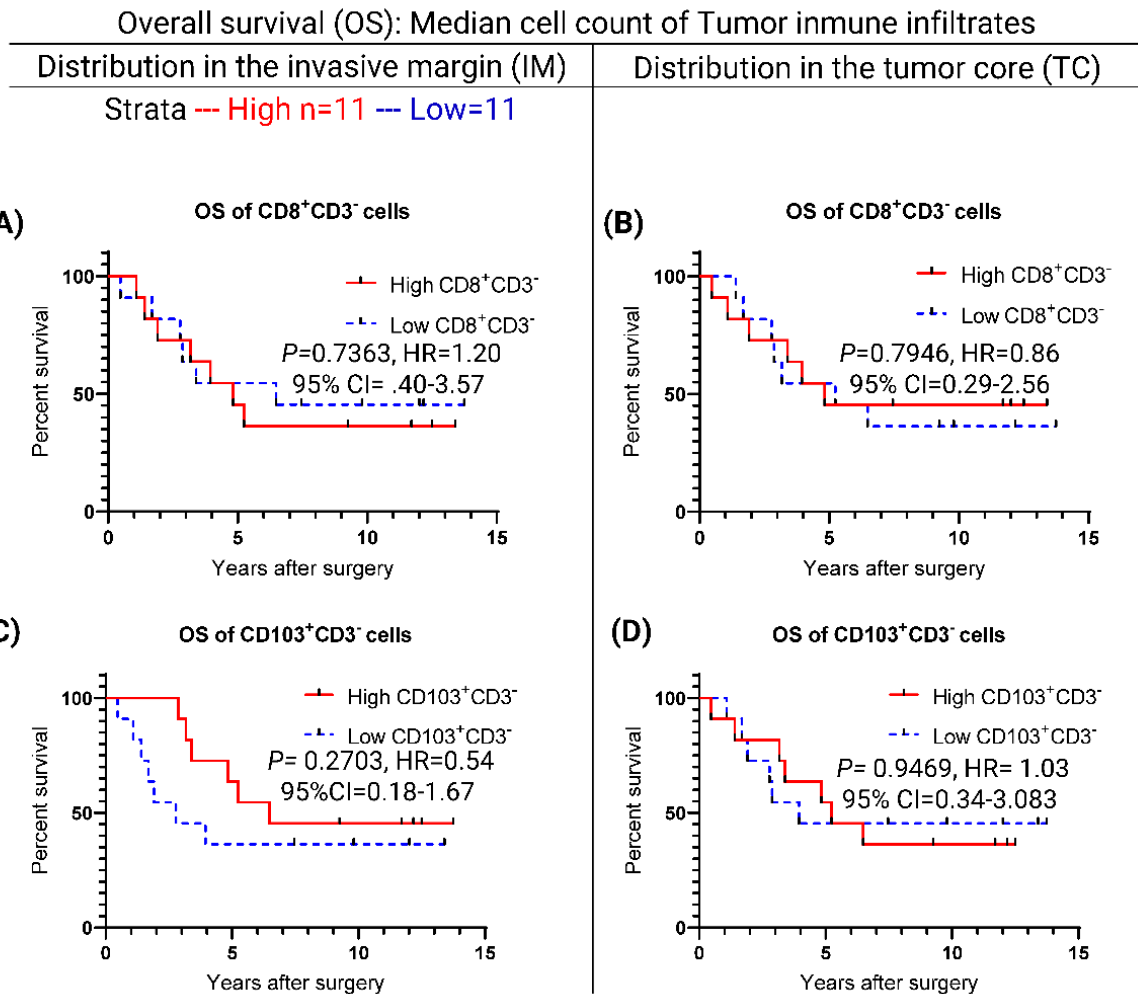


Figure 36. CLM patient survival post-liver resection univariate analysis using the median distribution of cell count per HPF and spatial distribution of non- T cell subpopulations. (A- B) CD8⁺ and (C-D) CD103⁺ populations that did not co-expressed CD3⁺T lymphocytes, according to the tissue segmentation. High cell count: red line (n=11), Low cell count: blue dash line (n=11). Log-rank (Mantel-Cox) test p-value is annotated.

6.3.4 Spatial expression patterns of α -SMA and E-cadherin in CLM

Next, the positivity and spatial distribution for α -SMA were examined. α -SMA expressed by cancer-associated fibroblasts (CAFs), which is usually prominent within desmoplastic reaction tissue (Mueller et al., 2007). Also, the expression of E-cadherin was assessed, which is well-documented as homotypic epithelial cell-cell adhesion marker (Adams et al., 1998), as EMT marker (Zeisberg & Neilson, 2009), and as heterotypic binding marker on epithelial tissues by T cells expressing the integrin CD103 (Shields et al., 2019). The results indicate that α -SMA is most strongly expressed in the IM and TC and significantly less in the LP (**Fig. 37A**). The pattern of

α -SMA staining indicates that not only CAFs in the tumour stroma were α -SMA-positive (**Fig. 37B**) but also pericytes on blood vessels (**Fig. 37C**).

The E-cadherin staining pattern was highly heterogeneous throughout the entire CLM cohort, with certain specimens displaying strong positivity and others minimal. Consequently, overall no significant difference was observed in the spatial distribution of E-cadherin expression across the liver-tumour segmentation (**Fig. 37D**). For instance, we found positive E-cadherin expression on the hepatocyte-to-hepatocyte junctions and in the junctions of epithelial tumour cells (**Fig. 37E**). Intriguingly, while most E-cadherin staining is associated with junctional staining of hepatocytes and tumour epithelial cells, a strong expression of E-cadherin was also found on the surface of some tumour infiltrating cells (**Fig. 37F**). This observation prompted us to investigate whether E-cadherin colocalizes on any of the immune cells, T lymphocytes or non-lymphocytes characterized above. A colocalization analysis discovered that a small proportion of CD8⁺ T cells intrinsically expressed E-cadherin (**Fig. 37G**).

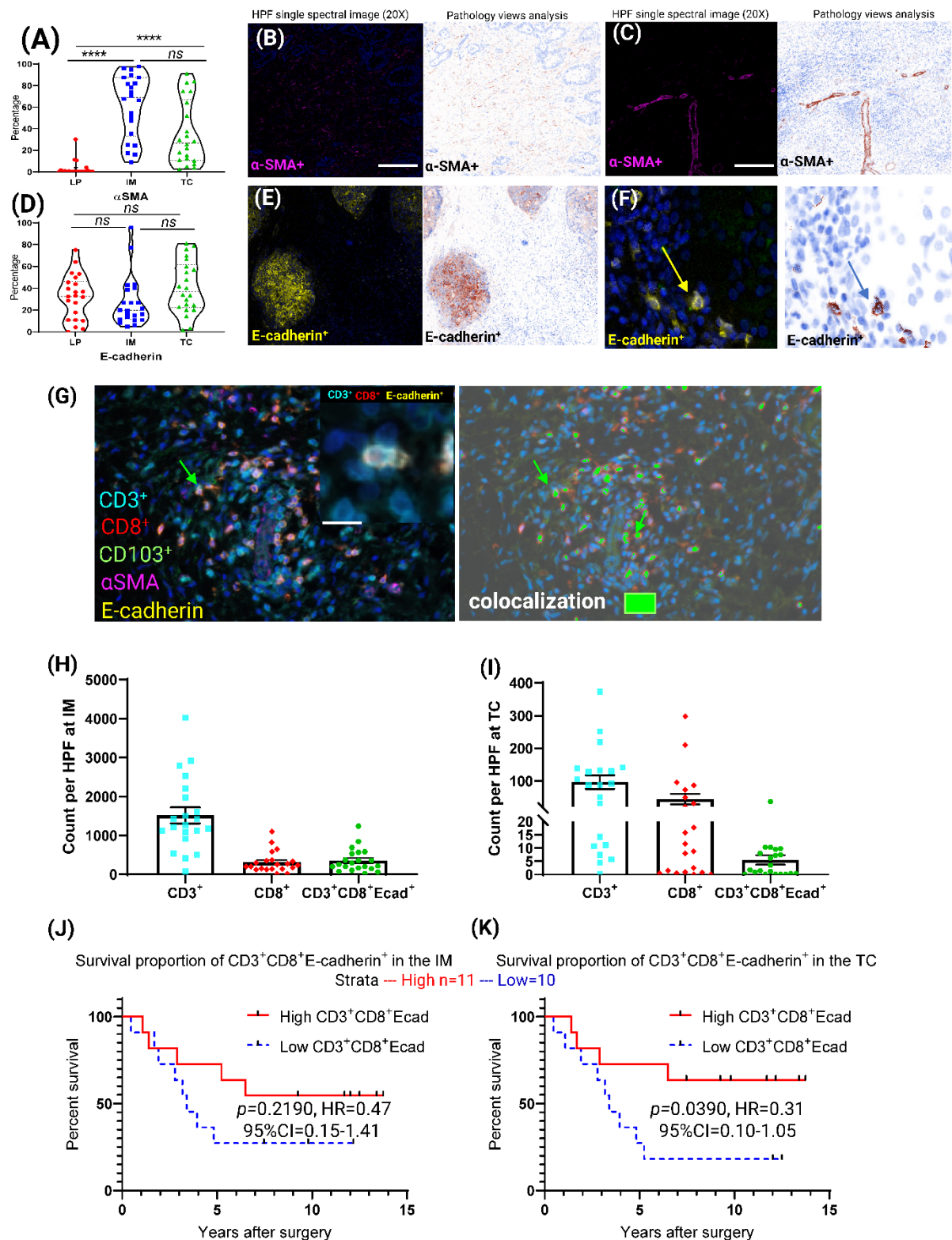


Figure 37. Spatial expression patterns of α-SMA and E-cadherin in CLM and colocalization of E-cadherin expression on CD8+ T cells. (A) Spatial quantification of α-SMA staining in the LP, IM, and TC (B) α-SMA positive cancer-associated fibroblast in the tumour stroma (scale bar: 100μm) and (C) α-SMA positive pericytes on blood vessels (scale bar: 100μm) Key: Bright pink: αSMA. (D) E-cadherin staining in the LP, IM, and TC (E) at the hepatocyte-to-hepatocyte junctions and in the junctions of epithelial tumour cells. Key: Yellow: E-cadherin (scale bar: 100μm). (F) E-cadherin expression by CD3+CD8+ T lymphocytes (G) Composite image displaying the expression of CD3+, CD8+, E-cadherin on the surface of CD8+ T cells (scale bar: 10μm). The colocalization map indicates in bright green the

position of the co-expression of all three markers. (H) Cell count per HPF was calculated in the invasive margin (IM) and (K) in the tumour core (TC). (L) Patient overall survival association with levels of E-cadherin positive CD8⁺ T cells in the IM and (M) the TC. Mann Whitney U test, High cell count: red line (n=11), Low cell count: blue dash line (n=10). Log-rank (Mantel-Cox) test; Data presented as mean $\pm$ SEM. Significantly different data represented by **** $p < 0.0001$.; not significant (n.s).

6.3.5 Prognostic value of E-cadherin expressing CD8⁺ T cells

Pixel-based colocalization analysis using inForm® (Perkin Elmer/Akoya, MA) (Stack et al., 2014) was applied to calculate the percentage of the positive pixel area of CD3⁺, CD8⁺ and E-cadherin (**Fig. 37G, H**). The colocalization of the three markers was determined statistically by quantitating overlapping components in all images based on each component's optical density thresholds (**Fig. 37H**) and visually, using the colour-based colocalization map (**Fig. 37G**). Intriguingly, one patient showed no expression of this subtype in any of the samples analysed. Subsequently, the precise location of CD3⁺, CD8⁺ and CD3⁺CD8⁺E-cadherin⁺ was analysed and found that the mean cell count per HPF for CD3⁺ was 1516 cells (SEM 206.8), for CD8⁺ (CD3⁻ CD103⁻) was 311.3 cells (SEM 59.12) and for CD3⁺CD8⁺E-cadherin⁺ was 356.2 cells (SEM 65.16) in the IM (**Fig. 37H**), whereas total cell count was significantly lower in the TC. CD3⁺ cell count was 96.39 (SEM 20.97), CD8⁺ was 44.30 (SEM 16.75) and CD3⁺CD8⁺E-cadherin⁺ 5.47 (SEM 1.79) in the TC (**Fig. 37I**). The prognostic value of CD8⁺ T cells expressing E-cadherin was evaluated. High numbers of T cells expressing CD3⁺CD8⁺E-cadherin⁺ in the IM had no significant impact in patient OS (**Fig. 37J**), while a significant difference was found in the TC (Log-rank $p = 0.0390$) (**Fig. 37K**). Then, to evaluate whether intrinsic expression E-cadherin on CD8⁺ T cells could indicate activation status (Morikawa et al., 2002), a small subset of whole tissue sections of samples with CD3⁺, CD8⁺, E-cadherin and Forkhead box P3 was stained (FoxP3). The average colocalization percentage of CD3⁺, CD8⁺, E-cadherin and FoxP3⁺ was significantly lower than the co-expression of all three markers in a CLM subset (0.0002%); hence the expression of FoxP3⁺ on CD3⁺CD8⁺E-cadherin⁺ was barely detectable (**Fig.S10**; see Supplementary Figures and Tables section, page number 296).

6.3.6 Spatial near neighbour relationships of T Lymphocytes influence the survival of CLM patients

It was hypothesized that T-lymphocyte subpopulations in the TME communicate and exert their influence on their nearest neighbour cells and that their spatial engagement may be a marker of predictive value for survival (**Fig. 38A**). First, the colocalization analysis was implemented using inForm® to identify unique cells using cartesian coordinates (x and y) (Stack et al., 2014). Secondly, cell populations were labelled according to the immune markers expressed (i.e., CD3⁺ T Lymphocytes (CD3⁺ CD8⁻ CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺ CD103⁺), non T cells expressing CD8⁺ or CD103⁺ and cells expressing α-SMA or E-cadherin. After cells were labelled for each inForm® (Perkin Elmer/Akoya, MA) (Stack et al., 2014) raw data file, the spatial relationships for all the types of cells were investigated, including E-cadherin expressing CD8⁺ T cells, to understand their function and interaction with other cell phenotypes. These spatial engagement patterns were explored by evaluating cell population distances using the R package Inter-cellular Spatial Analysis Tool bioinformatic approach (Wang, 2018). Thus, the nearest neighbour (NN) distances were computed between the CD8⁺ T cells expressing E-cadherin and other cell phenotypes, including T lymphocytes CD3⁺ (CD8⁻CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺CD103⁺), CD8⁺(CD3⁻ CD103⁻), and CD103⁺ (CD3⁻ CD8⁻) (**Fig. 38B,C**). The cut-off distances for spatial engagement were set at 30µm. The NN median distance distribution of CD8⁺ T cells expressing E-cadherin identifying that they locate near CD103⁺ (CD3⁻) non-lymphocytes, closely followed by T lymphocytes CD3⁺ (CD8⁻CD103⁻) and CD8⁺(CD3⁻ CD103⁻) non-lymphocytes. The farthest median distance distribution was found between T_{RM} and CD8⁺ T cells (**Fig. 38B**). The pattern of engagement for CD8⁺ T cells that do not express E-cadherin largely displayed a similar NN distance with CD103⁺ (CD3⁻), T lymphocytes CD3⁺ (CD8⁻ CD103⁻) and CD8⁺(CD3⁻ CD103⁻), but still far from CD8⁺ T cells expressing E-cadherin. The spatial engagement between CD8⁺ T cells and T_{RM} was not possible to ascertain in the HPFs used in the colocalization analysis of CD3⁺, CD8⁺ and E-cadherin because no T_{RM} expressing cells were found in some of these samples to calculate the distance to CD8⁺ T cells (**Fig. 38C**).

In a separate analysis using cartesian coordinates (x and y) extracted with the cell phenotyping algorithm, NN distances were computed between all phenotypes included in the study to explore how they would spatially engage at 30µm (**Fig. 38D**). In addition to that, “the effective percentage of engagement” was quantified by analysing not only the mean distribution distance profile for each immune cell subtype identified in this study but also to estimate the difference in the proportion of each relationship (Huang et al., 2019). The distance of engagement was set 30µm, CD3⁺ T lymphocytes (box colour: cyan), predominantly engaged with CD8⁺(CD3⁻) (box colour: red), then CD103⁺(CD3⁻) (box colour: bright green) and CD8⁺ T cells (box colour: purple) (median above 25%); whereas the relationship between CD3⁺ T lymphocytes and T_{RM} (box colour: pink) showed a significantly decreased engagement (median lower than 25%) (**Fig. 38D**). In the case of CD8⁺ T cells, they seemed to largely engage with CD3⁺ T lymphocytes (median above 75%), followed by CD8⁺(CD3⁻) and CD103⁺ (CD3⁻) with a median effective percentage of engagement below 50%. Lastly, a significantly low proportion of CD8⁺ T cells engaged with T_{RM} (**Fig. 38D**). When comparing the spatial engagement profile of T_{RM}, they are closer to CD3⁺ T lymphocytes than CD8⁺ T cells, but they still engaged more effectively with T cell subtypes (median above 50%) than with CD8⁺(CD3⁻) and CD103⁺ (CD3⁻) (**Fig. 38D**).

On the other hand, the non-T cell populations expressing CD8⁺(CD3⁻) and CD103⁺ (CD3⁻) showed a comparable interaction pattern with T lymphocyte subtypes. For instance, both cell types engaged mainly with CD3⁺ T lymphocytes followed by CD8⁺ T cells, then with each other and lastly only poorly engaged with T_{RM} (**Fig. 38D**). No significant difference was found in any immune cell phenotype engagement pattern to cells expressing α-SMA (**Fig. 38D**), while their engagement with E-cadherin expressing cells was at different NN proximity for engagement depending on the particular phenotype (**Fig. 38D**). The median percentage of engagement between CD8⁺(CD3⁻), CD103⁺ (CD3⁻) and E-cadherin was above 50%, while CD8⁺ T cells and T_{RM} was equal to or below 50%. Other T Lymphocytes comprised within the heterogeneous population of CD3⁺ T (CD8⁻CD103⁻) seemed to engage at a higher proportion (**Fig. 38D**).

To assess the influence of spatial engagement profiles on CLM patients’ survival post-liver resection, first patients were stratified according to the NN median distribution distance (High n=11; Low n=11). In a univariate analysis of outcome, significant

survival differences were found when comparing CD8⁺ T cells (Log-rank $p=0.049$) (**Fig. 38E**), CD8⁺(CD3⁻) (Log-rank $p=0.045$) (**Fig. 38F**) and CD103⁺ (CD3⁻) (Log-rank $p=0.049$) (Fig. 30g) engagement with E-cadherin. The results indicated that less engagement or higher distribution distance to E-cadherin had a significantly better OS than those samples with a lower median distance distribution (**Fig. 38E, G**). In contrast to this, CD8⁺ T cells close to either α -SMA (Log-rank $p=0.022$) (**Fig. 38H**) or T_{RM} (Log-rank $p=0.07$) (**Fig. 38I**) indicated a better outcome than in those samples that had a greater median distance distribution. Likewise, high CD8⁺(CD3⁻) engagement with either α -SMA (Log-rank $p=0.049$) (**Fig. 38J**) or T_{RM} (Log-rank $p=0.023$) (**Fig. 38K**), showed a protective effect.

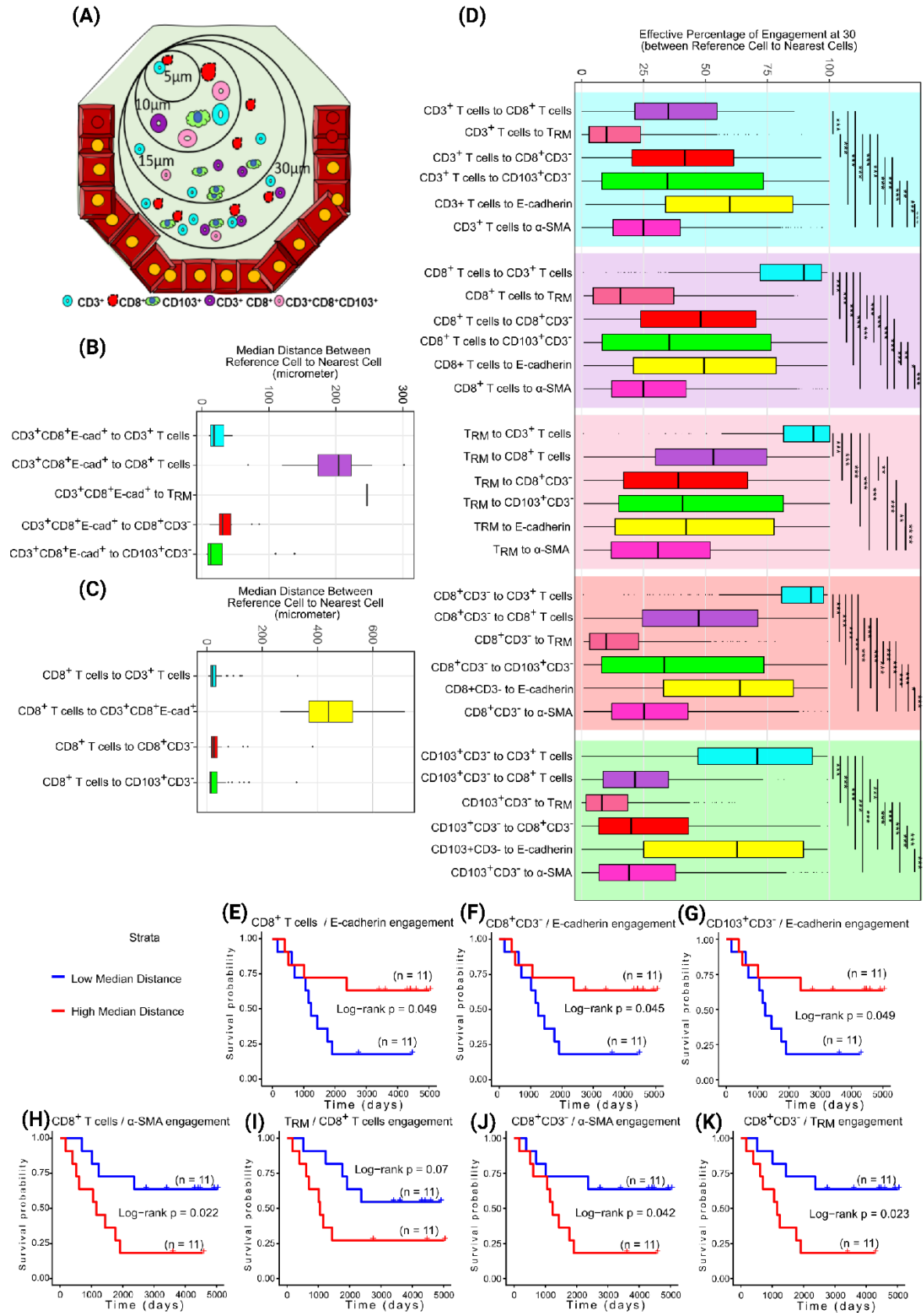


Figure 38. Nearest neighbour spatial relationships of T Lymphocytes and their influence on patient survival. a Schematic representation of T lymphocytes subpopulations in the TME and their nearest neighbour (NN) cells. (A) The median distribution distance was computed using ISAT (see *methods*); between (B) E-cadherin positive CD8⁺ T cells (CD3⁺CD8⁺E-cad⁺) to other immune cell phenotypes (C) CD8⁺ T cells to other immune cell phenotypes. (D) The effective percentage of engagement of CD3⁺T lymphocytes, CD8⁺ T cells, CD103⁺T_{RM}, and CD8⁺ CD3⁻ and CD103⁺ CD3⁻ non-T cells are shown within a cut-off of 30 μ m. Patient overall survival was interrogated stratifying according to the median distribution distance between E-cadherin and (E) CD8⁺ T cells, (F) CD8⁺CD3⁻ non-T cells, and (G) C103⁺ CD3⁻non-T cells, between CD8⁺ T cells and (H) α -SMA and (I) T_{RM}, between CD8⁺CD3⁻ non-T cells and (J) α -SMA and (K) T_{RM}. Log-rank *p*-value is annotated. Comparisons of cell populations or subpopulations were performed using the Kruskal–Wallis test, followed by a pairwise Mann–Whitney test adjusted by Bonferroni to identify the pairs significantly different. Statistical significance was calculated at a *p*-value of 0.05. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

6.3.7 Histologic growth patterns influence the spatial distribution of infiltrating immune cells and EMT markers

The second part of this study investigated whether the type of tumour growth influences spatial immune cell distribution. Histological evaluation by an anatomical pathologist (D.W) of the 22 CLM specimens defined seven desmoplastic, eight replacement and seven pushing growth patterns according to their tumour invasive interface (Fernández Moro et al., 2018) (**Table 8**). The desmoplastic growth pattern is characterized by extensive reactive stroma at the tumour liver interface, replacement growth pattern has the least stroma, and there is no clear boundary at the tumour-liver interface. In contrast, the pushing growth pattern is characterized by distorted, often flattened hepatocytes at the tumour-liver interface as seen in and depicted in the cartoon (**Fig.S11**; see Supplementary Figures and Tables section, page number 297). In all three growth patterns, the highest accumulation of CD3⁺ T lymphocytes was found in the IM (**Fig. 39A-C**), but the levels only reached a significant difference between the LP and the IM in the desmoplastic and replacement pattern (**Fig. 39A, B**), whereas a significant difference was observed between the IM and TC in the desmoplastic and pushing pattern (**Fig. 39A, C**).

Similarly, the highest cell counts of CD8⁺ T cells occurred in the IM for all three growth patterns (**Fig. 39A-C**), but only significantly different from the TC in the desmoplastic and pushing patterns (**Fig. 39A, C**). There was a significantly different T_{RM} distribution between the IM and the TC in the replacement pattern (**Fig. 39B**), while in the pushing pattern, there was distinct accumulation in the IM more than in the LP (**Fig. 39C**). No change was observed in the T_{RM} distribution in the desmoplastic pattern across the liver-tumour interface (**Fig. 39A**). A similar pattern of distribution for CD8⁺(CD3⁻) was

found in the desmoplastic and replacement histologic subtype indicating a more significant accumulation in the IM more than in the LP (**Fig. 39A, B**); whilst there was no difference in the accumulation of these cells within IM and TC in the pushing pattern (**Fig. 39C**). No significant difference was found in the distribution of CD103⁺(CD3⁻) across the liver-tumour interface. Altogether, there was no significant difference in the cell count of CD3⁺ T lymphocytes, CD8⁺ T cells and T_{RM} when the three different growth patterns were compared, or when compared with the average cell count of the entire cohort of CLM, neither in the IM (**Fig. 39D**) nor the TC (**Fig. 39E**). Regarding the other staining markers, only the replacement pattern showed a significantly higher E-cadherin expression in the TC compared with the IM (**Fig. 39F**). There was a trend for a higher expression α -SMA in the IM compared with the TC and LP for the desmoplastic and pushing patterns; but not in the replacement pattern (**Fig. 39G**), which seemed to have a similar α -SMA expression between the IM and TC (**Fig. 39G, H**).

cell phenotype count at the liver parenchyma (LP), the invasive tumour margin (IM) and the tumour core (TC). Key: Cyan: CD3⁺, Red: CD8⁺, Bright Green: CD103⁺, Purple: CD3⁺CD8⁺, light pink: CD3⁺CD8⁺CD103⁺, Bright pink: α SMA, and Yellow: E-cadherin. T lymphocyte count in the three main tumour growth patterns compared with the average cell count of the entire cohort of CLM (D) at the IM and (E) the TC. Spatial expression of (F) E-cadherin and (G-H) α -SMA in the three tumour growth patterns (scale bar: 100 μ m). Statistical analysis was performed across the cohort (n= 22) using Mann Whitney U test. Data presented as mean $\pm$ SEM. Significantly different data represented by * $p < 0.05$ and ** $p < 0.01$.

6.3.8 Correlation Between Frequency and Spatial Distribution in the histologic growth patterns

It was hypothesized that patterns of tumour immune cell infiltration might be dictated by the CLM histologic growth pattern, which is determined by the definitive presence of an invasive margin, and whether the tumour mass would comprise a vast area of tumour stroma or the core would consist mainly of tumour cells (Höppener et al., 2019; van Dam et al., 2017) (**Fig. S12**). Thus, to evaluate whether there was any correlation between the frequency of all cell phenotypes and their spatial distribution in the liver-tumour interface compared to the three distinct histologic growth patterns. A positive correlation between the distribution of CD3⁺ T lymphocytes in the IM and TC for the desmoplastic pattern was found (r 0.891, p =0.007) (**Fig. 40A**). Also, there was a strong correlation between the distribution of CD3⁺ T lymphocytes in the TC and CD8⁺ T cells in the TC for desmoplastic (r 0.922, p =0.003) (**Fig. 40A**) and replacement (r 0.956, p =2.072e-004) pattern (**Fig. 40B**). Interestingly, there was a strong positive correlation between CD8⁺ T cells and CD8⁺(CD3⁻) cells in the IM (r 0.949, p =3.142e-004) and the TC (r 0.961, p =1.448e-004) in the replacement pattern (**Fig. 40B**). Only the pushing pattern indicated a positive correlation between α -SMA in the TC and the T_{RM} in the IM (r 0.802, p =0.017) (**Fig. 40C**). Additionally, CD8⁺(CD3⁻) cells located in the IM displayed a positive correlation with CD8⁺(CD3⁻) cells in the TC in the pushing pattern (r 0.854, p =0.007) (**Fig. 40C**). Collectively, these results showed a strong positive correlation between the histologic growth pattern and the frequency of the cell populations included; therefore, the phenotypic immune profile needs to be assessed not only cell density and the spatial distribution across the tumour interface; but also their proximity between each other to infer on the prognostic relevance of these immunologic cell markers.

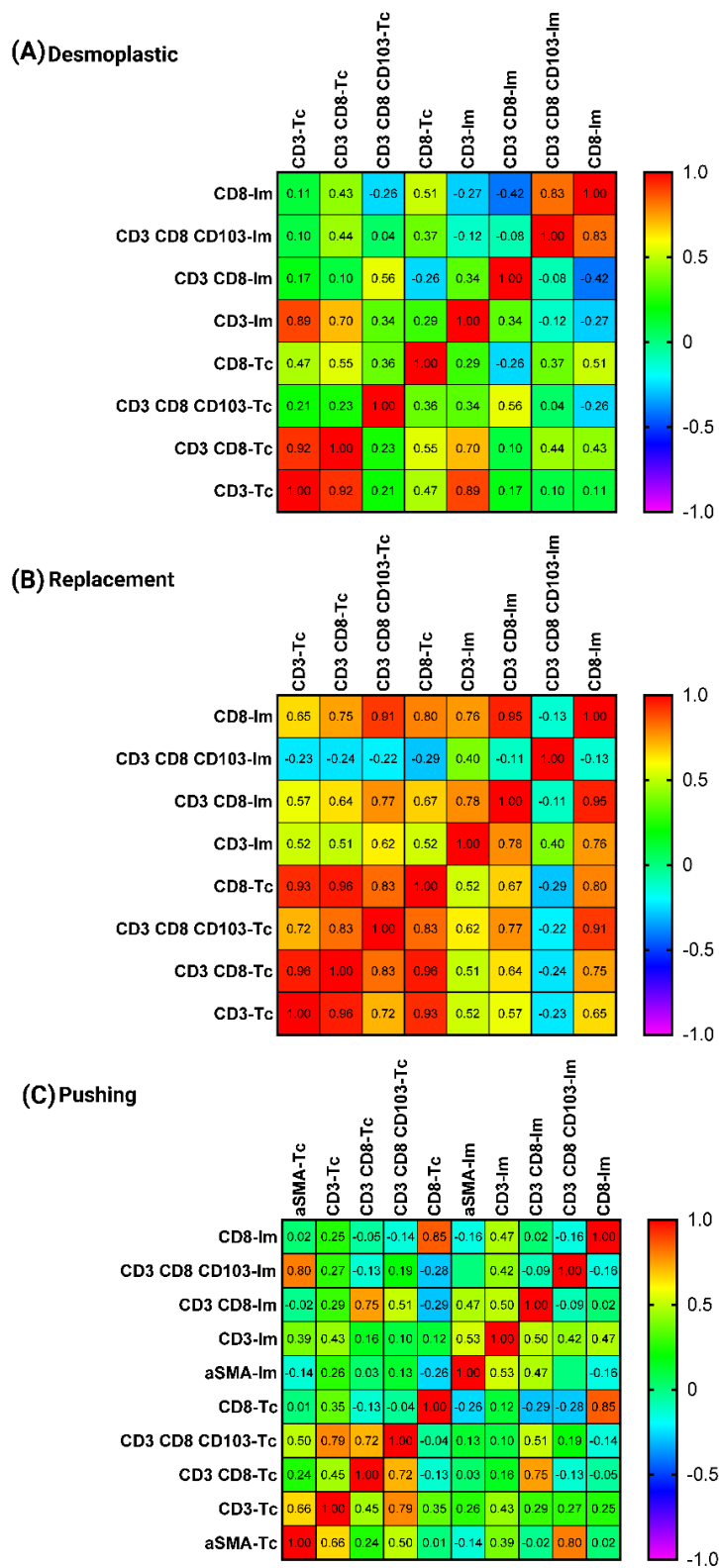


Figure 40. Correlation between cell phenotypes in the three dominant tumour growth patterns. Correlation matrix using Pearson r was computed using the frequency of all cell phenotypes and their spatial distribution for (A) desmoplastic (n=7), (B) replacement (n=8), and (C) pushing (n=7) growth pattern. Significant different correlations are annotated.

6.3.9 Spatial Engagement Profile of Desmoplastic and Pushing Pattern Differed from Replacement Pattern

Previously, the spatial relationships and their prognostic value within the CLM cohort were identified (**Fig. 38D, K**). According to histologic growth pattern, “the effective percentage” of cells paired at 30 μm (distance) was calculated for the spatial engagement analysis of immune cells. The spatial of engagement profile for CD3^+ T lymphocytes (box colour: cyan), CD8^+ T cells (box colour: purple), T_{RM} cells (box colour: pink), with $\text{CD8}^+(\text{CD3}^-)$ cells (box colour: red), and $\text{CD103}^+(\text{CD3}^-)$ cells (box colour: bright green) in the desmoplastic (**Fig. 41A**) and pushing pattern (**Fig. 41B**) was similar. In contrast, the effective percentage of engagement between CD3^+ T lymphocytes, CD8^+ T cells, $\text{CD8}^+(\text{CD3}^-)$ cells, $\text{CD103}^+(\text{CD3}^-)$ cells and T_{RM} cells was remarkably lower in the replacement pattern (**Fig. 41C**) when compared with the desmoplastic and pushing pattern (**Fig. 41A, B**). $\text{CD8}^+(\text{CD3}^-)$ cells predominantly engaged with CD3^+ T lymphocytes, then with CD8^+ T cells and $\text{CD103}^+(\text{CD3}^-)$ in the desmoplastic (**Fig. 41A**) and pushing patterns (**Fig. 41B**). There is a significantly larger proportion of $\text{CD8}^+(\text{CD3}^-)$ cells pairing with $\text{CD103}^+(\text{CD3}^-)$ in the replacement pattern (**Fig. 41C**). These results indicate that the desmoplastic and pushing pattern differed from the replacement pattern in the cell density distribution and TRM cells' spatial engagement profile (**Fig. 41**).

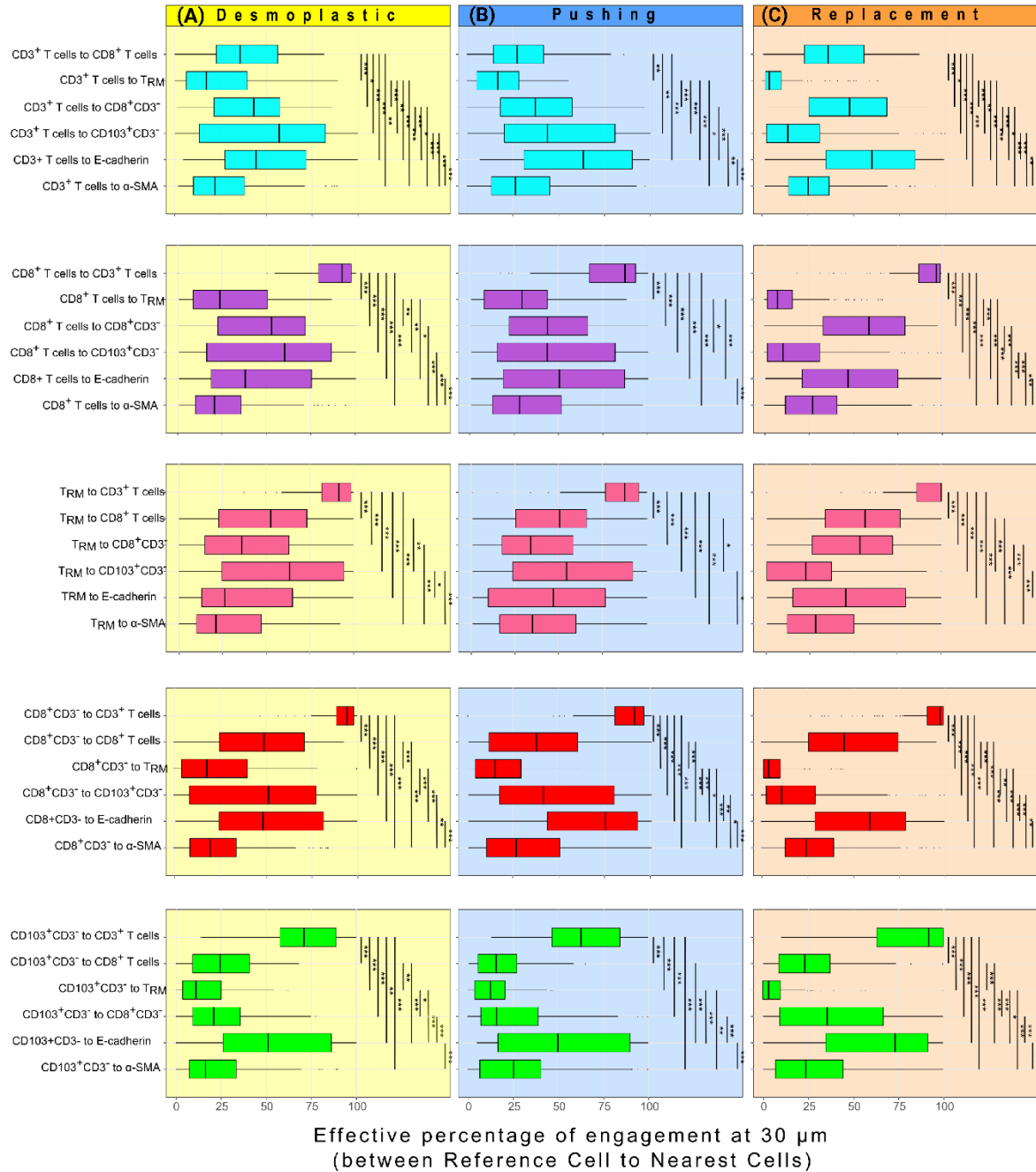


Figure 41. Immune cell spatial engagement in the three dominant tumour growth patterns. The percentage engagement between two phenotypes was calculated by counting the number of pair cells within the nearest distance cut-off of 30 μm . The effective percentage of engagement of T Lymphocytes (CD3^+), CD8^+ T cells ($\text{CD3}^+\text{CD8}^+$), T_{RM} ($\text{CD3}^+\text{CD8}^+\text{CD103}^+$), and $\text{CD8}^+\text{CD3}^-$ and $\text{CD103}^+\text{CD3}^-$ non-T cell populations was analyzed using ISAT (see *methods*) for (A) desmoplastic ($n=7$), (B) pushing ($n=7$), and (C) replacement ($n=8$). Comparisons of cell populations or subpopulations were performed using the Kruskal-Wallis test, followed by a pairwise Mann-Whitney test adjusted by Bonferroni to identify the pairs significantly different. Statistical significance was calculated at a p -value of 0.05. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

6.4 Discussion

Over the past decade, there has been a rapidly growing interest in delineating the prognostic value of T-cells, including CD3⁺ T lymphocytes, CD4⁺ T regulatory cells (Katz et al., 2013) and CD8⁺ cytotoxic cells (Lazarus et al., 2018) for the OS of patients with CLM (Kong et al., 2019). The heterogeneous nature of TILs and their uneven spatial distribution within the TME suggest that the localization-dependent prognostic relevance of T cell densities may be a novel predictive biomarker for survival, response to cancer chemotherapy (Falco et al., 2016) and immunotherapies (Berthel et al., 2017; Halama et al., 2011). Such studies require an intact tumour landscape, but until recently, traditional IHC was the only technique available and was limited to detecting single markers at a time. A recent study that characterized the TME of deficient mismatch repair CLM using mIHC demonstrated an increased infiltration of cytotoxic CD3⁺CD8⁺ cells, which were often engaged with epithelial cells and impacted OS (Lazarus et al., 2018), thus, demonstrating the power of mIHC to reveal predictive spatial relationships. In that study, however, randomly selected tissue microarray cores were utilized, limiting the positional verification of the immune infiltrates, size of each segment of the liver-tumour interface and spatial assessment (Parra et al., 2017).

In the present study, CLM sections were used to accurately define the spatial cell infiltrate composition and the spatial engagement between nearest neighbouring cells using mIHC. Initially, the distribution of three well-known lymphocyte populations, including CD3⁺ T lymphocytes, CD8⁺ T cells, and TRM, was determined by tumour control. For example, CD8⁺ T cells have been previously recognized as cytotoxic T cells (Kong et al., 2018), and several recent studies reported a significant role of T_{RM} in tumour immunosurveillance (Duhon et al., 2018). All three lymphocyte subtypes were found in significantly higher IM levels compared with the LP or TC, thus confirming previous findings (Berthel et al., 2017; Halama et al., 2011; Wang et al., 2018). While all three phenotypes occur in higher IM levels, they display a spatially differential significant impact in patient OS. Thus, a high density of CD3⁺ cells in the TC was predictive of better OS. This population included all CD4 expressing phenotypes (T-helper subpopulations and regulatory T cells) and the CD4⁺CD8⁻ DNT cells and possibly other unconventional T cells (Godfrey et al., 2018). To identify the

phenotype associated with the improved OS, additional investigation is warranted, given recent studies in an animal model of CLM demonstrating a significant increase of a novel DNT population in response to therapy (D. L. Vallejo Ardila et al., 2020). Nevertheless, focusing on markers that would differentiate the populations makes it feasible to determine the accountable T cell subtype accurately. In contrast, high CD8⁺ T cell levels in the IM were also associated with increased OS. Other studies also reported this association in CLM (Katz et al., 2013; Kong et al., 2019; Peng et al., 2019; Wang et al., 2018) and other solid cancers (Dieci et al., 2020; Idos et al., 2020). Also, high ratios of intratumoral CD8/CD3 T cells have been reported to be more sensitive predictors of survival than the density of either phenotype (Peng et al., 2019). There was no survival difference in those patients with high levels of T_{RM} expressing CD3⁺CD8⁺CD103⁺ compared to those with low levels at either the IM or the TC location. The distinct genetic signature of T_{RM} is often defined by the expression of several surface markers, including CD69, CD103, CD49a and CD44 (Cognac et al., 2018). While in other studies, the intratumoral presence of T cells expressing the integrin CD103 has been strongly correlated with a favourable prognosis for cancer patients (Dumauthioz et al., 2018), this phenotype was reported to occur in low levels in CLM (Duhén et al., 2018) and these findings support this. Despite this fact, in the univariate analysis of spatial engagement, a better prognosis was associated with T_{RM} and CD8⁺ T cells' proximity, which has not been previously reported.

Notably, a novel population of CD8⁺ T cells expressing E-cadherin was identified, which is associated with significant patient OS improvement. This is the first study to report this novel phenotype in tumours. A previous study reported E-cadherin positive CD8⁺ T lymphocytes promoting T_{RM} accumulation after systemic lymphocytic choriomeningitis virus (LCMV) infection in murine salivary glands (Hofmann & Pircher, 2011). The precise role of E-cadherin positive CD8⁺ T cells in tumour immunosurveillance remains to be further investigated.

Many CD8⁺ and CD103⁺ cells that did not co-express CD3⁺ were quantified. While CD8 and CD103 expression has been reported on human NK cells (Ahmad et al., 2014) and NK-like CD8 T cells (Barbarin et al., 2017), both CD8 and CD103 (Williford et al., 2019) expression has been reported on DC in mice (Fu & Jiang, 2018). Surprisingly, high levels of their expression have not been considered in many studies investigating T

lymphocytes' levels. Traditionally, in tumour studies, CD8 positivity is associated with T cells; thus, errors are introduced if the CD3 co-expression is not investigated, as in the case of most IHC (Angelova et al., 2018; Halama et al., 2011; Peng et al., 2019; Wang et al., 2018). While higher levels of these two cell populations did not predict better OS, the longer their spatial distance from E-cadherin, the better the clinical outcome OS, similar to CD8⁺ T cells. Further analysis is required to characterize the exact nature of these non-T cell populations and their implications in tumour growth. In other studies, a critical role was reported for CD103⁺ DC in the cross-priming of antigen-specific CD8⁺ T cells and represents a new perspective for tilting the balance between cell immunity versus tolerance to tumour antigens (Fu & Jiang, 2018) because the loss of CD103⁺ DC is known to increase seeding of metastases (Salmon et al., 2016).

The biomarkers α -SMA and E-cadherin were included as their expression have been linked to tumour aggressiveness, inflammation, and metastasis (Peng et al., 2019). It was suggested that the dense collagen matrix produced by α -SMA⁺ CAFs presents a physical barrier to the infiltration of T lymphocytes (Levental et al., 2009; Salmon et al., 2012) and compresses tumour blood vessels leading to hypoxia and acidosis in the tumour stroma (Levental et al., 2009) impacting the activity of T lymphocytes (Calcinotto et al., 2012). However, α -SMA⁺ stromal cell depletion promotes primary tumour progression by inducing immunosuppression in pancreatic cancer (Özdemir et al., 2014), while the effect of angiotensin receptor blocker reprogrammed CAF to an immune-supportive state (Chauhan et al., 2019). In this chapter, it was observed that a greater spatial engagement between CD8⁺ T cells and α -SMA⁺ predict better OS in CLM, with a similar finding for the proximity between CD8⁺(CD3⁻) cells and α -SMA⁺. These findings and the published literature indicate the complex relationships of α -SMA⁺ cells within the TME. Further studies are needed to elucidate their involvement with CD8⁺ T cells and antitumour activity, offering new therapeutic options for immune modulation in CLM.

Contrary to accepted opinion in the literature that strong tumour E-cadherin expression is associated with less aggressive and less metastatic tumours, current findings challenge this. Thus, it was found that better patient OS when tumour infiltrating cells (CD8⁺ T cells and CD8⁺ or CD103⁺ non-T cells) are not near E-cadherin

positive cells in the TME. Furthermore, the highest E-cadherin positive tumours display a replacement growth pattern associated with a worse prognosis than the other two CLM growth patterns (Eefsen et al., 2015).

Importantly, results indicate that CLM histological growth patterns do not dictate changes in overall cell density but display the distinct spatial distribution of T-cell subtypes and other immune cells across the tumour landscape. Previous reports had shown that desmoplastic growth pattern had higher infiltration than the pushing and replacement-type (Nielsen et al., 2014). However, there was no spatial and phenotypical characterization of T-cell subtypes (Nielsen et al., 2014). Thus, the desmoplastic pattern featured a lower density of CD8⁺ (CD3⁻) cells, whereas the replacement pattern had a lower density of CD103⁺ (CD3⁻) cells. Their engagement profile indicated that in the replacement pattern, T_{RM} would engage poorly with CD3⁺ T lymphocytes, CD8⁺ T cells, CD8⁺(CD3⁻) cells, and CD103⁺(CD3⁻) cells. This is the first study to report a detailed characterization of how T cell spatial distribution and engagement is correlated with the different histologic growth patterns of CLM.

This study's main limitations were the relatively small cohort of whole tissue specimens included because of the limited number of slides that can be manually stained using mIHC together with the high cost of the staining procedures and automated image analyses previously reported (Remark et al., 2016). Also, the retrieval of clinical information including mutation status of specific molecular prognostic markers (*KRAS*, *BRAF* and MMR), the primary tumour location of these patients and neoadjuvant therapies before CLM resection, was not available when requested to VCB. *KRAS* and *BRAF* genes are well-known molecular prognostic biomarkers associated with poor patient survival (Tsilimigras et al., 2018). The differences in neoadjuvant chemotherapy may have caused bias, yet no other study has reported any neoadjuvant chemotherapy associations with immune infiltration or patient survival (Hof et al., 2019).

6.5 Conclusion

Taken together in this study was confirmed published results on the predictive value of T-lymphocyte phenotypes in CLM and highlighted the importance of their spatial location. Additionally, a novel E-cadherin positive CD8⁺ T cell phenotype with significant OS prognostic value was identified. Several immune cell interactions with other immune or stromal cells were also shown to have predictive value. These results highlight the clinical importance of performing integrative analysis of the immune landscape to identify robust predictive markers, which may help stratify patients using the immune score and considering the histologic tumour growth patterns. The use of whole tumour sections and the long-term follow-up of these patients enabled identifying markers that should be further evaluated in future prospective studies with a larger cohort of patients.

Chapter 7

Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in human colorectal liver metastases

This chapter aims to investigate the effects of RASi on T lymphocyte composition and spatial distribution in human colorectal liver metastases using multiplex immunohistochemistry and machine-learning-based algorithms.

This chapter is presented as a regular thesis chapter which includes unpublished results.

CHAPTER 7

THE IMMUNOMODULATORY EFFECT OF RENIN- ANGIOTENSIN INHIBITOR ON T - LYMPHOCYTES IN HUMAN COLORECTAL LIVER METASTASES

7.1 Introduction

The effects of RASi have been evaluated based on cancer risk susceptibility and survival outcomes within large retrospective cohorts, systematic reviews and metaanalyses, concluding that there are no differences in new cancer incidence between patients on RASi medications compared to never-users (Bangalore et al., 2011; Pinter et al., 2017; Sun et al., 2017; Teo et al., 2011), which was then confirmed by the FDA (Perini et al., 2020). However, the potential benefits of RASi in cancer patients have been described by individual reports in some cancer types, including breast cancer (Chae et al., 2011; Coulson et al., 2017; Rhodes et al., 2009), pancreatic cancer (Liu et al., 2017; Mandilaras et al., 2017), gastric cancer (Kim et al., 2012), hepatocellular carcinoma (HCC) (Pinter et al., 2017), CRC (Chiang et al., 2014; Ozawa et al., 2019) influencing survival outcomes and decreasing the incidence of recurrence and metastases (Engineer et al., 2013). Additionally, other favourable effects such as preventing cancer treatment-related adverse events (Pinter et al., 2018) and enhanced response to bevacizumab on CLM have been associated with RASi (Osumi et al., 2015). Diverging findings of the potential anti-tumour benefits for patients on RASi as antihypertensive medication have been reported in retrospective cohorts studies (Sun et al., 2017), which omitted several confounding factors such as tumour heterogeneity, tumour stage, hormone receptor status, EGFR2 expression, and neoadjuvant treatment (Pinter & Jain, 2017).

Pre-clinical and clinical evidence (Perini et al., 2020; Pinter & Jain, 2017; Regulska et al., 2019) and computational studies (Nowak-Sliwinska et al., 2019; Pereira et al., 2019) support drug repositioning of RASi as a cancer treatment. Additionally, a meta-analysis suggested that RASi may improve survival rates depending on the cancer type and the class of RASi (Sun et al., 2017). Furthermore, robust functional evidence supports the rationale of repurposing both ACEi and ARBs to impair tumour progression, tumour vascularization, and metastasis using *in vitro* and *in vivo* experimental cancer models (Perini et al., 2020).

In the CLM mouse model, both captopril ACEi and irbesartan (ARB/AT1R antagonist) significantly decreased tumour growth and central microvascular density. However, there was no impact in survival or tumour necrosis reported by either of these drugs

in this study (Neo et al., 2007). More recently, using the same animal model, we have shown that captopril upregulates CD3⁺ T cells, decreases CD4⁺ T cells and CD8⁺ T cells in the adjacent liver segment while increasing CD8⁺ T cells and T_{RM} expressing CD103 in the tumour centre in mice with CLM (D. L. Vallejo Ardila et al., 2020). In another study, Nakamura et al. showed that valsartan and candesartan (ARB/AT1R antagonist) enhanced the induction of tumour antigen-specific CD8⁺ T cells (Nakamura et al., 2018). However, there was limited effect to complete the tumour destruction, leading to tumour recurrence (Neo et al., 2010; Neo et al., 2007; D. L. Vallejo Ardila et al., 2020). Consequently, RASi may potentially be used in combination with other cancer immunotherapeutic agents to enhance their efficacy and delivery (Martin et al., 2020; Mpekris et al., 2020).

In previous studies using the MoCR-CLM mouse model, we have shown that captopril changes T-cell phenotype levels and their spatial distribution (D. L. Vallejo Ardila et al., 2020). These results prompt us to investigate T-cell distribution in human CLM in specimens collected at CLM resection. The relevance of integrating spatial phenotypic immune profiling of CD3⁺ T lymphocytes, CD8⁺ T cells and T_{RM} on CLM has been investigated in chapter 6. The three phenotypes occurred in higher levels in the IM, and they showed a spatially differential significant impact in patient OS. Hence, a high density of CD3⁺ cells in the TC and a high density of CD8⁺ T cells in the IM were predictive of better OS on CLM.

Additionally, it was demonstrated how T cell spatial distribution was determined by the CLM growth pattern. This study hypothesised that T-cell density and spatial distribution within the TME are modulated by RASi (ACEi/ARBs) and could impact patient survival. Chapter 6 is the study's control arm, but as has been shown in that study, analysis of the spatial immune cell distribution in patients, even if therapy treatments and other confounding factors were not considered, yielded new significant and valuable information.

The current chapter is an extension of the findings in chapter 6. Initially, it was hypothesized that T-cell density and their spatial distribution might be modulated by RASi (ACEi/ARBs) across different human CLM tissue compartments. Moreover, T cell density and spatial dynamic were integrated to determine whether changes in density and distribution may influence patients' survival after CLM resection, using a

retrospective cohort that received RASi as antihypertensive medication before surgery, compared to patients who had not been on any antihypertensive medications. An integrative spatial analysis was performed using machine learning-based algorithms to determine differences in cell density and distribution, spatial engagement and CLM growth pattern between patients on RASi medications and non-treated patients after CLM resection. Moreover, it was investigated if these changes in the immune infiltration pattern were associated with improved OS.

7.2 Methods

A 7-plex OPAL™ protocol was used to assess T cell markers' composition and spatial distribution (CD3, CD8, FoxP3 and CD103) and the EMT markers (α -SMA and E-cadherin). The protocol was manually optimized and validated in two independent cohorts of FFPE whole tissue specimens of CLM using well-established antibodies, single spectral library, negative controls, and biological controls for corroborating staining pattern of α -SMA⁺ (sclerosed hemangioma samples) and immune infiltrates (normal liver tissue). The RASi group consisted of twenty-one CLM specimens collected from patients on RASi (ACEi and ARBs) for hypertension prior to liver resection. In contrast, the control group contained seventeen CLM specimens collected from patients who had not been on any antihypertensive medications. Some of these specimens were previously stained and analysed in chapter 6. All samples were analysed using the same batch analysis algorithm for tissue segmentation and cell phenotyping on inForm® software v2.4.2.

The specific antibodies used, the mIHC staining procedures, image analysis and cell density quantitation, spatial tumour region delineation and distribution of positively stained cells are fully described in chapter 2 (**Human Colorectal Liver Metastases immune profiling**, page numbers 48-55).

7.3 Statistical analysis

7.3.1 Cell density analysis

Cell density (cell/mm²) was calculated by combining cell count from all HPFs normalized according to the area of interest (HPF stamp size 3x3= 2046 x 1530 μ m,

resolution= 0.5 μm equivalent to 1 pixel, x 20 magnification). Data visualization was performed using functions in the *ggplot2* package for generating graphics and pairwise statistical comparisons (Wickham, 2016).

7.3.2 Survival analysis and KM curves

The patient's survival post-liver resection was interrogated in two separate univariate analysis to find differences in the immune pattern as follows: intergroup differences (RASi^{high} to Control^{high} and RASi^{low} to Control^{low}) and intragroup differences (RASi^{high} to RASi^{low} and Control^{high} to Control^{low}). The median cell density of each phenotype was used to stratify patients in whose tumours showed a low density for each group (RASi^{low} and control^{low}), defined as below the median cell density, with those that had high density (RASi^{high} and control^{high}), or above the median cell density in the whole tissue, the LP, IM or the TC. In the first univariate analysis, pairwise comparisons were made between RASi^{high} and control^{high}. Subsequently, RASi^{low} was compared to control^{low}. In the second univariate analysis, pairwise comparisons were performed between RASi^{high} and RASi^{low}. Successively, control^{high} and control^{low} were compared. This involved Log-rank (Mantel-Cox) test comparison between the two groups. Statistical analysis was performed using GraphPad Prism Statistics version 8.3 (GraphPad Software, Inc., San Diego CA). KM plots (for more than two groups) were performed using functions of *ggsurvminer* included in the *ggplot2* package for generating graphics and pairwise statistical comparisons (Wickham, 2016).

7.3.3 Code availability

Customized R functions to assess spatial analyses based (on the x, y coordinates) data provided by inForm® software v2.4.2 (Perkin Elmer/Akoya, MA) are available in the supplementary material section. The ISAT R package (Wang, 2018) is available online. The *ggplot2* package for generating graphics is available online (Wickham, 2016).

7.4 Results

7.4.1 Demographic and clinical characteristics

Two independent cohorts of FFPE whole CLM sections from thirty-eight resected specimens were evaluated by mlHC (OPAL™ protocol) to assess the presence and spatial distribution of T cell markers CD3⁺, CD8⁺, and CD103⁺ and EMT markers including α -SMA and E-cadherin. The RASi group consisted of twenty-one CLM specimens collected from patients (17 males and 4 females, mean age 63 years) who were submitted to liver resection at a Tertiary Hospital. These patients were selected because they had been on RASi (ACEi and ARBs) for hypertension prior to liver resection. The list of medications used by patients in the RASi group is shown in **Table 9**. The control group contained seventeen CLM specimens collected from patients who had not been on antihypertensive medications (14 males and 3 females, mean age 61 years). Using annotated patient data previously collected by VCB (**Table S4**), the OS was compared between the RASi cohort and controls. There was no significant survival difference between the group that had been on RASi for hypertension before liver resection and those that had not been using antihypertensive medications (Log-rank Mantel-Cox test $p=0.821$, HR= 1.104, 95% CI of ratio [0.461-2.639]) (**Fig. 42**). It was observed that up to five years of follow-up, the RASi group had better OS than the control group (Log-rank Mantel-Cox test $p=0.0143$, HR= 2.598, 95% CI of ratio [0.93-7.24], the number at risk: Control 9 and RASi 12).

Table 9. List of antihypertensive medications taken in the Group RASi before liver metastasectomy

ACE inhibitors	Sartans
Captopril	Candesartan
Enalapril	Eprosartan
Fosinopril	Irbesartan
Lisinopril	Losartan
Perindopril	Olmesartan
Quinapril	Telmisartan
Ramipril	Valsartan
Trandolapril	

The data retrieval was performed by VCB based on antihypertensive medications listed in the **Australian medicines Handbook (AMH) 2015 edition**.

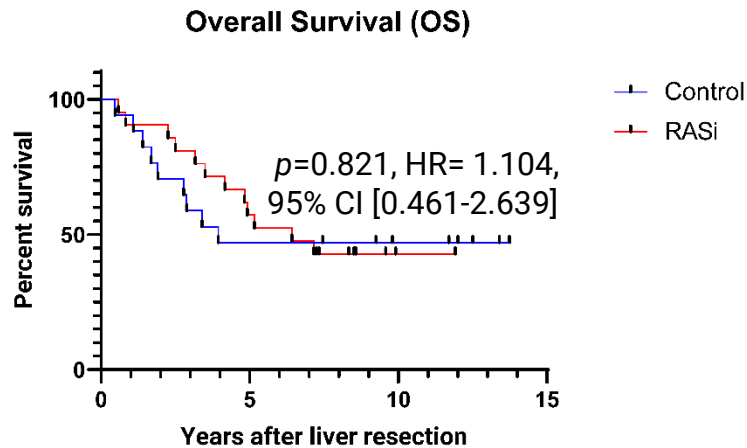


Figure 42. Overall survival between RASi and Control groups. RASi group is shown in red, and Control in blue continues lines. The cause of death was related to the advanced stage of the primary tumour (Colorectal cancer). Time is shown in years after CLM resection. Log-rank (Mantel-Cox test) p -value, Hazard ratio (HR) and 95% confidence interval (CI) are annotated.

7.4.2 Spatial and phenotypical characterization of CLM infiltrating immune cells within the tumour and adjacent liver parenchyma in patients using RASi medications

OPAL-stained sections were used to determine tissue segmentation for the control group (**Fig. 43A**), and RASi treated group (**Fig. 43B**). InForm® (Perkin Elmer/Akoya, MA) (Stack et al., 2014) software was used to perform supervised image analysis using the Tissue finder algorithm for trainable pattern recognition. Tissue segmentation was defined as follows: adjacent liver parenchyma depicted in red, invasive tumour margin in blue, and tumour core in green (**Fig. 43A, B**). Regions of interest were defined by fixed stamping (HPF stamp size 3x3) on the highest TIL density for both groups shown in the pathology view analysis; thus, cell density was calculated combining cell count from all HPFs normalized according to the area of interest (2046 x 1530 μm) (Halse et al., 2018; Huang et al., 2019) (**Fig. 43 A, D**).

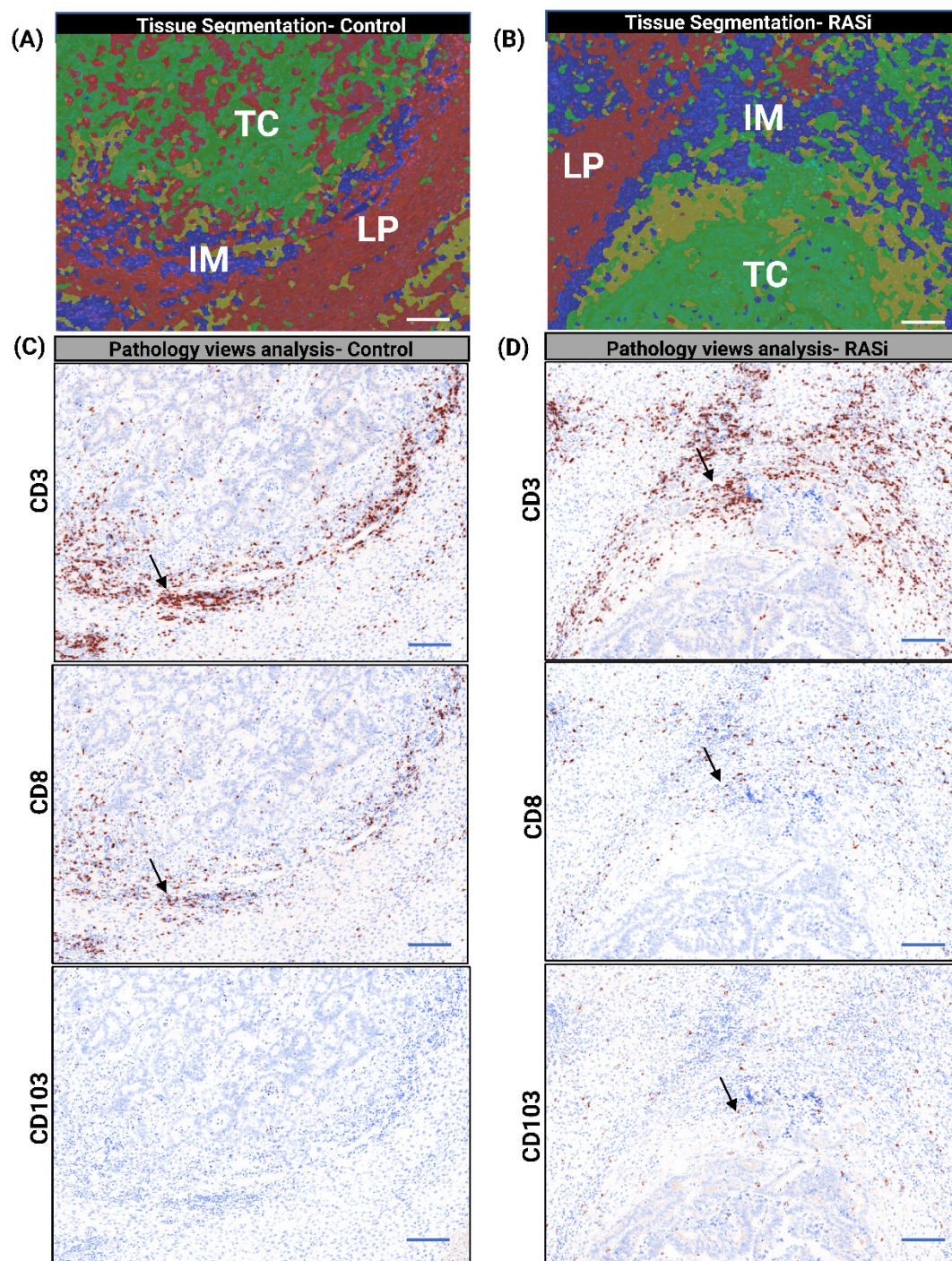


Figure 43. Representative microphotographs of the tissue segmentation using Vectra® imaging and inForm® pathology view analysis. Tissue segmentation displaying adjacent liver parenchyma (LP) in red, invasive tumour margin (IM) in blue, and tumour core (TC) (including tumour stroma and epithelial interface) in the green representative image for (A) control and (B) RASi group (scale bar: 100µm). Cell marker positivity CD3⁺, CD8⁺, CD103⁺ assess on Pathology view analysis using inForm in the (C) control and (d) RASi group (scale bar: 100µm).

Image quantitation analysis of the mIHC stained tissues was performed in two separate *batch* analyses using cell phenotyping on inForm® (Perkin Elmer/Akoya, MA)

(Stack et al., 2014). In the first batch, the following cell phenotypes were scored: CD3⁺ T lymphocytes (CD3⁺CD8⁻CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺CD103⁺), non-lymphocyte infiltrating cells expressing CD103⁺ and cells positive for α-SMA or E-cadherin within the LP, IM, and TC, was scored to compare cell density and distribution between the control group and RASi group (**Fig. 44A, B**). A separate phenotype scoring an additional phenotype was assigned to identify CD3⁺ expressing FoxP3⁺, which was investigated exclusively in the RASi group. Thus, CD3 positive cells in the first batch contained both the single marker and combination of markers CD3⁺ and FoxP3⁺ (**Fig. 44C**). T_{regs} express FoxP3, which is required for their development and function (Zou, 2006).

Based on the phenotypes above, cell density and spatial distribution within LP, IM, and TC in the RASi group were investigated. The highest T cell density corresponds to CD3⁺ T lymphocytes, which showed significantly higher levels in the IM (median cell density (MCD) ≈3300 cells/mm²) compared with TC (MCD ≈2750 cells/mm²; $p=0.0029$) and LP (MCD ≈2250 cells/mm²; $p=0.0045$). There was no significant difference in the number of CD3⁺ T cells accumulated within the LP and TC. Other T cell subpopulations had densities lower than CD3⁺ T cells (CD3⁺CD8⁻CD103⁻) (MCD ≤1000 cells/mm²), and their pattern of distribution differed for each subpopulation. Higher levels of CD8⁺ T cells were located in the IM ($p= < 0.0001$), followed by the LP and the TC ($p= < 0.0001$), whereas significantly higher levels of T_{RM} were found in the LP compared with IM ($p= < 0.0001$) and TC ($p= < 0.0001$). Lastly, the levels of CD3⁺FoxP3⁺(CD8⁻CD103⁻) were comparable in the LP and TC, while lower density was found in the IM (**Fig. 43C**). Altogether the cell density and spatial distribution of these three well-known lymphocyte subpopulations in the RASi group showed that among CD3⁺ T lymphocytes other than CD8⁺ T cells, T_{RM} and CD3⁺FoxP3⁺ need to be further phenotypically characterized since they only account for a rather small percentage of the total number of cells encompassed within this population.

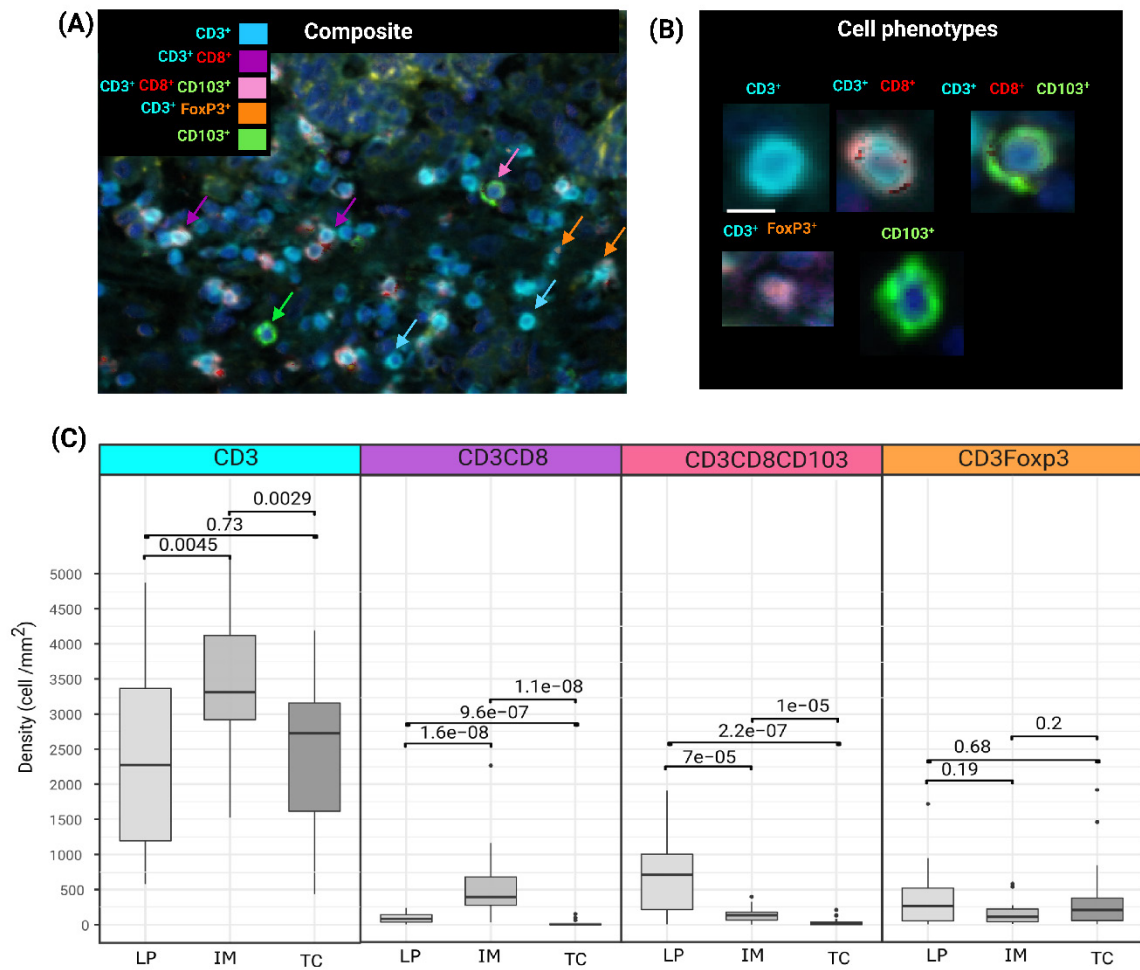


Figure 44. Cell density calculated for the RASi group relative to their distribution at the liver-tumour interface, including FoxP3 cell phenotypes. (A) Representative composite image displaying cell subpopulations CD3⁺, CD3⁺CD8⁺, CD3⁺CD8⁺CD103⁺, CD3⁺FoxP3⁺, CD103⁺. (B) Representative cell phenotypes stained by OPAL (scale bar: 10µm). (C) Immune profile of RASi plotted as cell density. Statistical analysis was performed comparing tissue segmentation using Mann Whitney U test, and significant different data is annotated. Key: Cyan: CD3⁺, Bright Green: CD103⁺, Purple: CD3⁺CD8⁺, light pink: CD3⁺CD8⁺CD103⁺ and Orange: CD3⁺FoxP3⁺

7.4.3 The effect of RASi on Human CLM alters cell density and spatial distribution of T cell populations

The cell density of CD3⁺ T lymphocytes, CD8⁺ T cells and T_{RM} was compared between the control group and RASi treated group within the adjacent LP (Fig. 45A, B), IM (Fig. 45C, D) and TC (Fig. 45E, F). In the adjacent LP, T_{RM} was found significantly higher in the RASi group than in control ($p = < 0.0001$), whereas no significant change was defined for CD3⁺ T lymphocytes and CD8⁺ T cells (Fig. 45B). In addition, CD8⁺ T cells and T_{RM} subpopulations were significantly higher in the RASi group in the IM when compared to control (Fig. 45C, D), while there was a significant reduction of CD8⁺ T

cells in the TC ($p=0.0014$) (**Fig. 45E, F**). In general, RASi did not change cell density or spatial distribution of CD3⁺ T lymphocytes within the liver-tumour interface. Also, these results demonstrate a broader variation in CD3⁺ T in the RASi group compared to the control (**Fig. 45**). Instead, the response to RASi was selective by upregulating T_{RM} and CD8⁺ T cells within the LP and IM (**Fig. 45A-D**), respectively, while only decreasing CD8⁺ T cells in the TC (**Fig. 45E, F**).

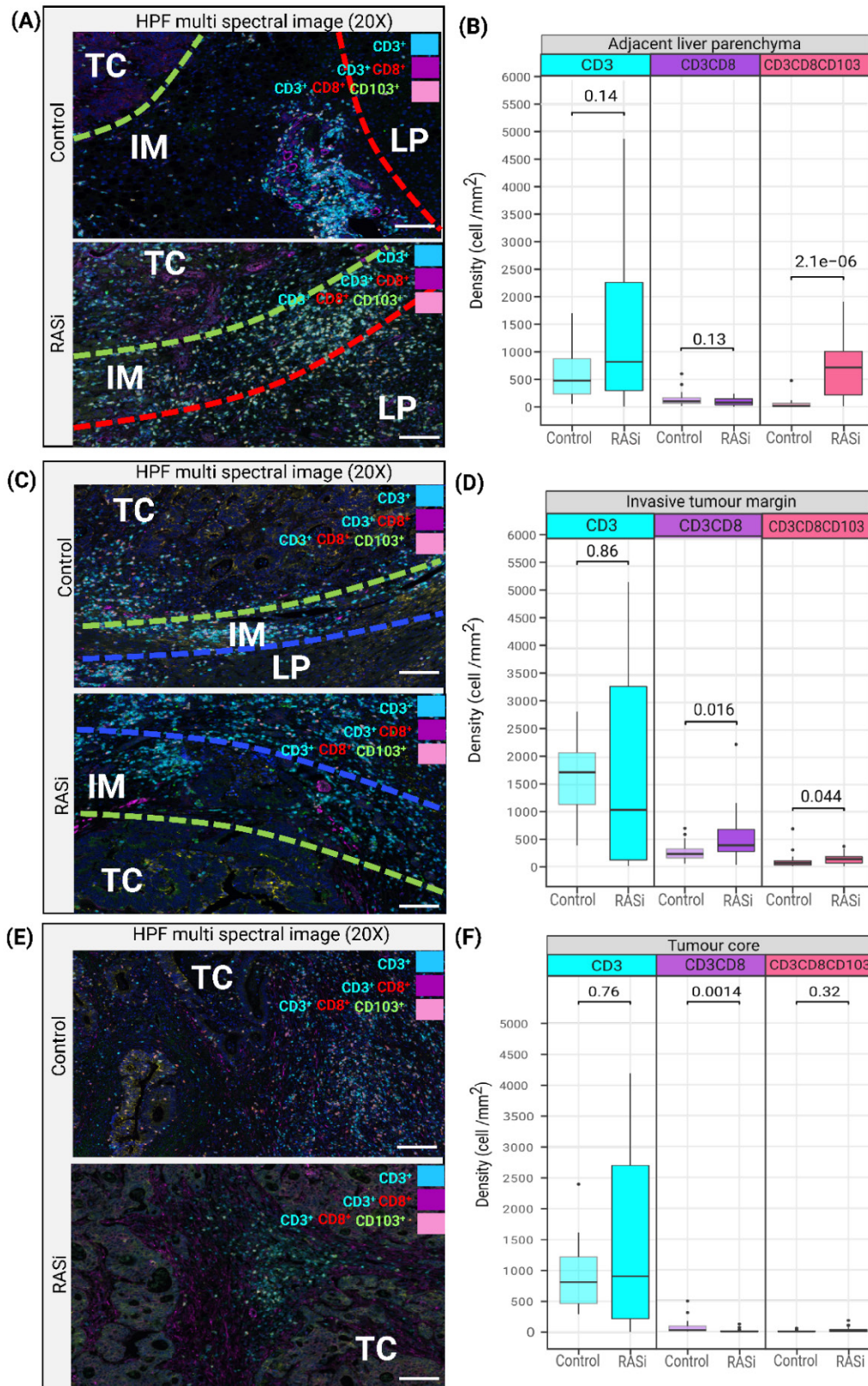


Figure 45. Comparison between T-cell density and spatial distribution in the control and RASi group. The cell density of T Lymphocytes (CD3⁺CD8⁺CD103⁺), CD8⁺ T cells (CD3⁺CD8⁺CD103⁺), T_{RM} (CD3⁺CD8⁺CD103⁺) was compared between control and RASi group in (A-B) the adjacent LP, (C-D) IM and (E-F) TC (scale bar: 100µm) Statistical analysis was performed using Mann Whitney U test, and significant different data is annotated. Key: Cyan: CD3⁺, Purple: CD3⁺CD8⁺ and light pink: CD3⁺CD8⁺CD103⁺.

7.4.4 The influence of RASi on CLM patient survival integrating T- cell density and their spatial distribution on the liver-tumour interface

A widespread T cell density was reported within the control and RASi group. Comparing the median T cell density of patients on RASi medications against controls to infer OS differences after liver resection would not account for such variability. To account for the heterogeneity in the immune response observed, both differences intergroup (RASi^{high} to Control^{high} and RASi^{low} to Control^{low}) intragroup (RASi^{high} to RASi^{low} and Control^{high} to Control^{low}) were interrogated to ascertain the location-dependent prognostic value for each phenotype.

The median cell density of each phenotype: CD3⁺ T Lymphocytes (CD3⁺CD8⁻CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺CD103⁺) and non-lymphocyte infiltrating cells expressing CD103⁺, was used to stratify patients. CLM that showed a low density for each group (RASi^{low} and control^{low}), defined as below the median cell count distribution, with those that had high density (RASi^{high} and control^{high}), or above the median cell density in the whole tissue (WT), the LP, IM or the TC. Using annotated patient data previously collected by VCB, univariate OS was carried out (**Table S5**). The median follow-up for the RASi group was 77 months (range 7-143 months), and for the control group was 47 months (range of 5- 160 months). The effect of RASi on cell density and spatial location of immune infiltration on OS was calculated. In a univariate analysis comparing RASi^{high} to control^{high} and RASi^{low} to control^{low}, the parameters taken into consideration were overall cell density and spatial distribution for each group. No significant differences in OS were found when CD3⁺ T cell density was compared between RASi^{high} and control^{high} in any of the tissue segments (Log-rank *p-value*: WT= 0.28; LP= 0.31; IM= 0.64 and TC= 0.91). Similarly, no significant difference in survival was observed for CD3⁺ T cells density and distribution across all tissue segments in the pairwise comparison between RASi^{low} to control^{low} (Log-rank *p-value*: WT= 0.71; LP= 0.53; IM= 0.87 and TC= 0.78) (**Fig. 46A-E**).

The effect of RASi on cell density and spatial distribution of immune infiltration on OS was calculated. In a univariate analysis comparing RASi^{high} to RASi^{low} and control^{high} to control^{low}, the parameters considered were overall cell density and spatial distribution for each group. Thus, high density of CD3⁺ T cells had a significant impact on RASi^{high} patients compared to RASi^{low} in the WT (Log-rank *p*=0.0064) (**Fig. 46A**),

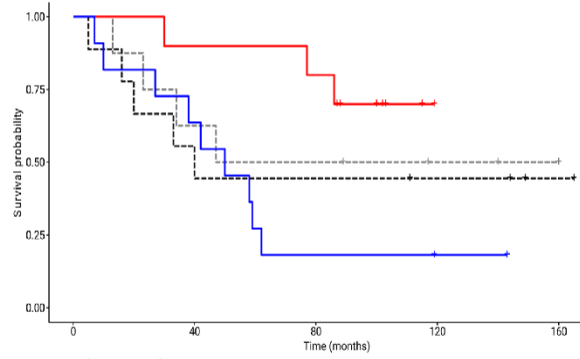
while no significant difference in survival was observed for CD3⁺ T cells in the pairwise comparison and control^{high} to control^{low} (Log-rank $p=0.7$) (**Fig. 46A**). Likewise, high levels of CD3⁺ T cells in the RASi group have a significant impact compared to RASi^{low} in the LP (Log-rank $p=0.021$), whereas no significant difference was found in the control group (**Fig. 46B**). In IM, CD3⁺ T cells showed no significant difference in survival in either group (**Fig. 46C**). In the TC, CD3⁺ T cells showed no significant difference in survival in either group (**Fig. 46D**). OS pairwise comparisons for CD3⁺ T cells density for each segment are shown in **Fig. 46E**.

Overall survival (OS): Median cell count of CD3⁺T-lymphocytes

Spatial distribution across all tissue segments

Strata — RASi High — RASi Low -- Control High -- Control Low

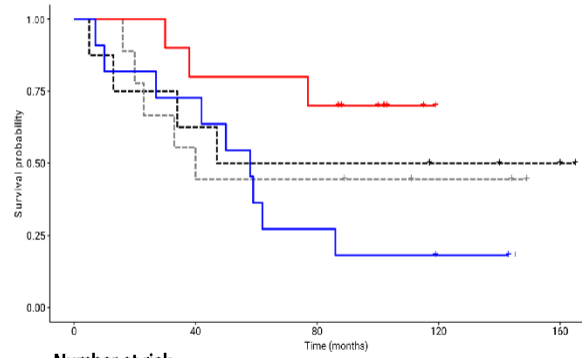
(A) Whole tissue



Number at risk

Control low	8	5	4	2	1
Control high	9	5	4	3	1
RASi high	10	9	8	0	0
RASi low	11	7	2	1	0

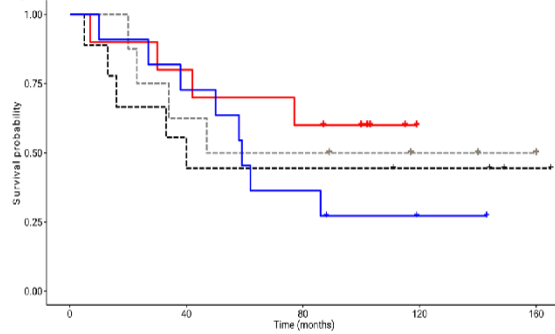
(B) Adjacent liver parenchyma



Number at risk

Control high	8	5	4	3	2
Control low	9	5	4	2	0
RASi high	10	8	7	0	0
RASi low	11	8	3	1	0

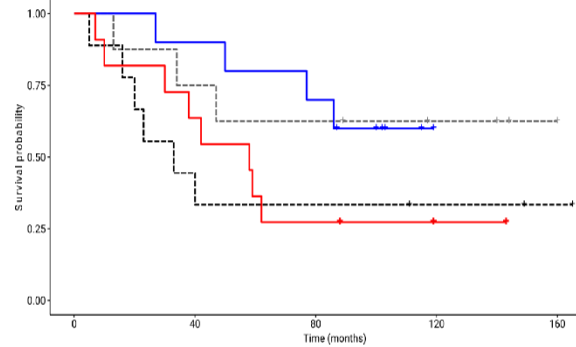
(C) Invasive tumour margin



Number at risk

Control low	8	5	4	2	1
Control high	9	5	4	3	1
RASi high	10	8	6	0	0
RASi low	11	8	4	1	0

(D) Tumour core



Number at risk

Control low	8	6	5	3	1
Control high	9	4	3	2	1
RASi low	10	9	7	0	0
RASi high	11	7	3	1	0

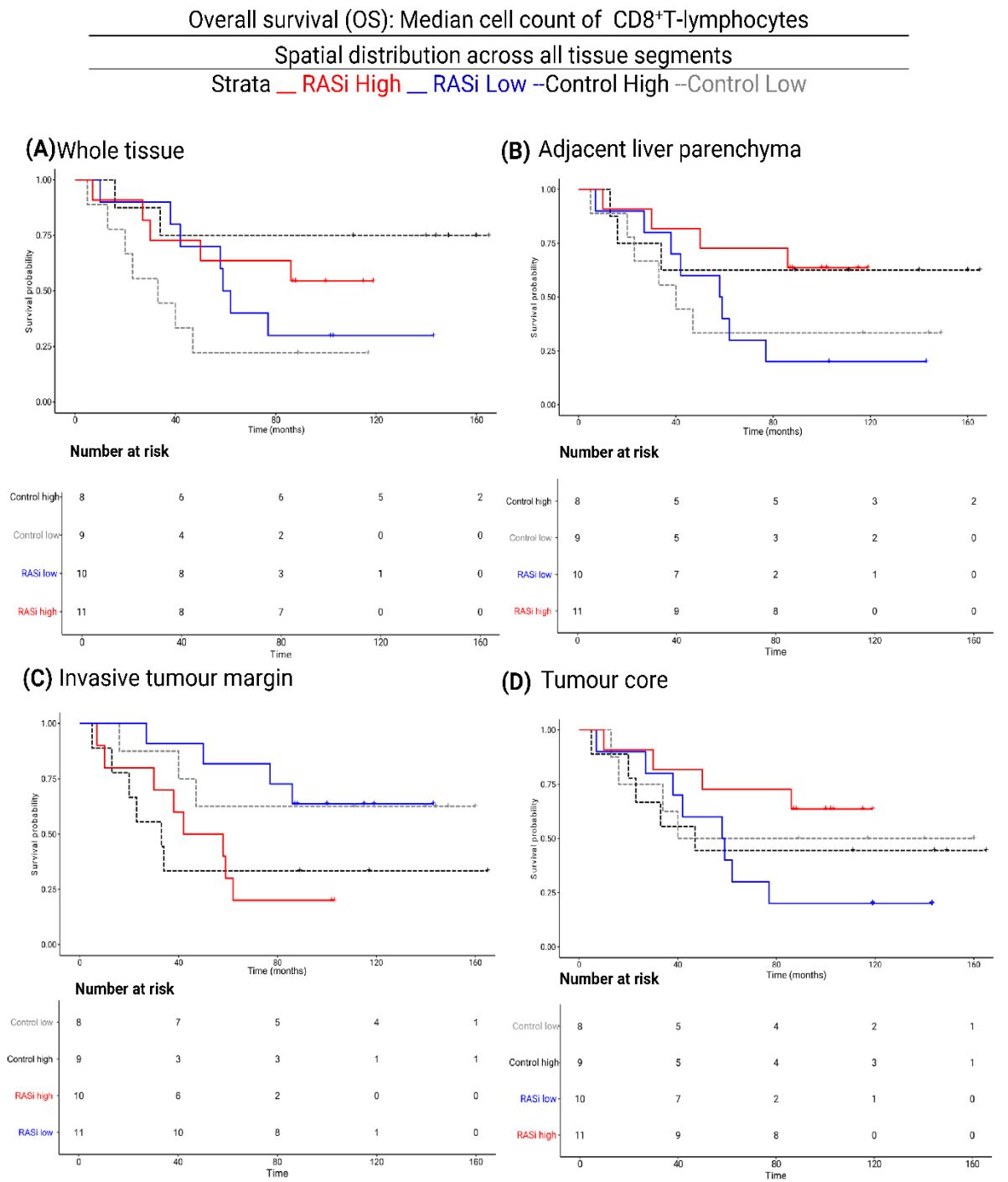
(E) OS pairwise comparisons for CD3⁺ T cells density

Pairwise Comparison	Tissue Segmentation	Log-rank (Mantel-cox) p-value
RASi High to Control High	WT	0.28
	LP	0.31
	IM	0.64
	TC	0.91
RASi Low to Control Low	WT	0.71
	LP	0.53
	IM	0.87
	TC	0.78
RASi High to RASi low	WT	0.0064
	LP	0.021
	IM	0.20
	TC	0.078
Control High to Control Low	WT	0.7
	LP	0.84
	IM	0.63
	TC	0.18

Figure 46. The influence of RASi on CLM patient survival post-liver resection univariate analysis using CD3⁺ T-cell density and spatial distribution. The patient's overall survival was interrogated using the median distribution of CD3⁺ T cell density in (A) the whole tissue (WT), (B) adjacent liver parenchyma (LP), (C) invasive margin (IM) and (D) tumour core (TC) for the control (control high in black and control low in discontinuous grey line) and RASi group (RASi high in red and RASi low in blue continues lines). The number at risk (time in months) (E) Log-rank (Mantel-Cox test) p-value for pairwise comparisons were calculated.

To determine whether the spatial distribution of CD8⁺ T-cells would impact the prognosis after liver resection, the prognostic value of CD8⁺T- cell density was explored within the WT, adjacent LP, IM, and TC. No significant differences in OS were found when CD8⁺ T cell density was compared between RASi^{high} and control^{high} in any of the tissue segments (Log-rank *p-value*: WT= 0.12; LP= 0.2; IM= 0.11 and TC= 0.46) (**Fig. 47A-E**). Comparably, no significant difference in survival was observed for CD8⁺ T cells density and distribution in any of the tissue segments in the pairwise comparison between RASi^{low} to control^{low} (Log-rank *p-value*: WT= 0.086; LP= 0.13; IM= 0.066 and TC= 0.3) (**Fig. 47A-E**). Moreover, no significant effect was observed in the CD8⁺ T cell density when comparing RASi high to RASi low, whereas improved survival in the Control was^{high} to control^{low} at the WT (Log-rank *p*=0.04) (**Fig. 47A**). High levels of CD8⁺ T cells show a trend of improved survival compared to RASi^{low} (Log-rank *p*=0.053), whereas no significant difference was found in the control group (Log-rank *p*=0.34) at the LP (**Fig. 47B**). Intriguingly, low levels of CD8⁺ T cells in the IM show an improved survival compared to RASi^{high} (Log-rank *p*=0.019), while no

significant difference was found in the control group (Log-rank $p=0.15$) (**Fig. 47C**). In contrast, High levels of CD8⁺ T cells had an impact on survival compared to RASi^{low} (Log-rank $p=0.053$), whereas no significant difference was found in the control group (Log-rank $p=0.83$) in the TC (**Fig. 47D**). OS pairwise comparisons for CD8⁺ T cells density for each segment shown in **Fig. 47E**.



(E) OS pairwise comparisons for CD8⁺ T cells density

Pairwise Comparison	Tissue Segmentation	Log-rank (Mantel-cox) p-value
RASi High to Control High	WT	0.12
	LP	0.2
	IM	0.11
	TC	0.46
RASi Low to Control Low	WT	0.086
	LP	0.13
	IM	0.066
	TC	0.3
RASi High to RASi low	WT	0.38
	LP	0.053
	IM	0.019
	TC	0.053
Control High to Control Low	WT	0.04
	LP	0.34
	IM	0.15
	TC	0.83

Figure 47. The influence of RASi on CLM patient survival post-liver resection univariate analysis using CD8⁺ T- cell density and their spatial distribution. The patient's overall survival was interrogated using the median distribution of CD8⁺ T cell density in (A) the whole tissue (WT), (B) adjacent liver parenchyma (LP), (C) invasive margin (IM) and (D) tumour core (TC) for the control (control high in black and control low in discontinuous grey line) and RASi group (RASi high in red and RASi low in blue continues lines). The number at risk (time in months) (E) Log-rank (Mantel-Cox test) *p*-value for pairwise comparisons were calculated.

To define whether the spatial distribution of CD103⁺ T_{RM} cells would impact the prognosis after liver resection, their prognostic value was explored within the WT, adjacent LP, IM, and TC. No significant differences in OS were found when CD103⁺ T_{RM} cell density was compared between RASi^{high} and control^{high} in any of the tissue segments (Log-rank *p*-value: WT= 0.34; LP= 0.73; IM= 0.44 and TC= 0.5) (**Fig. 48A-E**). Comparably, no significant difference in survival was observed for CD103⁺ T_{RM} cell density and distribution in any of the tissue segments in the pairwise comparison between RASi^{low} to control^{low} (Log-rank *p*-value: WT= 0.18; LP= 0.45; IM= 0.29 and TC= 0.39) (**Fig. 48A-E**). Furthermore, CD103⁺ T_{RM} cells across the liver-tumour segmentations showed no significant difference in survival in either of the groups (**Fig. 48A-E**).

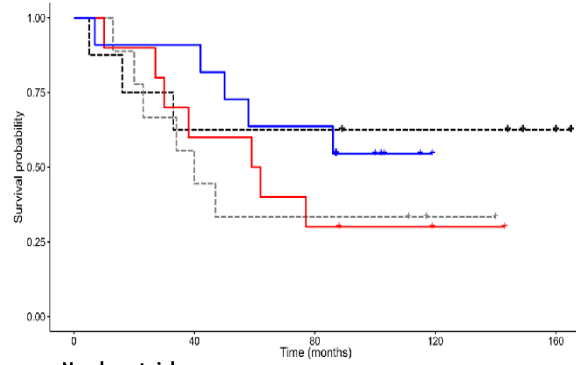
In summary, there is a significant improvement in the survival of RASi treated patients when high levels of CD3⁺ T cells and CD8⁺ T cells are found in the WT and LP, and CD8⁺ T cells high levels at the TC. There is no influence in survival changes over CD103⁺ T_{RM} cells in any of the spatial locations.

Overall survival (OS): Median cell count of T_{RM} CD103+

Spatial distribution across all tissue segments

Strata — RASi High — RASi Low — Control High — Control Low

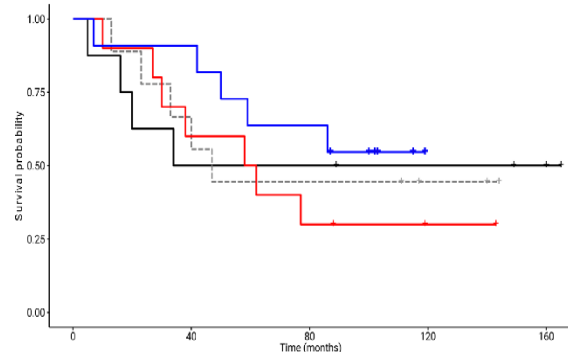
(A) Whole tissue



Number at risk

Control high	8	5	5	4	2
Control low	9	5	3	1	0
RASi high	10	6	3	1	0
RASi low	11	10	7	0	0

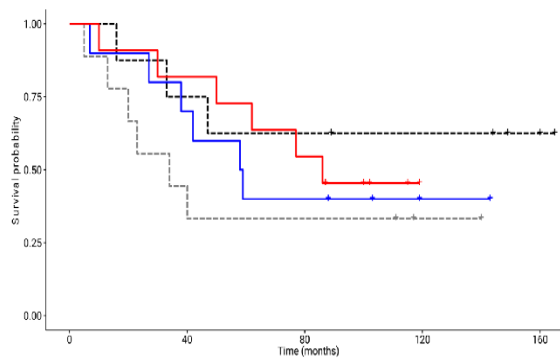
(B) Adjacent liver parenchyma



Number at risk

Control high	8	4	4	3	2
Control low	9	6	4	2	0
RASi high	10	6	3	1	0
RASi low	11	10	7	0	0

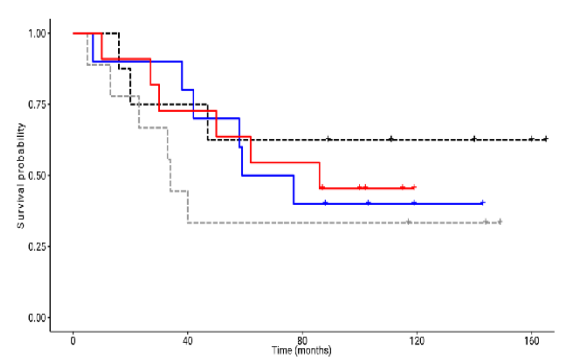
(C) Invasive tumour margin



Number at risk

Control high	8	6	5	4	2
Control low	9	4	3	1	0
RASi low	10	7	4	1	0
RASi high	11	9	6	0	0

(D) Tumour core



Number at risk

Control high	8	6	5	3	2
Control low	9	4	3	2	0
RASi low	10	8	4	1	0
RASi high	11	8	6	0	0

(E) OS pairwise comparisons for T_{RM} CD103⁺ cells density

Pairwise Comparison	Tissue Segmentation	Log-rank (Mantel-cox) p-value
RASi High to Control High	WT	0.34
	LP	0.73
	IM	0.44
	TC	0.5
RASi Low to Control Low	WT	0.18
	LP	0.45
	IM	0.29
	TC	0.39
RASi High to RASi low	WT	0.25
	LP	0.23
	IM	0.58
	TC	0.58
Control High to Control Low	WT	0.91
	LP	0.95
	IM	0.19
	TC	0.19

Figure 48. The influence of RASi on CLM patient survival post-liver resection univariate analysis using T_{RM} CD103⁺ T- cell density and their spatial distribution in the IM. The patient's overall survival was interrogated using the median distribution of CD8⁺ T cell density in (A) the whole tissue (WT), (B) adjacent liver parenchyma (LP), (C) invasive margin (IM) and (D) tumour core (TC) for the control (control high in black and control low in discontinuous grey line) and RASi group (RASi high in red and RASi low in blue continues lines). The number at risk (time in months) (E) Log-rank (Mantel-Cox test) p-value for pairwise comparisons were calculated.

7.4.5 The effect of RASi on Human CLM modifies cell density and spatial distribution of non-T cells expressing CD103

The expression of CD103 is also found on DCs (Williford et al., 2019) and tissue-resident NK cells in humans (Marquardt et al., 2019). A high frequency of non-T cells expressing CD103 had been previously reported in human CLM (see chapter 6); therefore, it was included in this analysis. Furthermore, FoxP3 had been reported in other studies on T_{regs} expressing CD103 (Allakhverdi et al., 2006; Leithäuser et al., 2006), but no report of non-T cells was expressing CD103 that co-express FoxP3⁺. Herein, it was discovered a novel non-T cell expressing CD103⁺ FoxP3⁺ (**Fig. 49A**). This population was not explored in the control group. The changes in cell density and spatial distribution of all phenotypes found for CD103⁺ (CD3⁻) due to RASi were explored. In the RASi group a significant reduction of CD103⁺ (CD3⁻) was found in the IM ($p= 0.00046$) and TC ($p= < 0.0001$) compared to the control group (**Fig. 49B, C**).

Overall, these results indicate that the cell density and the spatial location of another type of immune infiltrates besides T-cells were altered by RASi.

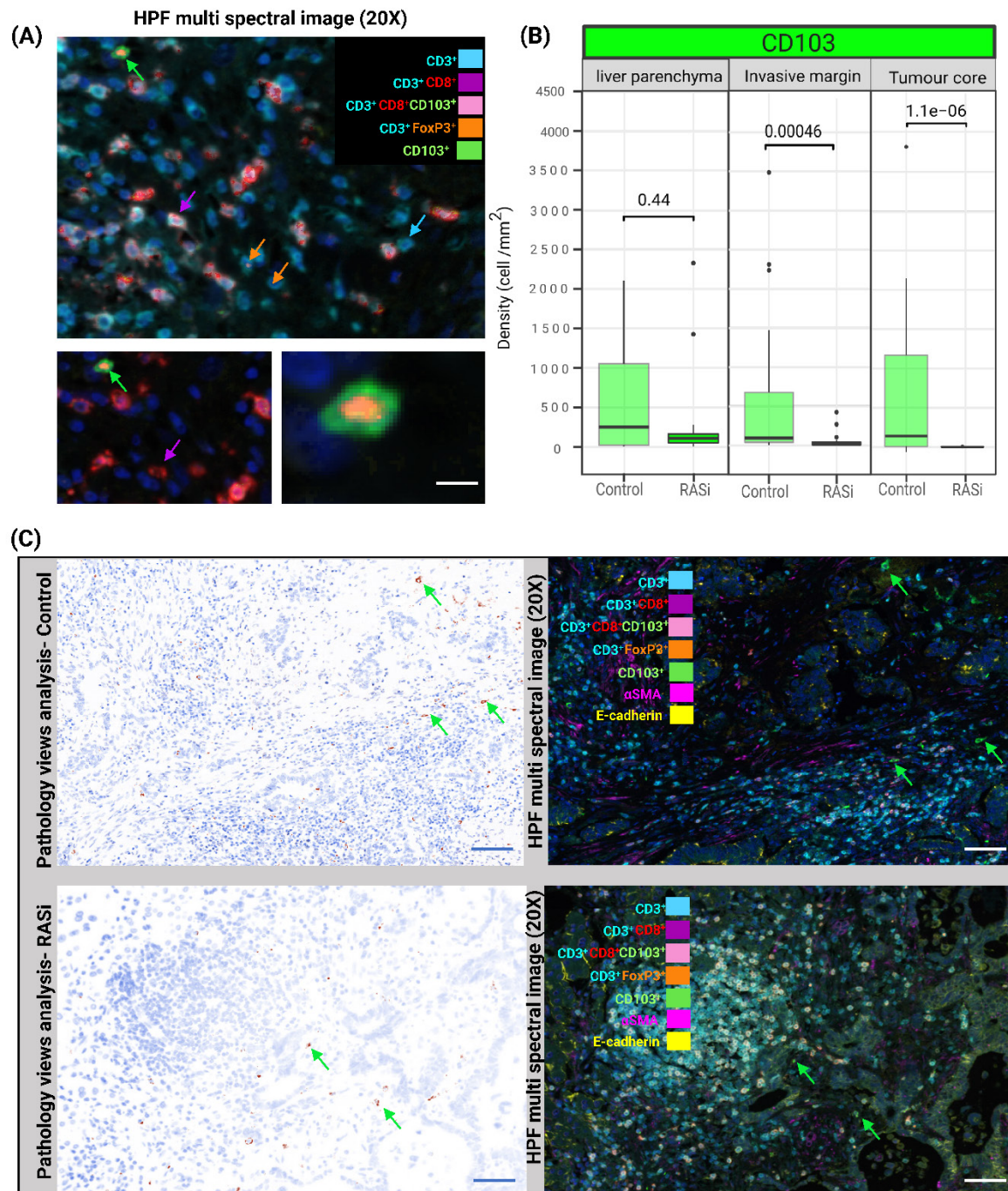


Figure 49. Comparison between cell density and spatial distribution of CD103+(CD3-) in the control and RASi group. (A) Representative composite indicating novel non-T cells expressing CD103+ FoxP3+ phenotype (scale bar: 10µm). (B) the density of CD103+(CD3-) was compared between the control and RASi group in the adjacent LP, IM, and TC. (C) The representative HPF displayed a CD103+(CD3-) comparison between control and RASi groups (scale bar: 100µm).

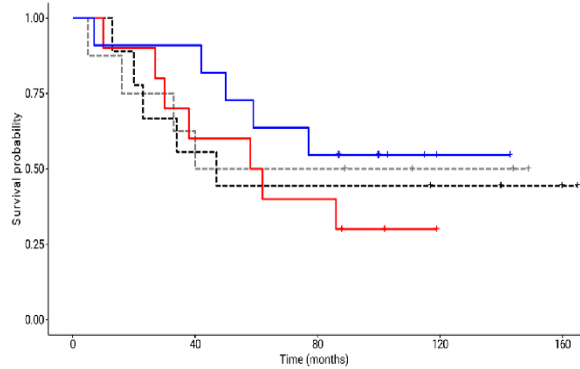
To ascertain whether the spatial distribution of non-T cells expressing CD103⁺ cells would impact the prognosis after liver resection, their prognostic value was explored at the WT, adjacent LP, IM, and TC. No significant differences in OS were found when CD103⁺ cell density was compared between RASi^{high} and control^{high} in any of the tissue segments (Log-rank *p-value*: WT= 0.63; LP= 0.85; IM= 0.96 and TC= 0.74) (**Fig. 49A- E**). Additionally, no significant differences in survival were observed for CD103⁺ cell density and distribution in any of the tissue segments in the pairwise comparison between RASi^{low} to control^{low} (Log-rank *p-value*: WT= 0.45; LP= 0.64; IM= 0.78 and TC= 0.51) (**Fig. 49A- E**). Furthermore, to determine the prognostic significance of the cell density changes of non-T cells expressing CD103⁺, it was compared RASi^{high} to RASi^{low} (Log-rank *p-value*: WT= 0.26; LP= 0.59; IM= 0.79 and TC= 0.39) and control^{high} to control^{low} (Log-rank *p-value*: WT= 0.91; LP= 0.91; IM= 0.91 and TC= 0.91), the parameters taken into consideration were median cell density and spatial distribution. There was no impact in survival caused by the changes over non-T cells expressing CD103⁺ cells in any tissue segments (**Fig. 49A- E**).

Overall survival (OS): Median cell count of non T cell expressing CD103+
Spatial distribution across all tissue segments

Strata — RASi High — RASi Low -- Control High -- Control Low

KM Plot survival analysis

(A) Whole tissue segments

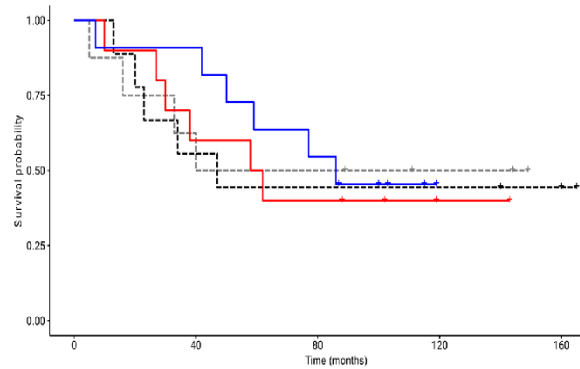


Number at risk

	0	40	80	120	160
Control low	8	5	4	2	0
Control high	9	5	4	3	2
RASi high	10	6	4	0	0
RASi low	11	10	6	1	0

Time

(B) Adjacent liver parenchyma

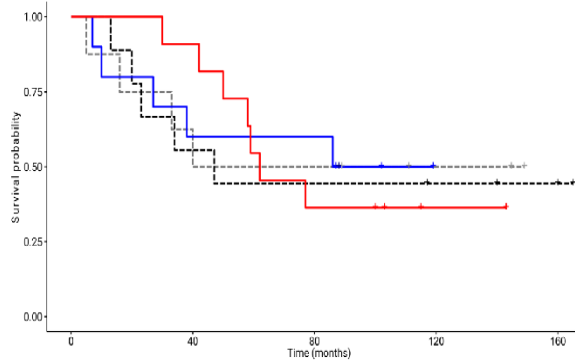


Number at risk

	0	40	80	120	160
Control low	8	5	4	2	0
Control high	9	5	4	3	2
RASi high	10	6	4	1	0
RASi low	11	10	6	0	0

Time

(C) Invasive tumour margin

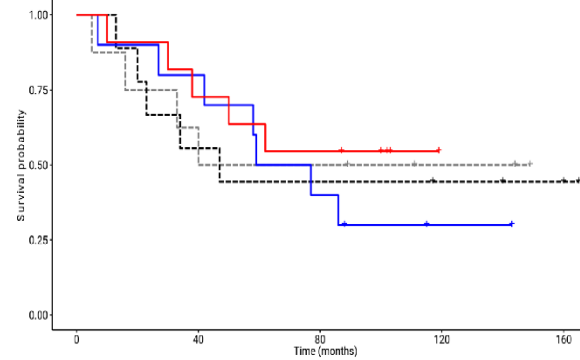


Number at risk

	0	40	80	120	160
Control low	8	5	4	2	0
Control high	9	5	4	3	2
RASi low	10	6	6	0	0
RASi high	11	10	4	1	0

Time

(D) Tumour core



Number at risk

	0	40	80	120	160
Control low	8	5	4	2	0
Control high	9	5	4	3	2
RASi low	10	8	4	1	0
RASi high	11	8	6	0	0

Time

(E) OS pairwise comparisons for non- T cells CD103⁺ cells density

Pairwise Comparison	Tissue Segmentation	Log-rank (Mantel-cox) p-value
RASi High to Control High	WT	0.63
	LP	0.85
	IM	0.96
	TC	0.74
RASi Low to Control Low	WT	0.45
	LP	0.64
	IM	0.78
	TC	0.51
RASi High to RASi low	WT	0.26
	LP	0.59
	IM	0.79
	TC	0.39
Control High to Control Low	WT	0.91
	LP	0.91
	IM	0.91
	TC	0.91

Figure 50. The influence of RASi on CLM patient survival post-liver resection univariate analysis using CD103⁺ (CD3⁻) cell density and their spatial distribution. The patient's survival was interrogated using the median distribution of cell density of CD103⁺ (CD3⁻) (A) the whole tissue (WT), (B) adjacent liver parenchyma (LP), (C) invasive margin (IM) and (D) tumour core (TC) for the control (control^{high} in black and control^{low} in discontinuous grey line) and RASi group (RASi^{high} in red and RASi^{low} in blue continues lines). The number at risk (time in months) (E) Log-rank (Mantel-Cox test) *p*-value for pairwise comparisons were calculated.

7.4.6 The effect of RASi on spatial expression patterns of E-cadherin and α -SMA

The changes in E-cadherin's positivity and spatial distribution (**Fig. 51**) and α -SMA (**Fig. 51B**) were assessed in the RASi group and control group. The results indicate that E-cadherin expression was significantly reduced across the liver-tumour segmentation in the RASi group compared to the control ($p = < 0.0001$) (**Fig. 51C, D**). Instead, α -SMA positive staining was only significantly increased in the LP ($p = < 0.0001$) (**Fig. 51C, D**). Overall, the changes in the expression of α -SMA positive in the RASi group can be associated with an increased number of CAFs in the tumour stroma and increased density of pericytes on blood vessels (**Fig. 51B**). In contrast, E-cadherin staining mainly was associated with a reduced expression of the junctional hepatocytes and tumour epithelial cells (**Fig. 51A**).

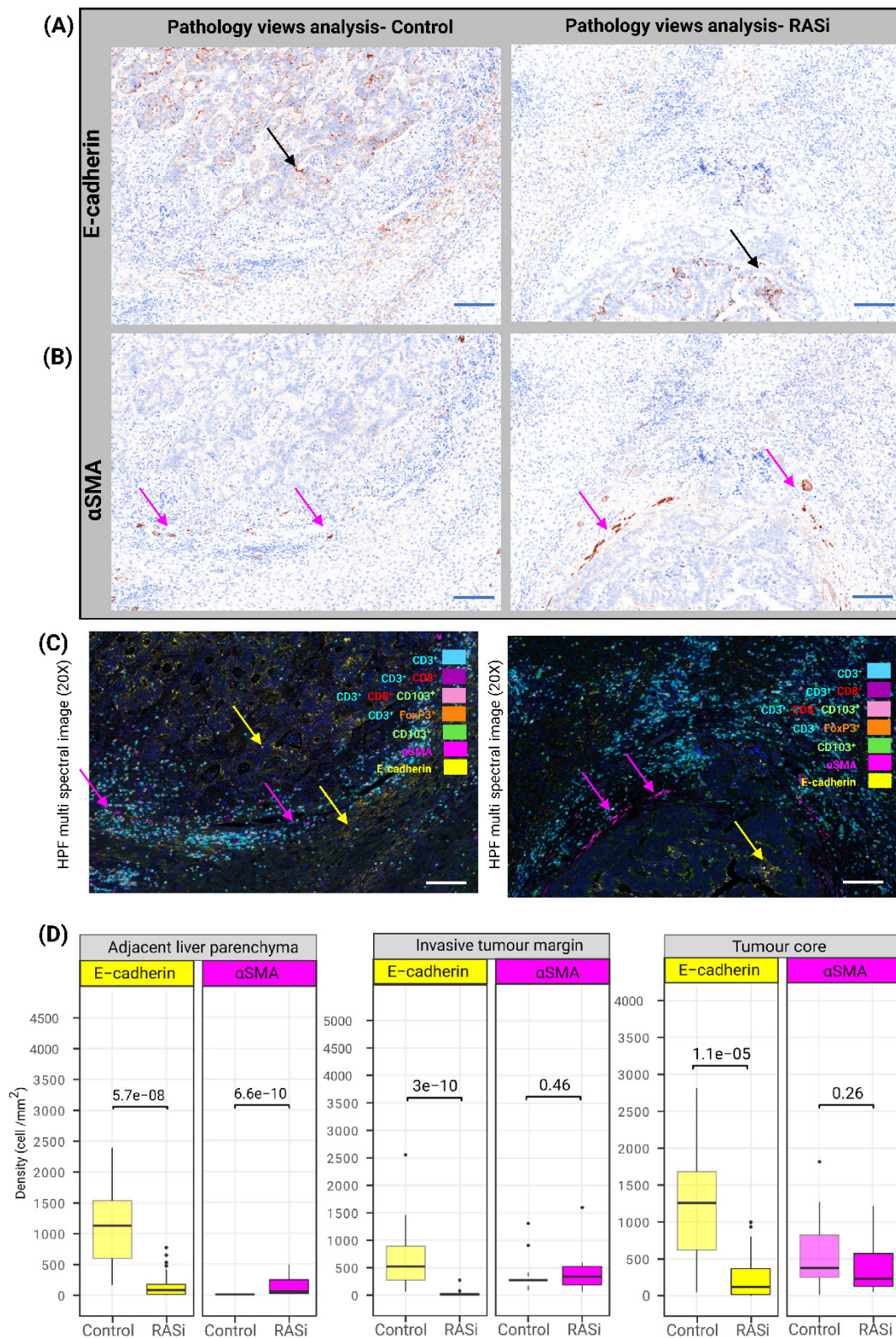


Figure 51. Comparison between cell density and spatial distribution of E-cadherin and α -SMA in the control and RASi group. Representative HPF is displaying (A) E-cadherin expression of the junctional hepatocytes and tumour epithelial cells and (B) α -SMA comparison between control and RASi group (scale bar: 100 μ m). Arrows indicate CAFs in the tumour stroma and pericytes on blood vessels. (C) Representative composite displaying E-cadherin and α -SMA in control and RASi group (scale bar: 100 μ m). (D) The density of E-cadherin and α -SMA were compared between the control and RASi group in the adjacent LP, IM, and TC.

To determine the prognostic significance of the changes on the positivity and spatial distribution of E-cadherin and α -SMA, the OS of patients in the RASi group and control group was assessed in a univariate analysis. The parameters taken into consideration were median cell density and spatial distribution of E-cadherin and α -SMA. No significant differences in OS were found when E-cadherin⁺ cell density was compared between RASi^{high} and control^{high} in any of the tissue segments (Log-rank *p-value*: WT= 0.59; LP= 0.93; IM= 0.8 and TC= 0.9) (**Fig. 52A- E**). Additionally, no significant differences in survival were observed for E-cadherin⁺ cell density and distribution in any of the tissue segments in the pairwise comparison between RASi^{low} to control^{low} (Log-rank *p-value*: WT= 0.81; LP= 0.71; IM= 0.95 and TC= 0.55) (**Fig. 52A- E**).

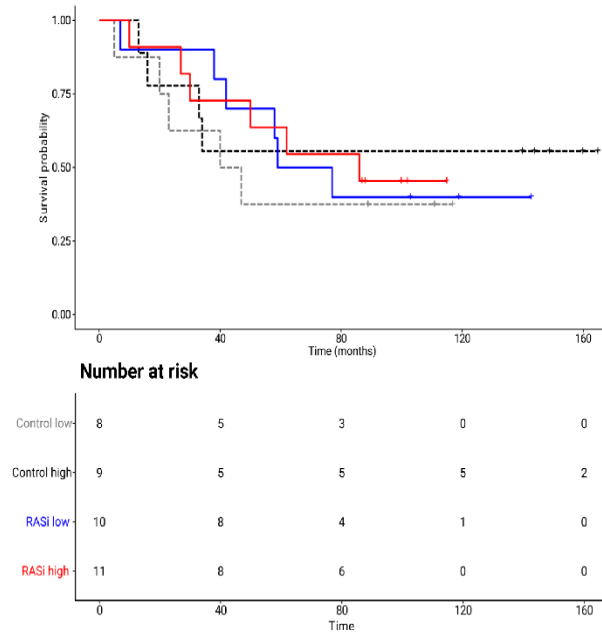
There was no impact in survival caused by the changes over E-cadherin in any of the tissue segments when it was compared RASi^{high} to RASi^{low} (Log-rank *p-value*: WT= 0.83; LP= 0.8; IM= 0.36 and TC= 0.77) and control^{high} to control^{low} (Log-rank *p-value*: WT= 0.57; LP= 0.77; IM= 0.57 and TC= 0.2) (**Fig. 52A- E**), whilst high levels of α -SMA at the LP significantly improved survival in the RASi group (**Fig. 53B**). No significant differences in OS were found when α -SMA⁺ cell density was compared between RASi^{high} and control^{high} in any of the tissue segments (Log-rank *p-value*: WT= 0.38; LP= 0.36; IM= 0.81 and TC= 0.78) (**Fig. 53A- E**). In addition, no significant differences in survival were observed for α -SMA⁺ cell density and distribution in any of the tissue segments in the pairwise comparison between RASi^{low} to control^{low} (Log-rank *p-value*: WT= 0.25; LP= 0.26; IM= 0.61 and TC= 0.97) (**Fig. 53A- E**). There was no difference in survival caused by the cell density changes on α -SMA in the other of the tissue segments when it was compared RASi^{high} to RASi^{low} (Log-rank *p-value*: WT= 0.53; IM= 0.7 and TC= 0.53), while control^{high} to control^{low} showed a significant difference in OS α -SMA⁺ cell density is calculated for the entire tissue (Log-rank *p-value*: WT= 0.049) (**Fig. 53A- D**).

Overall survival (OS): Median cell count of E-cadherin⁺ cells

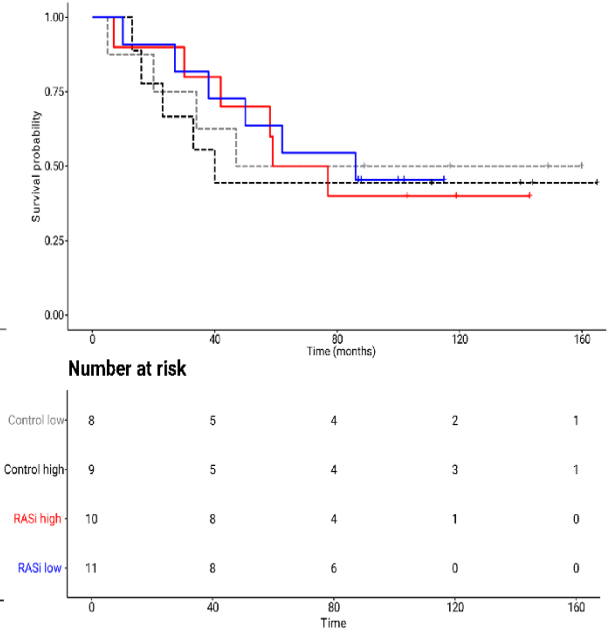
Spatial distribution across all tissue segments

Strata — RASi High — RASi Low — Control High - - - Control Low

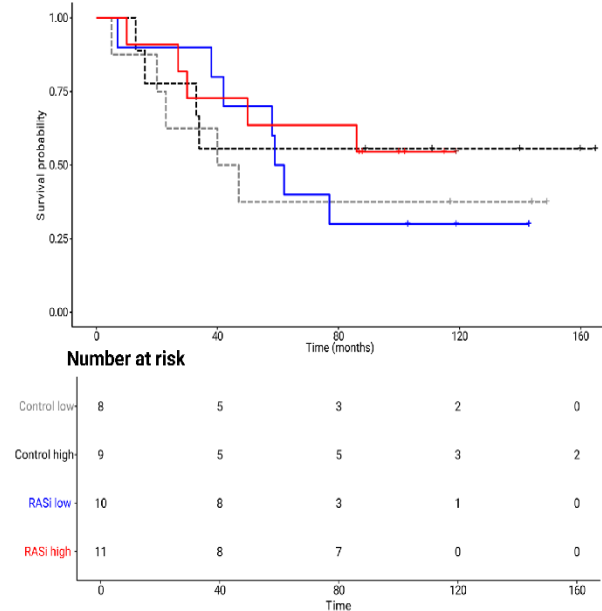
(A) Whole tissue segments



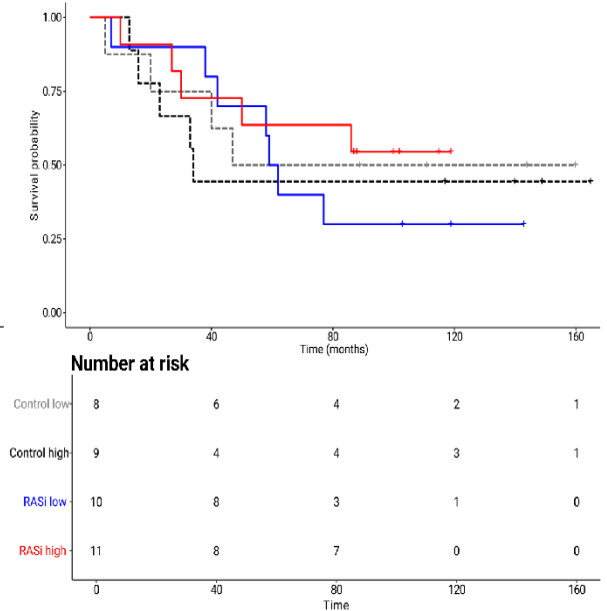
(B) Adjacent liver parenchyma



(C) Invasive tumour margin



(D) Tumour core



(E) OS pairwise comparisons for E-cadherin density

Pairwise Comparison	Tissue Segmentation	Log-rank (Mantel-cox) p-value
RASi High to Control High	WT	0.59
	LP	0.93
	IM	0.8
	TC	0.7
RASi Low to Control Low	WT	0.81
	LP	0.71
	IM	0.95
	TC	0.55
RASi High to RASi low	WT	0.83
	LP	0.8
	IM	0.36
	TC	0.77
Control High to Control Low	WT	0.57
	LP	0.77
	IM	0.57
	TC	0.2

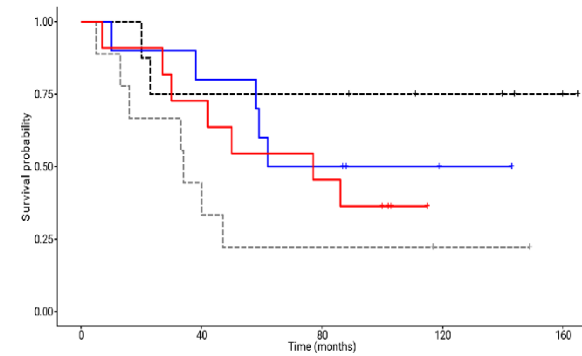
Figure 52. The influence of RASi on CLM patient survival post-liver resection univariate analysis using E-cadherin cell density and spatial distribution. The patient's survival was interrogated using the median distribution of cell density of E-cadherin in the (A) WT, (B) LP, (C) IM and (D) TC for the control (control^{high} in black and control^{low} in discontinuous grey line) and RASi group (RASi^{high} in red and RASi^{low} in blue continues lines). The number at risk (time in months) (E) Log-rank (Mantel-Cox test) *p*-value for pairwise comparisons were calculated.

Overall survival (OS): Median cell count of α SMA+ cells

Spatial distribution across all tissue segments

Strata — RASi High — RASi Low — Control High — Control Low

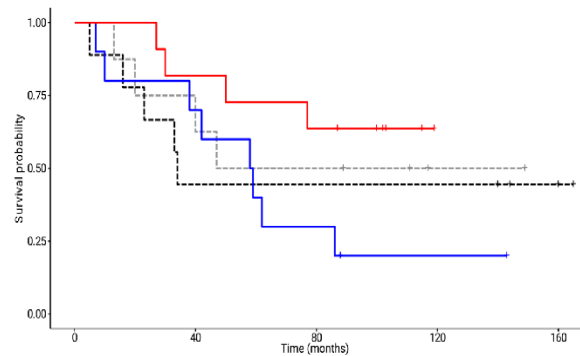
(A) Whole tissue segments



Number at risk

Control high	8	6	6	4	2
Control low	9	4	2	1	0
RASi low	10	8	5	1	0
RASi high	11	8	5	0	0

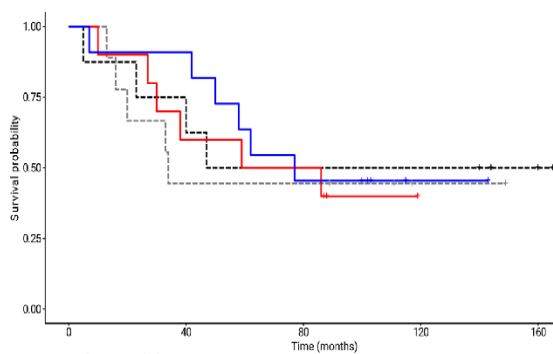
(B) Adjacent liver parenchyma



Number at risk

Control high	8	6	4	1	0
Control low	9	4	4	4	2
RASi low	10	7	3	1	0
RASi high	11	9	7	0	0

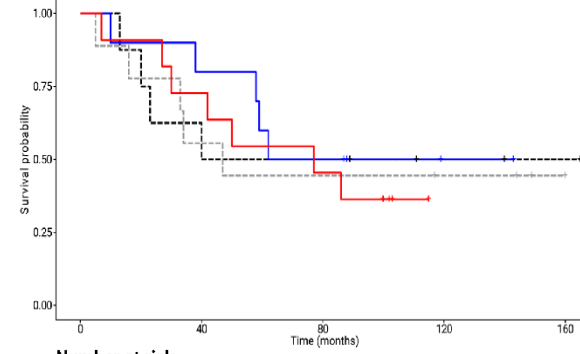
(C) Invasive tumour margin



Number at risk

Control high	8	6	4	4	2
Control low	9	4	4	1	0
RASi high	10	6	5	0	0
RASi low	11	10	5	1	0

(D) Tumour core



Number at risk

Control high	8	5	4	2	1
Control low	9	5	4	3	1
RASi low	10	8	5	1	0
RASi high	11	8	5	0	0

(E) OS pairwise comparisons for α -SMA density

Pairwise Comparison	Tissue Segmentation	Log-rank (Mantel-cox) p-value
RASi High to Control High	WT	0.38
	LP	0.36
	IM	0.81
	TC	0.78
RASi Low to Control Low	WT	0.25
	LP	0.26
	IM	0.61
	TC	0.97
RASi High to RASi low	WT	0.53
	LP	0.05
	IM	0.7
	TC	0.53
Control High to Control Low	WT	0.049
	LP	0.69
	IM	0.68
	TC	0.89

Figure 53. The influence of RASi on CLM patient survival post-liver resection univariate analysis using α -SMA cell density and spatial distribution. The patient's survival was interrogated using the median distribution of cell density of α -SMA in the (A) WT, (B) LP, (C) IM and (D) TC for the control (control^{high} in black and control^{low} in discontinuous grey line) and RASi group (RASi^{high} in red and RASi^{low} in blue continues lines). The number at risk (time in months) (E) Log-rank (Mantel-Cox test) p-value for pairwise comparisons were calculated.

7.4.7 The changes of spatial engagement profile of immune cells and EMT markers after RASi

To evaluate whether RASi modifies the spatial engagements of the immune cells with other cells as an indicator of immune functional relationships, a colocalization analysis was implemented using inForm® (Perkin Elmer/Akoya, MA) (Stack et al., 2014) to identify the relative position of each cell phenotype using their cartesian coordinates (x and y). Next, they were labelled according to the immune markers expressed (i.e., CD3⁺ T Lymphocytes (CD3⁺ CD8⁻ CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺ CD103⁺), non T cells expressing CD103⁺ and cells expressing α -SMA or E-cadherin for each treatment group and cell population distances were measured using the ISAT bioinformatic approach (Wang, 2018). The cut-off distances for spatial engagement were set at 30 μ m. The NN median distance was quantified as "effective percentage of engagement", analysing the mean

distribution distance profile for each immune cell subtype identified and estimating the difference in each relationship's proportion (Huang et al., 2019). The results indicate a dramatic reduction in the percentage of engagement between CD3⁺ T lymphocytes with CD103⁺(CD3⁻) cells (box colour: bright green) in the RASi group (median= 25%) compared to the control group (median= 5%); whereas the engagement of CD3⁺ T lymphocytes to T_{RM} cells (box colour: pink) increased in the RASi group compared to control (median difference ≈10%) (**Fig. 54A**). The engagement of CD3⁺ T lymphocytes to CD8⁺ T cells (box colour: purple) decreased approximately 10% in the RASi group, whilst engagement of CD3⁺ T with E-cadherin (box colour: bright yellow) was markedly reduced in the RASi group compared to control approximately 40% (**Fig. 54A**). The profile of engagement CD8⁺ T cells to CD103⁺ (CD3⁻) cells was also diminished in the RASi group (median difference = 25%) compared to the control group (median= 5%); while their engagement with T_{RM} cells improved (**Fig. 54B**). Interestingly, no significant variation in the pattern of engagement was found for CD8⁺ T cells to CD3⁺ T lymphocytes in any of the groups. In addition, the percentage of engagement of CD8⁺ T cells to E-cadherin was less in the RASi group (**Fig. 54B**). Intriguingly, the profile T_{RM} cells to CD103⁺ (CD3⁻) showed a similar trend as the other T-cells, but with a less pronounced decline in their spatial engagement (**Fig. 54C**). Still, T_{RM} cells engaged less to CD8⁺ T cells in the RASi group (median difference ≈ 25%) when compared to control (median ≈ 50%), while no significant difference was observed in the pattern of spatial engagement of T_{RM} cells to CD3⁺ T cells between RASi and control group (**Fig. 54C**). Also, the percentage of T_{RM} cells' engagement to E-cadherin was noticeably lowered in the RASi group compared to the control (median difference ≈ 25%) (**Fig. 54C**).

Conversely, the non-T cell populations expressing CD103⁺ (CD3⁻) had a distinct interaction pattern with T lymphocyte subtypes, particularly compared to T_{RM} (**Fig. 54D**). CD103⁺ (CD3⁻) engagement to CD3⁺ T cells was increased from a median of ≈ 75% to 90%, whereas no change in the engagement between CD103⁺ (CD3⁻) to CD8⁺ T cells was found when comparing RASi to the control group (**Fig. 54D**). In addition, the percentage of engagement of CD103⁺ (CD3⁻) to T_{RM} cells was higher in the RASi group, while their pattern of engagement to E-cadherin was lessened when compared with other T-cell populations (**Fig. 54D**). Altogether, RASi influences the spatial interaction

of T-cells and non- T cells, which may also reflect cell density and spatial distribution changes.

7.4.8 RASi influences cell density of immune cells and EMT markers in the different histologic growth patterns

Histological evaluation by an anatomical pathologist (D.W) defined the 38 CLM specimens as five desmoplastic, eight replacement and eight pushing growth patterns in the RASi group. In contrast, four desmoplastic, eight replacement and five pushing growth patterns were classified in the control group according to their tumour invasive interface (Fernández Moro et al., 2018) (**Table S4**). The desmoplastic growth pattern has an extensive reactive stroma at the tumour liver interface; the replacement growth pattern has very little stroma and no clear boundary of the tumour-liver interface. In contrast, the pushing growth pattern is characterized by distorted, often flattened hepatocytes at the tumour-liver interface as depicted in the cartoon (**Fig.S10**; see Supplementary Figures and Tables section, page number 296).

The density of infiltrating immune cells (T-cells and non-T cells) and the EMT mentioned above markers was determined according to the three main growth patterns comparing the control and RASi groups (**Fig. 55**). There was no significant difference in density of CD3⁺ T lymphocytes (box colour: cyan), and CD8⁺ T cells (box colour: purple) between RASi treated group and control in any of the growth patterns (**Fig. 55**), whereas T_{RM} (box colour: pink) showed a significant increase in the pushing ($p=0.028$) (**Fig. 55B**) and replacement ($p= < 0.0001$) (**Fig. 55C**) in the RASi group compared to control. However, the desmoplastic pattern did not reach significance but indicated a similar trend ($p=0.077$) (**Fig. 55A**). On the contrary, the results showed a significant reduction of CD103⁺ (CD3⁻) (box colour: bright green) in the RASi group compared to control in all the growth patterns (**Fig. 55A-C**). Similarly, there was a significant reduction in the expression of E-cadherin (box colour: bright yellow) in the three growth patterns (**Fig. 55A-C**), being more pronounced in the replacement pattern (**Fig. 55C**). Lastly, the expression of α -SMA positive in the RASi group compared to the

control group showed no significant difference in any growth pattern, the only trend of increased positivity in the desmoplastic growth pattern (**Fig. 55A**).

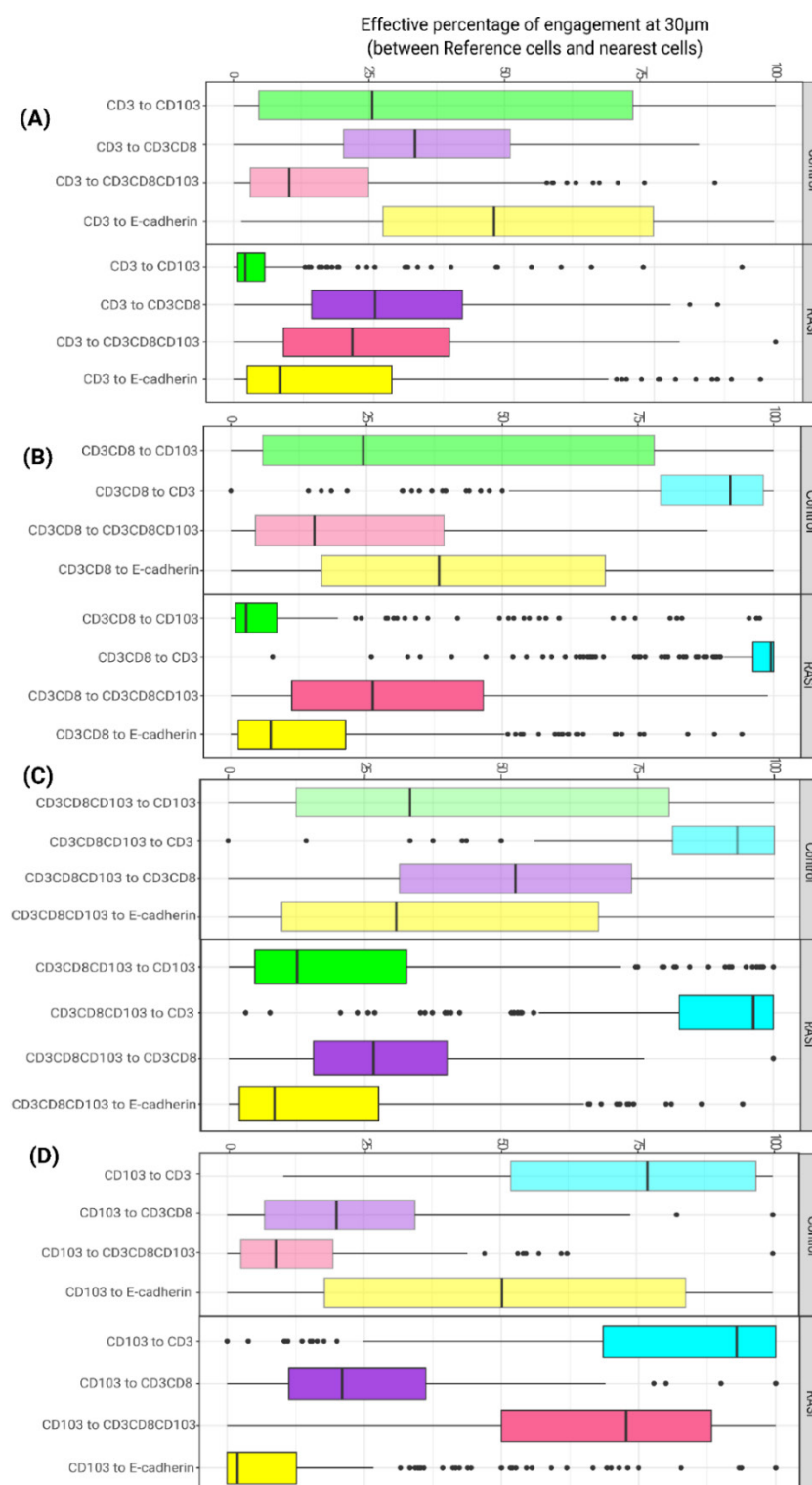


Figure 54. Nearest neighbour spatial relationships of T Lymphocytes and non-T lymphocytes. The effective percentage of engagement of (a) T Lymphocytes (CD3⁺), (b) CD8⁺ T cells (CD3⁺CD8⁺), (c) T_{RM} (CD3⁺CD8⁺ CD103⁺), and (d) CD103⁺ populations that did not co-express CD3⁺T lymphocytes are shown within a cut-off of 30 µm.

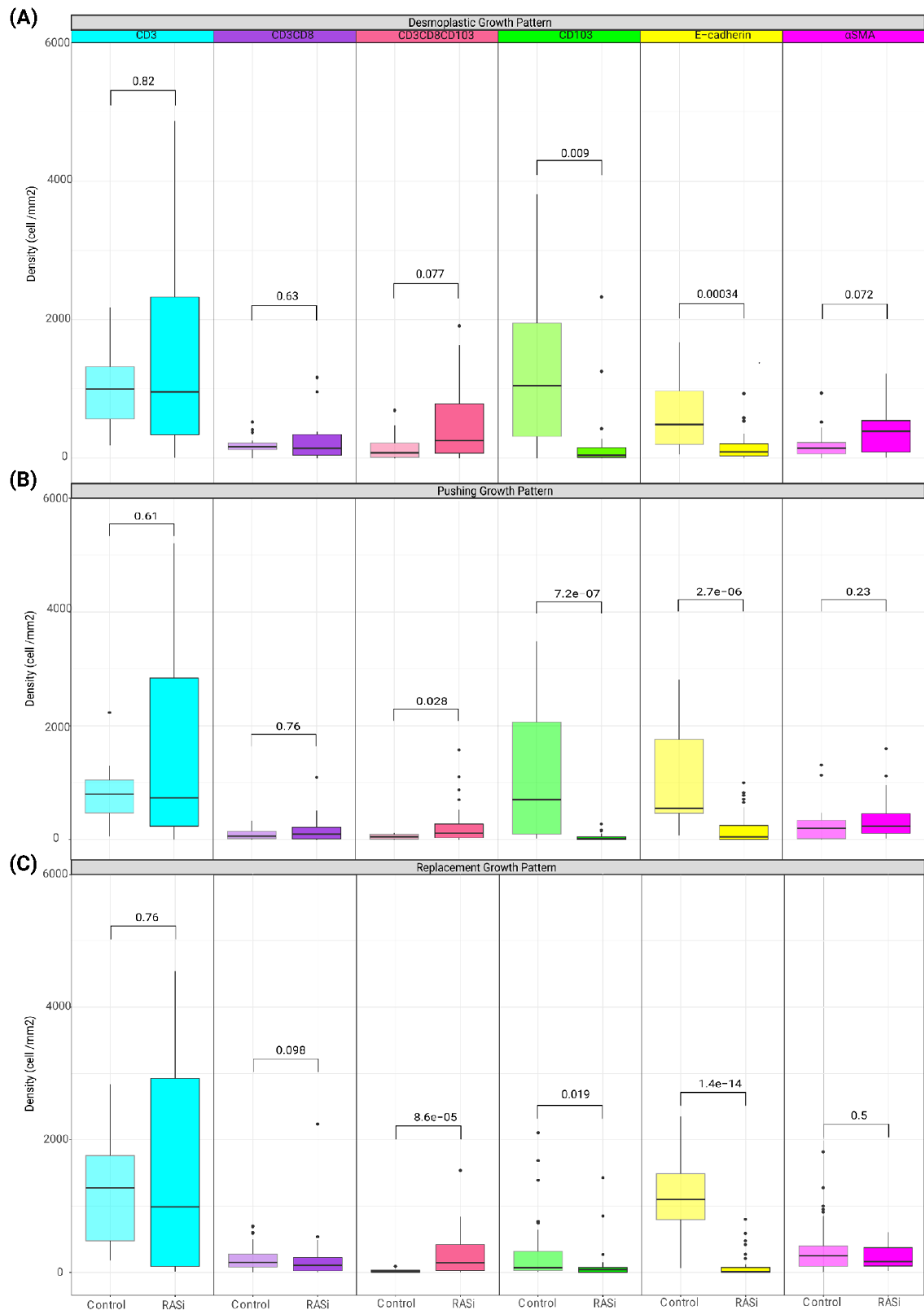


Figure 55. The effect of RASi on cell density of immune cells and EMT markers in the different histologic growth patterns. Cell density profile of (a) desmoplastic, (b) pushing, (c) replacement growth patterns, comparing between control and RASi cohort of CLM calculated for the whole tissue. Data is display in cell count/mm².

7.5 Discussion

Accumulating experimental and clinical evidence over the last years suggest that RASi have anti-tumour effects that influence overall survival, disease free-survival and progression free-survival in cancer patients using RASi as antihypertensive medication (Sun et al., 2017). The characterisation of RASi on T-lymphocytes in the MoCR-CLM mouse model uncovered the temporal dynamic induced by captopril on distinct T-cell subpopulations, including CD3⁺ T lymphocytes, CD8⁺ T cells, CD4⁺ T cells and T_{RM} (CD3⁺CD8⁺CD103⁺). Unexpectedly, the most responsive population was DNTs. Captopril treatment markedly boosted CD3⁺ lymphocytes in the adjacent liver parenchyma and the tumour region at the early angiogenic stage of tumour development, while the CD4⁺ T cell population was diminished. In addition, a selective up-regulation of tumour-specific T_{RM} (CD3⁺CD8⁺CD103⁺) was also found (D. L. Vallejo Ardila et al., 2020).

Several limitations interfere with characterizing the effect of pharmacological treatments on immune subsets infiltrating populations and their complex interactions with TME. First, the lack of studies describing the distribution of T-cell populations within the liver-tumour interface. Second, the absence of exclusive markers between populations (Wong et al., 2016), and third the unavailability of a reliable approach that allows spatial compartmentalization in an automated, unbiased manner (Komura & Ishikawa, 2018). The breakthrough of mIHC to conduct in-depth immunophenotyping incorporating up to seven markers enables capturing the complex network relationships among the different tissue compartments. Furthermore, supervised imaging analysis using inForm™ help incorporating additional variables to cell segmentation (nuclear and cytoplasmic compartment), allowing for the Tissue finder algorithm's training to compartmentalize according to the presence of an invasive margin interface (Stack et al., 2014). Also, mIHC enabled identifying novel immune cell types (For example, CD8⁺ T cells expressing E-cadherin and CD103⁺ expressing Foxp3⁺), detailing their recruitment patterns as shown in chapter 6 and the response behind immunomodulatory treatments (Chuah & Chew, 2020; Gnjatic et al., 2017). In this chapter, the results demonstrate that a wide variation in density and distribution for each T cell phenotype exists within the control and RASi groups. However, the

spatial context of T-lymphocyte subpopulations and EMT markers on human CLM using mIHC and machine learning-based algorithms was described, to spatially resolve their association with post-liver resection outcome after treatment with RASi as antihypertensive medication.

First, a comprehensive spatial phenotypic profile of the specimens included as part of the RASi treated group was performed, which indicated that the highest density corresponds to CD3⁺ T lymphocytes (CD3⁺CD8⁺CD103⁻Foxp3⁻). CD3⁺ T lymphocytes are accumulated mainly at the IM compared to the TC and LP. The control specimens were stained separately under the same conditions as the RASi group, except for the Foxp3 marker, which was not included in the staining panel (see chapter 6). Cell phenotype scoring was performed for both groups using the identical training set of images for the following phenotypes: CD3⁺ T Lymphocytes (CD3⁺CD8⁺CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺CD103⁺) and non-lymphocyte infiltrating cells expressing CD103⁺, using the same tissue segmentation. The computational capacity to perform batch analyses limited the number of assigned phenotypes, training images, and tissue segmentation accuracy (91%).

In this chapter and chapter 6, it was observed that the T-cell compartment was highly heterogeneous, and these may comprise not only conventional but also unconventional T-cells. Among those, $\gamma\delta$ TCR⁺ unconventional T cells reside in a high frequency in the liver (3-5%) and respond to different types of antigens (Banerjee et al., 2019; Godfrey et al., 2015). A similar response was observed on CLM mice, in which DNT (CD3⁺CD4⁺CD8⁻) were significantly upregulated in response to captopril treatment within the tumour (D. L. Vallejo Ardila et al., 2020). No differences in the immune pattern were found in the intergroup comparisons (RASi^{high} to Control^{high} and RASi^{low} to Control^{low}). In contrast, some markers indicated differences intragroup (RASi^{high} to RASi^{low} and Control^{high} to Control^{low}) that impacted OS in CLM patients after liver metastasectomy. These differences may suggest that only some individuals may respond to the T-cell immunomodulatory effect of RASi.

Intriguingly, no significant differences were observed when comparing the median cell density of CD3⁺ T lymphocytes across all the tissue segments between the RASi and the control groups, but there was a marked heterogeneity within the RASi treated group. The immune-modulatory effect of RASi on CD3⁺ T lymphocytes became evident

when RASi^{high} with elevated levels of CD3⁺ T showed an improved OS compared to those with low CD3⁺ T cell density within the whole tissue assessed. More importantly, this effect was not observed in the control group. Despite no changes in CD3⁺ T density, the OS was significantly correlated with increased infiltration at adjacent LP. In chapter 6, it was reported that a higher density of CD3⁺ T lymphocytes in the TC influence OS after CLM resection.

On the other hand, CD8⁺ T cells were found in a higher density in the IM, while their density was lower in the TC in the RASi treated group than the control. Some studies reported an association of high CD8⁺ T cell levels and better OS in CLM (Katz et al., 2013; Kong et al., 2019; Peng et al., 2019; Wang et al., 2018). However, the specific location of these immune cells within the liver-tumour interface was not stipulated, which can cause discrepancies with the observed localization-dependent prognostic relevance of CD8⁺ T cells (see chapter 6). High levels of CD8⁺ T cells in the RASi treated group showed significantly better OS than those with low levels in the LP and TC, whereas the IM was the opposite. In some studies, CD8⁺ T cells that accumulate at the IM can be considered as poorly immunogenic or *infiltrated-excluded* (Aki et al., 2010; Hegde et al., 2016), the majority of these CD8⁺ T cells displayed feature of “exhaustion” (Lanitis et al., 2017). Further functional phenotyping may resolve the clinical significance of these findings (Bindea et al., 2013).

Another study showed that increased infiltration of cytotoxic CD3⁺CD8⁺ cells engaging with epithelial cells impacted OS in deficient mismatch repair human CLM (Lazarus et al., 2018). In this study, the epithelial interface was identified by the E-cadherin staining, which in the spatial engagement between nearest neighbouring cells showed a decreased percentage of engagement between CD8⁺ T cells and E-cadherin in the RASi group. E-cadherin expression was markedly reduced across all the tissue segments in the RASi treated group. In the past, low expression of E-cadherin had been associated with invasive metastases development in some epithelial cancer types (Berx et al., 1995; Padmanaban et al., 2019; Perl et al., 1998; Vleminckx et al., 1991), including CRC (S. A. Kim et al., 2016). Furthermore, there is evidence that E-cadherin regulates cell-to-cell adhesion independent of its surface expression (Brieher & Gumbiner, 1994); therefore, losing E-cadherin is not required to either undergo an EMT or to promote metastases, as it has been widely believed (Na et al., 2020). Instead,

multiple processes in the metastatic cascade are regulated by the activation of E-cadherin. The signalling pathways associated with this process are the Hippo signalling pathway (Gumbiner & Kim, 2014; Kim et al., 2011), Wnt pathway (Mendonsa et al., 2018), the small GTPases (Nelson & Nusse, 2004), Rac and Rho or PI3Kinase (Yap & Kovacs, 2003), which supports the findings that a reduced expression of E-cadherin by the tumour core epithelial interface may inhibit the activation of pro-metastatic pathways.

E-cadherin's immunological functions in Langerhans cells' maturation levels, a type of DC found in the skin epidermis, showed that more than being an “anchor” to keratinocytes, losing E-cadherin mediated adhesion allowed for their maturation (Riedl et al., 2000). Another study reported that the disruption of E-cadherin adhesion prompted the elevation of MHCII and activation of antigen processing features in other DCs under inflammatory conditions (Jiang et al., 2007). In the present study in the RASi treated group, a distinct pattern of decreased E-cadherin engagement was found for T-cell and CD103⁺ non-T cells. In chapter 6, an improved CLM patient survival was observed when tumour infiltrating cells (CD8⁺ T cells and CD8⁺ or CD103⁺ non-T cells) were located far from E-cadherin positive cells in the TME. Cell density changes are generally reflected directly on the spatial engagement when all tissue compartments indicated a significant depletion of CD103⁺ non-T cells in the RASi treated group. These cells are most likely to be CD103⁺ DCs. In chapter 6, it was reported that a significant level of CD103⁺ non-T cells is uniformly distributed across the liver-tumour interface and showed no significant correlation with post-liver resection OS, which was corroborated by this study.

α -SMA expression has been used as a signature marker of myofibroblast and an indicator of fibrogenic processes (Layton et al., 2020). Besides, α -SMA is one marker that identifies CAFs activation (Sahai et al., 2020). In the RASi treated group, higher levels of α -SMA in the adjacent LP, which were positively correlated with OS. Notably, at this specific location, the expression may correspond to other cells such as pericytes on blood vessels instead of α SMA⁺ myofibroblast-type CAFs. CAFs can contribute to either a tumour immunosuppressive milieu (Özdemir et al., 2014) by secreting factors such as CXCL12/CXCR4 (Chen et al., 2019; Feig et al., 2013), or immune-supportive (Kraman et al., 2010; Lo et al., 2015) milieu depending on their

state of activation. Further phenotypic characterizations of α -SMA⁺ CAF using fibroblast activation protein (FAP) can ascertain the functional impact on the activity of cytotoxic CD8⁺ T (Costa et al., 2018). Additionally, increased levels of α -SMA⁺ CAF may build up to act as a physical barrier to prevent T-cell infiltration (Salmon et al., 2012) and increase the ECM stiffness in CLM (Shen et al., 2020). Therefore, therapeutic targeting of CAFs aims to reprogram from an active state to a quiescent myofibroblast instead of reducing their density, which has been successful using AT1R blockers (Chauhan et al., 2013). Furthermore, the immunostimulatory shift of CAF leads to active cytotoxic T cell infiltration (Chauhan et al., 2019). These findings indicate the complex spatial dynamic of α -SMA⁺ positive CAF with CD8⁺ T cells within the TME, and further studies are required to manipulate α -SMA⁺ CAF using ACEi therapeutically.

Several studies support the association between more prolonged survival and T_{RM} (CD3⁺CD8⁺CD103⁺) infiltration in solid tumours such as breast (Z.-Q. Wang et al., 2016), lung (Djenidi et al., 2015), endometrial (Workel et al., 2016), ovarian (Webb et al., 2014), cervical (Komdeur et al., 2017), urothelial (Wang et al., 2015), melanoma (Edwards et al., 2018) and CRC (Quinn et al., 2003). However, the CD103⁺T_{RM} subpopulation in this study demonstrated no significant impact after liver resection, despite their increased density in the adjacent LP and IM in the RASi group. This effect of RASi was consistent across species since expression CD103 on CD8⁺ T cells was found in a higher proportion in the tumour in captopril treated mice (D. L. Vallejo Ardila et al., 2020). The enrichment of CD103⁺ on CD8⁺ T-infiltrating lymphocytes displayed an enhanced T cell receptor (TCR) antigen sensitivity (Le Floc'h et al., 2007), which allowed for faster tumour recognition and improved their cytotoxic activity (Abd Hamid et al., 2020). In a previous study, a lower proportion of CD103⁺T_{RM} was found in CLM than in HNSCC, lung, ovarian, rectal and CRC microsatellite instable (MSI) high, which showed an increased expression of CD39 (Duhon et al., 2018). In a separate study, CD103⁺T_{RM} were found in a more significant number within the tumour epithelium than in CRC patients' normal epithelium, particularly in those MSI high tumours (Quinn et al., 2003). MMR status of the primary tumour was not analysed; therefore, there is a limited understanding of the association between MMR status and the distribution of the CD103⁺T_{RM} and clinical significance for the RASi group.

In this chapter, it was reported a novel non-T cell expressing CD103⁺ FoxP3⁺. FoxP3 had been reported in other studies on T_{regs} expressing CD103 (Allakhverdi et al., 2006; Leithäuser et al., 2006), but there was no report of non-T cells expressing CD103 that co-express FoxP3⁺. However, this phenotype needs to be further investigated as it was only explored in the RASi group, not included in the phenotype scoring algorithm.

It must be acknowledged that the limitations of the retrospective cohort design including the information and classification biases, which involve not having age-matched controls, and differentiating between the specific effects of ACEi and ARBs, not knowing about *KRAS*, *BRAF* and MMR status and primary tumour location of these patients. The clinical information about administering neoadjuvant chemotherapy and these patients' primary tumour location was not available for data retrieval. The differences in neoadjuvant chemotherapy may have caused bias, yet no other study has reported any neoadjuvant chemotherapy associations with immune infiltration or patient survival (Hof et al., 2019). Additionally, only a few of the samples used had been tested for the presence of mutations on *KRAS* and *BRAF* genes, which are well-known molecular prognostic biomarkers associated with poor patient survival (Tsilimigras et al., 2018); however, their influence on anti-tumour immune response and RASi response remains unclear. Additionally, the RASi cohort was selected because they had been on RASi (ACEi and ARBs) for hypertension before liver resection, whereas the control cohort contained specimens collected from patients who had not been on any antihypertensive medications; still, it was not possible to age-match controls, or to separate between the specific effects of ACEi and ARBs (Perini et al., 2020).

7.6 Conclusion

This chapter described the effects of RASi on antitumour immunity in human CLM for the first time using a manually validated mIHC approach and machine learning-based algorithms for imaging analysis. Therefore, the differences in cell density, spatial distribution and engagement can be attributed to RASi treatment. While this study needs to be extended to include a larger cohort of CLM patients using automated staining procedures and image analysis, the results suggest that RASi could modulate

T-cell when used as an antihypertensive medication anti-tumour response. Of additional importance, considering disease stage, phenotype, kinetics, and sequence of neoadjuvant treatment for CLM may help untangle the resistance to current immunotherapies. Adopting mIHC, incorporating complementary single-cell RNA sequencing expression patterns and mass cytometry (CyTOF) of patient tissues can provide a comprehensive characterization of the immune landscape of CLM patients and may inform on treatment regimens. Future prospective studies are necessary to delineate better the protective effects and the immunomodulation by RASi in CLM patients.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE DIRECTIONS

8.1 Contributions of the thesis using the MoCR-CLM mouse model

CRC is the second leading cause of cancer-related death for both genders combined in high-income countries (Keum & Giovannucci, 2019). More than 50% of CRC patients die from liver metastatic disease development (Tomlinson et al., 2007). A relatively small group of CLM patients are considered amenable to surgical resection, which remains the only potentially curative treatment (Petrowsky et al., 2020), while most CLM patients are usually treated with chemotherapy on a palliative basis (Chiorean et al., 2020). Innovative approaches to tackle liver metastases development and recurrence are needed to improve CRC patients' survival outcomes; therefore, advances in the understanding of anti-tumour immune response became critical to tailor approaches to current cancer immunotherapy strategies and for the discovery of novel prognostic biomarkers.

Our lab's previous findings demonstrated that RAS inhibition significantly decreased tumour growth and central microvascular density in a dose-dependent manner (Neo et al., 2010; Neo et al., 2007). In another study, the importance of TAMs in tumour control at early-stage liver metastases indicated the potential of captopril to enhance the anti-tumour response in mice with CLM (Wen et al., 2013). Furthermore, the expression of RAS components had been previously characterized in the various immune cells within TME, which include M Φ (Cortez-Retamozo et al., 2013), myeloid-derived cells (Shen et al., 2014), T-lymphocytes (Crowley & Rudemiller, 2017) and DCs (Meng et al., 2017), and in other cell types such as smooth muscle cells (Chen et al., 2016) and precursor stromal fibroblast (Incio et al., 2016; Masamune et al., 2013; Okazaki et al., 2014). Thus, the regulation of the basal gene expression of tumour infiltrating immune cells by RASi offers an inroad to expand cancer immunotherapies for CLM beyond targeting CD4⁺Foxp3 T_{reg} and cytotoxic CD8⁺ TILs.

This study provides extensive evidence of the potent immunomodulatory effects of RASi using an immunocompetent orthotopic mouse model, which recapitulates CLM immunological features. The tumour kinetics in the MoCR-CLM model had been previously characterized (Kuruppu et al., 1996), which led to the discovery that the immunomodulatory effects of RASi occurred before the angiogenic switch that drives the exponential growth phase, as shown in chapter 3. The effects of captopril appear

mainly through restraining tumour growth because it affects both the number and the size of metastatic foci (D. L. Vallejo Ardila et al., 2020). While RASi immunomodulatory effects on T lymphocytes were evident at the early angiogenic stage, tumour control's effect became significant at a later time point when a greater number of metastatic foci have gone through the exponential growth stage (D. L. Vallejo Ardila et al., 2020). The advantages of controlling the genetic and environmental factors of CLM mice allowed for precise observations in modulating the anti-tumour immune responses by RASi. In this sense, MoCR-CLM can be considered a powerful immune-oncology model.

In Chapter 3, we confirmed previous reports from our research team that captopril treatment significantly inhibited tumour growth. Additionally, we demonstrated significant modulation of the infiltrating T cell population in both cell density and activation. Captopril treatment significantly increased CD3⁺ T cells in liver and tumour tissues, primarily due to the significant increase of DNTs. This study highlights the potential role of DNTs in CLM anti-tumor immunity, which until now had only started to be investigated in NSCLC (Fang et al., 2019). The immune-suppressive effect of human DNTs on CD4⁺ T cells proliferation was corroborated and attributed to impair metabolic activation via mTOR signalling in the context of alloreactive immune response (Haug et al., 2019). We also found a significant decrease of CD4⁺ T cells due to captopril treatment. While CD8⁺ T cells expressing PD1⁺ and CD103⁺ were significantly upregulated by captopril within the tumour, which may offer an opportunity for increase efficacy and durability in response to anti-PD1 treatment when combined with RASi (Webb et al., 2014). Improved mouse mIHC panels including CD25, FOXP3, CD127 and CD39 can facilitate in-depth phenotypic characterization of CD4⁺ T cells. Also, an accurate assessment of function CD8⁺ T cell is required to gain clues into reducing effector T cell populations observed after captopril treatment. FACS sorting can be used to isolate CD8⁺ T cell from the tumour, then using *in vitro* immune cytotoxic assays such as chromium (51Cr) release assay, the activity of cytotoxic CD8⁺ T cells can be measured in response to captopril. Further functional phenotypic characterization of unconventional T lymphocyte populations of TcRαβ⁺, TcRγδ⁺ and other liver resident subpopulations like CD103⁺ T_{RM} is needed to identify their involvement in tumour control in response to RASi.

Apart from T lymphocytes, many other infiltrating immune cells contribute to tumour progression, including myeloid cells that differentiate into MΦ, DCs, and granulocytes (Gabrilovich et al., 2012). However, deconvoluting the concept that all myeloid-derived cells contribute to immune suppression requires individual assessment of myeloid populations. In chapter 4, the characterization of myeloid-derived cells distributed within the liver and tumour tissues and those that expressed CD31 and co-inhibitory molecules like PD-L1 enables a better understanding of their function and phenotype in CLM. Moreover, the effect of captopril on myeloid populations in mice with CLM was investigated. In that chapter, myeloid cells expressing CD11c⁺ seem to accumulate in the tumour preferentially and then migrate into secondary lymph node organs. At the same time, CD11b⁺ myeloid cells were significantly reduced in the liver. ACE inhibition regulates maturation, migration and function of both DCs (Meng et al., 2017) and myelomonocytes (Lin et al., 2011). CD31 had been previously characterized at different stages of maturation of myeloid cells (Ling et al., 1997) and myeloid progenitor cells (Bronte et al., 2000), a contradictory criterion that both mature F4/80⁺ MΦ in the liver and TAMs also expressed CD31⁺. We further investigated the phenotypic composition of CD11b⁺ myeloid cells, and we found that CD31⁺ myeloid cells were significantly diminished in captopril treated mice, particularly the subset expressing F4/80⁻ Ly6C^{intermediate/High}. Another study suggested that CD31⁺ endothelial cells were a source of contamination when preparing hepatic MΦ for FACS because endothelial cells with parts of MΦ membranes attached to them and are a manifestation of isolation procedures (Lynch et al., 2018). More recently, CD31⁺MDSC had been reported in the context of late sepsis (Brudecki et al., 2012; Dai et al., 2015), chronic hepatitis B virus infection (Chen et al., 2011) and some tumour-bearing mouse models (Connolly et al., 2010; Yang et al., 2004). Altogether these results suggest that captopril may have a dual mechanism by impeding tumour angiogenesis and reversing TME immunosuppression by targeting the expression CD31. However, implementing specific lineage markers is warranted to differentiate between the effect of RASi on endothelial progenitors and myeloid cells expressing CD31. Other myeloid subsets including MDSC expressing Ly6C^{high} (CD31⁻F4/80⁻) were also markedly reduced by captopril in the liver and tumour, same as MΦ expressing PD-L1⁺, which also support the previous findings that RASi reprograms the TME towards an

immunostimulatory milieu by decreasing the inhibitory action of these subsets over T-lymphocytes. Further assessment the immunosuppressive activity of MDSC can be evaluated by measuring circulating secreting factors like IFN- γ and IL-10 (Groth et al., 2019) using FACS for detection of cytokine secreting cells. These may provide insights into the role of myeloid cells enabling the formation of the pre-metastatic niche of CLM and the effect of RASi to prevent liver metastasis and recurrence.

In chapter 5, the global proteome's characterisation showed that captopril differentially regulated the immunoproteasome cluster PSMB8, PSMB10, PSMB5 and PSME1. Subsequently, we found that PSMB8 showed a trend of an increased level of expression within the tumour in the captopril treated group, while there was no detectable expression of PSMB5 within the tumour of mice with CLM. The expression of the immunoproteasome has been characterized in several immune cells, including DCs, M Φ and activated T-lymphocytes in the context of bacterial infection (Guimarães et al., 2018) and viral infection control (Krüger & Kloetzel, 2012; Pang et al., 2006). In addition, recent reports have shown the importance of the expression of antigen presentation machinery genes to determine additional prognostic factors in the anti-tumour response and immunotherapy response in solid tumours and hematologic malignancies (Kalaora et al., 2020; Kiuchi et al., 2020; Rouette et al., 2016; Tripathi et al., 2016). Furthermore, the expression of the immunoproteasome's inducible subunits is required to process MHC-I immunogenic peptides and antigen presentation for tumour cell recognition (Lee et al., 2020). The underlying principle is that downregulation of the immunoproteasome expression may lead to tumour evasion of the immune response because it impairs effective cytotoxic CD8⁺ T cells response (Jhunjunwala et al., 2021). However, there was no previous evidence that the tumour can alter the proteasome and immunoproteasome's constitutive expression, reducing the presentation of tumour-specific antigens (Jhunjunwala et al., 2021).

In chapter 3, we have demonstrated that RASi modulated T-cell mediated anti-tumour responses in CLM, congruent with the upregulation of the inducible subunits of immunoproteasome the tumour enhancing antigen presentation and tumour recognition. Also, we found that the signalling pathways attributed to the captopril effect were ubiquitin-dependent and independent proteasomal degradation pathways in CLM. Similarly, another study found that ACEi imidaprilat prevents muscle atrophy

by regulating the ubiquitin-proteasome proteolytic pathway (Sanders et al., 2005). A comparison between the proteome and phosphoproteome changes induced by captopril may help predict other candidate proteins and signalling pathways. These phosphosites would still need to be validated and correlated with the immunomodulatory effect of RASi on T-cell anti-tumour function. Of additional importance, the effect of RASi on the tumour immunoproteasome expression and the impact on the peptide repertoire may improve T cell therapies targeting a single epitope.

8.2 Contributions of the thesis using high-dimensional immune-profiling of human CLM

In chapter 7, we used archival human CLM specimens to investigate the effects of RASi on tumour immune infiltration, particularly T lymphocytes and EMT markers.

As a background study, we used a cohort of whole human CLM FFPE sections to manually optimize and validate a customized panel of highly specific monoclonal primary antibodies. In addition, we assess the composition and spatial distribution of T cell markers (CD3, CD8 and CD103) and EMT markers (α -SMA and E-cadherin), paired to TSA Opal technology following general recommendations to ensure quality data results (Masucci et al., 2016; Taube et al., 2020) (Appendix 3). Multiplexing technologies allow for simultaneous targeting of multiple proteins expressed on a single cell level with high precision and accuracy on a single section (Feng et al., 2015). Conceptually, mIHC using TSA Opal to immune-profile CLM offers an efficient and reliable striping of a broad array of antibodies while maintaining the integrity of whole tissue FFPE sections after several cycles of staining. However, the primary constraints are the limited number of slides that can be manually stained and the high cost of the staining procedures and automated image analyses (Remark et al., 2016).

Using Vectra 3.0™ multispectral detection platforms, image acquisition can deconvolute only between 6-8 plex assays (Stack et al., 2014). Moreover, quantitative mIHC is a powerful immunological discovery tool that enables phenotypic characterization of novel immune and stroma cells in the TME using inferences from well-known phenotypes (Blom et al., 2017; Halse et al., 2018; Lazarus et al., 2018;

Stack et al., 2014; Taube et al., 2020; Tsujikawa et al., 2017). Traditionally, the histopathological diagnosis was performed by trained humans observing the stained specimens on a glass slide using a microscope. New procedures for digital pathological image analysis use computer-assisted diagnosis (Komura & Ishikawa, 2018). Machine learning enables classification accuracy by reducing the variability in the interpretations and preventing overlooking essential features in whole slide images (Bhargava & Madabhushi, 2016).

In chapter 6, we performed in-depth CLM immune profiling using machine-learning-based algorithms. We identified a novel population of CD8⁺ T cells expressing E-cadherin with this state-of-the-art methodology, associated with significant improvement in CLM patient's OS. In a previous report, CD8⁺ T cells expressing E-cadherin induced T_{RM} accumulation after LCMV infection in murine salivary glands (Hofmann & Pircher, 2011). Additionally, we found that a high density of CD8⁺ T lymphocytes in the invasive tumour margin was associated with better patient survival in CLM, confirming previous studies (Katz et al., 2013; Kong et al., 2019; Peng et al., 2019; Wang et al., 2018).

Unexpectedly, we identified significant levels of CD8⁺ populations that did not co-express CD3⁺. Moreover, CD8 positivity has also been reported on human NK cells (Ahmad et al., 2014) and NKT like CD8 T cells (Barbarin et al., 2017), which implies that CD8 positivity is not exclusively associated with cytotoxic T cells; thus, inaccuracies in the identification of the phenotype may be introduced if the CD3 co-expression is not investigated (Angelova et al., 2018; Halama et al., 2011; Millen et al., 2020; Peng et al., 2019; Wang et al., 2018).

T cells' localisation-dependent prognostic relevance after liver metastasectomy was investigated using the ISAT bioinformatic approach (Wang, 2018). A recent study characterized T lymphocytes' spatial engagement in gastric cancer using the exact nearest neighbour spatial relationships approach (M. Wang et al., 2020). This study demonstrated that CD4⁺FoxP3⁺ preferentially interact with CD8⁺ T cells and not the tumour interface, which could be an independent prognostic marker for gastric cancer patients (M. Wang et al., 2020). Additional mIHC panels of Abs, including CD25, FoxP3, CD127 and CD39, can facilitate in-depth phenotypic characterization of CD4⁺ T cells in human CLM tissues.

Lastly, in that chapter, we found that the histologic growth pattern of CLM determines the spatial distribution and engagement of immune cells. Another report had shown that the desmoplastic growth pattern had higher infiltration than the pushing and replacement pattern (Nielsen et al., 2014). Instead, we outlined that CLM histological growth patterns do not dictate changes in overall cell density but display the distinct spatial distribution of T-cell subtypes. In summary, these findings highlight the importance of the spatial location and immune cell interactions within the tumour in identifying potential prognostic markers and therapeutic targets.

Chapter 7 is an extension of the findings in chapter 6. We implemented the previously optimized 7-plex OPALTM protocol to assess the composition and spatial distribution of T cell markers (CD3, CD8, FoxP3 and CD103) and the EMT markers (α -SMA and E-cadherin) in twenty-one CLM specimens from patients who had been on RASi (ACEi and ARBs) for hypertension before liver resection. In contrast, the control group contained seventeen CLM specimens collected from patients who had not been on any antihypertensive medications. Some of these specimens were previously stained and analysed in chapter 6. All samples were analysed using the same batch analysis algorithm for tissue segmentation and cell phenotyping on inForm[®] software v2.4.2. In that study, we found that RASi modifies T cell density and spatial distribution of CD8⁺ T cells and CD103⁺T_{RM}, and high densities of CD3⁺ T cells and CD8⁺ T cells in the RASi treated group showed significantly better OS than those with low levels in the adjacent LP and TC, respectively. However, there was no significant survival difference between the group that had been on RASi for hypertension before liver resection and those that had not been using antihypertensive medications. In general, the function and prognostic value of CD8⁺ T cells and CD103⁺T_{RM} have been associated with their location within the residency tissue (Szabo et al., 2019). Hence a sustained immunomodulatory response may require a deeper insight into the spatial dynamic of intratumoral and peripheral T-cells because, at different tumour sites, they may have a variable degree of tumour reactivity (Bindea et al., 2013).

In the RASi group, we found that the CD3⁺ T cell densities varied widely compared to control. A similar response was observed on CLM mice, in which DNTs (CD3⁺CD4⁺CD8⁻) were significantly increased in response to captopril treatment within the tumour (D. L. Vallejo Ardila et al., 2020). CD3⁺ T lymphocyte compartment comprises both

conventional and unconventional T-cells; therefore, functional phenotypic characterization of unconventional T lymphocyte populations is needed to identify their involvement in tumour control in response to RASi.

In both chapter 6 and chapter 7, we found that the CD103⁺T_{RM} subpopulation had no significant impact on OS post-liver resection. Previous studies correlated high levels of T_{RM} with favourable survival outcomes for a patient with CRC and CLM (Duhon et al., 2018). High levels of CD103⁺T_{RM} subpopulation were described in the RASi group in the adjacent LP and IM (chapter 7). Also, this finding was consistent across species since the expression CD103 on CD8⁺ T cells was found in a higher proportion in the tumour in captopril treated mice (D. L. Vallejo Ardila et al., 2020). Therapeutic options to target T_{RM} are also context-specific, and there is still limited understanding of their biology (Sasson et al., 2020). CD103⁺T_{RM} cells most likely play a role in antitumour cytotoxic response through $\alpha\text{E}\beta_7$ -E-cadherin interactions (Le Floc'h et al., 2007).

There are several limitations in the human CLM studies, which had a retrospective cohort design. The information and classification biases may have influenced the results in chapter 6 and chapter 7. The clinical information about administering neoadjuvant chemotherapy and these patients' primary tumour location was not available for data retrieval. However, no other study has reported any associations of neoadjuvant chemotherapy with immune infiltration or patient survival (Hof et al., 2019). Additionally, only a few of the samples used had been tested for the presence of *KRAS* and *BRAF* mutations, which are molecular prognostic biomarkers associated with poor patient survival (Tsilimigras et al., 2018). The influence of anti-tumour immune response and RASi response remains unclear *KRAS* and *BRAF* mutations. The RASi cohort was selected based on the criteria that they had been on RASi (ACEi and ARBs) for hypertension before liver resection, whereas the control were patients who had not been on any antihypertensive medications. The small sample size of both cohorts is constrained to age-match controls or separation between the specific effects of ACEi and ARBs (Perini et al., 2020).

Finally, we highlight that the usage of whole tumour sections of CLM and the long-term follow-up of these patients enabled us to discover robust predictive markers. However, automated stained procedures and automated image analysis are necessary to evaluate a larger cohort of patients.

8.3 Clinical translation potential of MoCR-CLM mouse model to benefit patients with CLM

Numerous challenges have been described in the effort of translating cancer immunotherapy discoveries from the current pre-clinical models to human immunity, which include that the pre-clinical model needs to prioritize in the following: the mechanism of action, safety, delivery approaches and treatment dose/schedule (Hegde & Chen, 2020). The translatability of these findings into human applications can be limited due to the wide variability in the anti-tumour immune responses to drug treatments, which are largely influenced by the genetic composition of the individual and the tumour (Li & Auwerx, 2020).

The MoCR-CLM mouse model is a chemically induced CRC animal model like MC38 (Mosely et al., 2017). The caveat, however, is that the MoCR model uses the carcinogen DMH to induce primary colon carcinoma in CBA animals, which is commonly used to induce mutations primarily CRC that resembles human CRC (Newell & Heddle, 2004). Like MC38, which was isolated from a colon tumour in a C57BL/6 mouse following long term exposure to DMH (Corbett et al., 1975). Chemically induced colon carcinogenesis leads to a high mutational burden (Corbett et al., 1975). Emerging evidence suggests that a high tumour mutational burden correlates with the presence of more neoantigens, thereby a better chance of triggering an immunogenic T cell response (Jardim et al., 2020). Further studies using the MoCR-CLM mouse model should account for the molecular characterization of the differences in MSI High and microsatellite stable CLM patients' immune response.

A strong sexual dimorphism in immune responses may influence the effect of RASi (Capone et al., 2018) as some components of the RAS pathway display a sex-biased differential expression; hence inequality between the sexes response to RASi treatments can be induced (Sullivan, 2008). However, the animal experiments used inbred single strain male mice to overcome genetic and sexual dimorphism variability. RASi continues to be widely prescribed worldwide despite the low-quality evidence that supports their efficacy and safety compared to other first-line antihypertensive medications (Chen et al., 2018). The safety constraints for RASi (ACEi/ARBs) to be repurposed as a cancer medication because of the presentation of dose-dependent side effects including hypotension as a systemic adverse reaction not only in

normotensive and borderline hypertensive individuals but also in patients with heart failure with systolic blood pressure below 100 mmHg, ischemic heart disease, cerebrovascular disease, hyponatremia, high dose diuretic therapy, renal dialysis, or severe volume and sodium depletion (Abraham et al., 2015; Rosano et al., 2018). A less common side effect like pulmonary toxicity has also been reported after using ACEi at the standard dosage as an antihypertensive medication (Kostis et al., 2005). The clinical safety and efficacy could be considered relatively easy for drug candidates repurposed, particularly when the same dosage and delivery route have been used in preclinical studies and clinical studies (Nowak-Sliwinska et al., 2019). Nevertheless, preclinical studies conducted using the MoCR-CLM model demonstrated a dose-dependent tumour reduction when captopril (ACEi) was administered daily via IP adjusted to the bodyweight of each mouse (750mg/kg), from the day one post-tumour induction to endpoint (Neo et al., 2010). The majority of ACEi are prodrugs that undergo hydrolysis after oral administration to release the active metabolite, except for captopril, a sulfhydryl compound, and can interact with the ACE catalytic Zinc ion *via* their thiol moiety (Sharman et al., 2018). The usual dose range for hypertension is 25 to 150 mg twice per day or three times per day. In humans, the maximum daily dose of 450 mg should not be exceeded (Chen et al., 2018), whereas the MoCR-CLM model tolerated at least thirty times the recommended dosage and had no observable detrimental side effects (Neo et al., 2007; D. L. Vallejo Ardila et al., 2020).

A previous study compared the effect of ACEi, and ARBs administered on different routes. Captopril (ACEi; dose: 750mg/kg) was administered daily via IP, while (ARB/AT1R antagonist; dose: 50mg/kg) was administered daily via irbesartan via subcutaneous injections in mice with CLM (Neo et al., 2007). Both significantly decreased tumour growth and central microvascular density. However, we found no report of impacting survival or tumour necrosis by either of these drugs in this study (Neo et al., 2007). Recent animal research about the feasibility of translating findings recommends for the delivery route to be optimized from systemically administered using IP injection (D. L. Vallejo Ardila et al., 2020) or subcutaneous injections (Neo et al., 2007) to a locally applied strategy (Martin et al., 2020). Current strategies involved systemic administration of drug-loaded nanoparticles (1–1,000 nm in diameter)

known as nanomedicine to enhance the tumour-priming effect (Schmid et al., 2018) and local (extratumoral) administration to induce anti-tumour immunity (for example, NCT02410733). The application of these strategies has shown improvement to overcome resistance to immune-checkpoint inhibition and other immunotherapies (Goldberg, 2019; Riley et al., 2019) and may potentiate the effects of systemic administration of RASi as a cancer immunotherapy agent while avoiding systemic side effects.

The complexity of the translatability of the effect RASi from the CLM mouse model into a human was limited by the statistical generalization of individuals immune response profile into treatment groups, which represents a challenge to interpret the clinical significance of the findings (Fisher et al., 2018). In both mouse and human CLM, RASi selectively modulated T-lymphocytes' cell density and spatial distribution (Vallejo-Ardila et al., 2018) (Chapter 7). The density changes within each T-cell subpopulation in the mouse and human RASi groups and controls may reflect the aggregation effect instead of the individual immune pattern profile. A thorough interpretation of OS influenced by T-cell density and distribution changes in each group is advised due to potential information and selection bias (Chapter 7). These biases are typical limitations of collecting clinical information to compare retrospective cohorts (Sedgwick, 2014).

In conclusion, the immune responses to RASi treatments for CLM need to be further investigated in both genders' animal models, considering how their genetic background affects the immune cell composition. Uncovering the temporal dynamics of these effects requires extensive analysis about changes in T-cell composition and tumour progression and recurrence that can be related to survival in CLM. Further prospective clinical studies are necessary to elucidate genetic and gender differences to RASi (ACEi and ARBs) response to define the patient cohort likely to benefit.

8.4 Conclusions and Future directions

The mechanisms by which RASi reprogram the TME involves modulating the spatiotemporal dynamic of diverse immune cell compartments and the pattern of expression of immune-related proteins by the epithelial tumour core. The potent

immunomodulatory effect of RASi modifies features that limit the efficacy and delivery of current cancer immunotherapies in liver metastases. The results presented here may be of significant clinical relevance to understand how changes in T-cell density and their spatial distribution patterns can be therapeutically manipulated at an early angiogenic stage of metastases development.

The accurate assessment of function CD8⁺ T effector cells isolated from captopril treated tumours is necessary using *in vitro* immune cytotoxic assays to measure the activity of CD8⁺ T cells in response to captopril. Besides, further functional phenotypic characterization of unconventional T lymphocyte populations of TcRαβ⁺, TcRγδ⁺ and other liver resident subpopulations like CD103⁺ T_{RM} is needed to identify their involvement in tumour control in response to RASi. RASi modulated additional factors that influence T-cell antitumour efficacy and antigen presentation. The phosphorylation-driven patterns of activation of the immunoproteasome need to be better outlined because the overexpression of *PSMB8* can be a stronger predictor to immunotherapy in certain types of cancer, in CLM remains to be determined.

The exact molecular mechanism associated with different densities and localization-dependent prognostic relevance of T cells in the RASi remains unclear. Future studies should consider the disease stage, phenotype, kinetics, and sequence of neoadjuvant treatment for CLM, which may help untangle current immunotherapies' resistance. Also, incorporating complementary single-cell RNA sequencing expression patterns and mass cytometry (CyTOF) of peripheral blood from selected patients can provide a comprehensive characterization of the immune landscape of CLM patients and may inform on treatment regimens.

CHAPTER 9

SUMMARY

RASi have shown anti-tumour effects that may have a significant impact to enhance the efficacy and delivery of current cancer immunotherapies. In chapter one, the components of the renin-angiotensin system including both, conventional and alternative axis, appear to have divergent effects on types of cancer. The compiling evidence about the mechanisms by which RASi impair tumour growth beyond their function of modulating tumour vasculature and tumour solid stress. The mechanisms by which RASi can reprogram the TME involves impairing hypoxia and acidosis within the tumour stroma, regulating inflammatory signalling pathways and oxidative stress, modulating the function of the non-cellular components and immune cells, and regulating the crosstalk between the kallikrein-kinin system and RAS. In chapters three to five, the immunomodulatory effect of RASi on T-cells (**chapter 3**), myeloid-derived cells expressing CD31 (**chapter 4**), inducible immune proteasome expression (**chapter 5**) were investigated using the MoCR-CLM mouse model.

Moreover, T-cell distribution and the spatial location was explored in human CLM in specimens collected at the time of liver resection (**chapter 6**). Finally, T-cell density and distribution changes were compared between a retrospective cohort that received RASi as antihypertensive medication before surgery and patients who had not been on any antihypertensive medications. Also, it was investigated if these changes in the immune infiltration pattern were associated with a better outcome.

Pre-clinical studies have shown that RASi reduced tumour progression and tumour viability in CLM mice. RAS components are expressed by most immune cells and adult hematopoietic cells, thus are potential targets for modulating tumour infiltrating immune cells and provide a mechanism of tumour control by RASi. The liver's organotropism as the most common site for metastases reflects on the anergic status before any pro-inflammatory triggering event occurs. The effect of RASi on immune landscape and the complex spatiotemporal relationships of T-cell populations (**chapter 3, 6 and 7**), MDSC (**chapter 4**), MΦ (**chapter 4**) and DCs (**chapter 4, 6 and 7**) was investigated in CLM.

The changes in composition and distribution of the immune infiltrate were compared between the early angiogenic metastatic stage and the exponential tumour growth stage. Captopril treatment significantly increased the infiltration of CD3⁺ T cells into the liver and tumour and altered the phenotypic composition of infiltrating CD4⁺ and

CD8⁺ T cells and DNT cells. Captopril treatment also increased checkpoint receptor PD-1 on CD8⁺ and DNT subsets analyzed (**chapter 3**). Also, RASi influenced the maturation status and changes in the phenotypical composition of myeloid-derived populations. RASi significantly reduces CD31⁺ monocyte-derived subset F4/80⁻ Ly6C^{intermediates/High}, which may have an immunosuppressive function. Also, captopril treatment decreased the expression of PD-L1 in mature MΦ, which may reprogram them towards an immune stimulatory state (**chapter 4**). Additional factors can determine the efficacy of T-cell anti-tumour function, including the expression of immunoproteasome subunits. Captopril treatment differentially regulated the immunoproteasome cluster. These proteins are functional subunits of the immunoproteasome with major roles in generating peptides for antigen presentation and other immunological functions. Captopril upregulates β5i (LMP7 or PSMB8) expression while downregulates the constitutive expression β5 (PSMB5) within the tumour. Furthermore, functional enrichment inferred that the biological processes and pathways modulated were neddylation, proteasomal ubiquitin-independent and proteasome-mediated ubiquitin-dependent protein catabolic processes (**chapter 5**).

Several prognostic factors have been associated with the patient's outcome after liver resection, including T lymphocytes' frequency and phenotype. A novel population of memory CD8⁺ T cells expressing CD103⁺ known as resident memory T cells T_{RM} showed a strong correlation with a favourable prognosis in some cancers. T-cells expressing the integrin CD103 were shown to bind to E-cadherin, an epithelial cell junctional protein, enabling their retention and activation. Functionally, the dense collagen matrix produced by α-SMA⁺ cancer-associated fibroblast may represent a physical barrier to infiltrating T lymphocytes. Spatial distribution of all three T lymphocyte phenotypes was seen in significantly higher levels in the invasive tumour margin compared to their density within the liver parenchyma or the tumour centre. The density and spatial locations of these phenotypes were related to overall survival. Thus, high CD3⁺ density in the TC or high CD8⁺ density in the tumour IM predicts a significantly better OS. Neither the distribution nor the density of TRM correlated with better patient outcome in this study. The E-cadherin staining pattern was highly heterogeneous among this cohort of CLM patients. However, a novel population of CD8⁺ T cells expressing E-cadherin was identified, which was significantly correlated

with better OS. α -SMA⁺ staining was strong in the TC and IM and significantly less in the LP. However, the high spatial engagement between CD8⁺ T cells and α -SMA⁺ cells predicts better OS in CLM patients (**chapter 6**). These findings highlight the relevance of using spatial and phenotypic characterization to investigate immune cell interactions within the tumour microenvironment, identify predictive immune patterns and potential therapeutic targets and inform future treatments that can modulate the number and distribution of immune cells.

T-cell density and their spatial location were modulated by RASi (ACEi/ARBs) across different human CLM tissue compartments (**chapter 7**). Integrating T cell density and spatial dynamic, it was found that changes in CD3⁺T- cell density and distribution at the adjacent LP influence patient survival after CLM resection in patients that received RASi as antihypertensive medication (**chapter 7**).

Further characterization of unconventional T-cells like the novel E-cadherin positive CD8⁺ T cells, non-T cells expressing CD103⁺ FoxP3⁺ may offer insights into CRM immune surveillance.

CHAPTER 10

REFERENCES

- Abd Hamid, M., Colin-York, H., Khalid-Alham, N., Browne, M., Cerundolo, L., Chen, J.-L., Yao, X., Rosendo-Machado, S., Waugh, C., Maldonado-Perez, D., Bowes, E., Verrill, C., Cerundolo, V., Conlon, C. P., Fritzsche, M., Peng, Y., & Dong, T. (2020). Self-Maintaining CD103+ Cancer-Specific T Cells Are Highly Energetic with Rapid Cytotoxic and Effector Responses. *Cancer Immunol Res*, 8(2), 203-216. <https://doi.org/10.1158/2326-6066.cir-19-0554>
- Abraham, H. M. A., White, C. M., & White, W. B. (2015). The comparative efficacy and safety of the angiotensin receptor blockers in the management of hypertension and other cardiovascular diseases. *Drug safety*, 38(1), 33-54. <https://doi.org/10.1007/s40264-014-0239-7>
- Adams, C. L., Chen, Y. T., Smith, S. J., & Nelson, W. J. (1998). Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *The Journal of cell biology*, 142(4), 1105-1119. <https://doi.org/10.1083/jcb.142.4.1105>
- Affara, N. I., Ruffell, B., Medler, T. R., Gunderson, A. J., Johansson, M., Bornstein, S., Bergsland, E., Steinhoff, M., Li, Y., Gong, Q., Ma, Y., Wiesen, J. F., Wong, M. H., Kulesz-Martin, M., Irving, B., & Coussens, L. M. (2014). B cells regulate macrophage phenotype and response to chemotherapy in squamous carcinomas. *Cancer Cell*, 25(6), 809-821. <https://doi.org/10.1016/j.ccr.2014.04.026>
- Ager, E. I., Neo, J., & Christophi, C. (2008). The renin-angiotensin system and malignancy. *Carcinogenesis*, 29(9), 1675-1684. <https://doi.org/10.1093/carcin/bgn171>
- Ahmad, F., Hong, H. S., Jäckel, M., Jablonka, A., Lu, I. N., Bhatnagar, N., Eberhard, J. M., Bollmann, B. A., Ballmaier, M., Zielinska-Skowronek, M., Schmidt, R. E., & Meyer-Olson, D. (2014). High frequencies of polyfunctional CD8+ NK cells in chronic HIV-1 infection are associated with slower disease progression. *Journal of virology*, 88(21), 12397-12408. <https://doi.org/10.1128/JVI.01420-14>
- Aki, K., Shimizu, A., Masuda, Y., Kuwahara, N., Arai, T., Ishikawa, A., Fujita, E., Mii, A., Natori, Y., Fukunaga, Y., & Fukuda, Y. (2010). ANG II receptor blockade enhances anti-inflammatory macrophages in anti-glomerular basement membrane glomerulonephritis. *Am J Physiol Renal Physiol*, 298(4), F870-882. <https://doi.org/10.1152/ajprenal.00374.2009>
- Albini, A., Bruno, A., Noonan, D. M., & Mortara, L. (2018). Contribution to Tumor Angiogenesis From Innate Immune Cells Within the Tumor Microenvironment: Implications for Immunotherapy. *Frontiers in Immunology*, 9, 527-527. <https://doi.org/10.3389/fimmu.2018.00527>

- Aliberti, J., Viola, J. P., Vieira-de-Abreu, A., Bozza, P. T., Sher, A., & Scharfstein, J. (2003). Cutting edge: bradykinin induces IL-12 production by dendritic cells: a danger signal that drives Th1 polarization. *J Immunol*, 170(11), 5349-5353.
- Allakhverdi, Z., Fitzpatrick, D., Boisvert, A., Baba, N., Bouguermouh, S., Sarfati, M., & Delespesse, G. (2006). Expression of CD103 identifies human regulatory T-cell subsets. *J Allergy Clin Immunol*, 118(6), 1342-1349. <https://doi.org/10.1016/j.jaci.2006.07.034>
- Alsaab, H. O., Sau, S., Alzhrani, R., Tatiparti, K., Bhise, K., Kashaw, S. K., & Iyer, A. K. (2017). PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Frontiers in Pharmacology*, 8, 561. <https://doi.org/10.3389/fphar.2017.00561>
- Anderson, K. G., Stromnes, I. M., & Greenberg, P. D. (2017). Obstacles Posed by the Tumor Microenvironment to T cell Activity: A Case for Synergistic Therapies. *Cancer Cell*, 31(3), 311-325. <https://doi.org/10.1016/j.ccell.2017.02.008>
- Angelova, M., Mlecnik, B., Vasaturo, A., Bindea, G., Fredriksen, T., Lafontaine, L., Buttard, B., Morgand, E., Bruni, D., Jouret-Mourin, A., Hubert, C., Kartheuser, A., Humblet, Y., Ceccarelli, M., Syed, N., Marincola, F. M., Bedognetti, D., Van den Eynde, M., & Galon, J. (2018). Evolution of Metastases in Space and Time under Immune Selection. *Cell*, 175(3), 751-765.e716. <https://doi.org/10.1016/j.cell.2018.09.018>
- Angulo, I., de las Heras, F. G., García-Bustos, J. F., Gargallo, D., Muñoz-Fernández, M. A., & Fresno, M. (2000). Nitric oxide-producing CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood*, 95(1), 212-220.
- Arendse, L. B., Danser, A. H. J., Poglitsch, M., Touyz, R. M., Burnett, J. C., Llorens-Cortes, C., Ehlers, M. R., & Sturrock, E. D. (2019). Novel Therapeutic Approaches Targeting the Renin-Angiotensin System and Associated Peptides in Hypertension and Heart Failure. *Pharmacol Rev*, 71(4), 539-570. <https://doi.org/10.1124/pr.118.017129>
- Arrieta, O., Pineda-Olvera, B., Guevara-Salazar, P., Hernandez-Pedro, N., Morales-Espinosa, D., Ceron-Lizarraga, T. L., Gonzalez-De la Rosa, C. H., Rembao, D., Segura-Pacheco, B., & Sotelo, J. (2008). Expression of AT1 and AT2 angiotensin receptors in astrocytomas is associated with poor prognosis. *Br J Cancer*, 99(1), 160-166. <https://doi.org/10.1038/sj.bjc.6604431>
- Arrieta, O., Villarreal-Garza, C., Vizcaino, G., Pineda, B., Hernandez-Pedro, N., Guevara-Salazar, P., Wegman-Ostrosky, T., Villanueva-Rodriguez, G., & Gamboa-Dominguez, A. (2015). Association between AT1 and AT2 angiotensin II receptor expression with cell proliferation and angiogenesis in operable

- breast cancer. *Tumour Biol*, 36(7), 5627-5634.
<https://doi.org/10.1007/s13277-015-3235-3>
- Bailey, A. S., Willenbring, H., Jiang, S., Anderson, D. A., Schroeder, D. A., Wong, M. H., Grompe, M., & Fleming, W. H. (2006). Myeloid lineage progenitors give rise to vascular endothelium. *Proceedings of the National Academy of Sciences*, 103(35), 13156-13161. <https://doi.org/10.1073/pnas.0604203103>
- Balkwill, F. R., Capasso, M., & Hagemann, T. (2012). The tumor microenvironment at a glance. *Journal of Cell Science*, 125(23), 5591-5596.
<https://doi.org/10.1242/jcs.116392>
- Ballbach, M., Dannert, A., Singh, A., Siegmund, D. M., Handgretinger, R., Piali, L., Rieber, N., & Hartl, D. (2017). Expression of checkpoint molecules on myeloid-derived suppressor cells. *Immunology Letters*, 192, 1-6.
<https://doi.org/https://doi.org/10.1016/j.imlet.2017.10.001>
- Banerjee, S., Dudeja, V., & Saluja, A. (2019). Unconventional T Cells in the Pancreatic Tumor Microenvironment: Thinking Outside the Box. *Cancer Discov*, 9(9), 1164-1166. <https://doi.org/10.1158/2159-8290.Cd-19-0722>
- Bangalore, S., Kumar, S., Kjeldsen, S. E., Makani, H., Grossman, E., Wetterslev, J., Gupta, A. K., Sever, P. S., Gluud, C., & Messerli, F. H. (2011). Antihypertensive drugs and risk of cancer: network meta-analyses and trial sequential analyses of 324,168 participants from randomised trials. *Lancet Oncol*, 12(1), 65-82.
[https://doi.org/10.1016/s1470-2045\(10\)70260-6](https://doi.org/10.1016/s1470-2045(10)70260-6)
- Barbarin, A., Cayssials, E., Jacomet, F., Nunez, N. G., Basbous, S., Lefèvre, L., Abdallah, M., Piccirilli, N., Morin, B., Lavoue, V., Catros, V., Piaggio, E., Herbelin, A., & Gombert, J.-M. (2017). Phenotype of NK-Like CD8(+) T Cells with Innate Features in Humans and Their Relevance in Cancer Diseases [Hypothesis and Theory]. *Frontiers in Immunology*, 8(316).
<https://doi.org/10.3389/fimmu.2017.00316>
- Beitia, M., Solano-Iturri, J. D., Errarte, P., Sanz, B., Perez, I., Etxezarraga, M. C., Loizate, A., Asumendi, A., & Larrinaga, G. (2019). Altered expression of renin-angiotensin system receptors throughout colorectal adenoma-adenocarcinoma sequence [Research Paper]. *International Journal of Medical Sciences*, 16(6), 813-821. <https://doi.org/10.7150/ijms.32599>
- Belkina, A. C., Ciccolella, C. O., Anno, R., Halpert, R., Spidlen, J., & Snyder-Cappione, J. E. (2019). Automated optimized parameters for T-distributed stochastic neighbor embedding improve visualization and analysis of large datasets. *Nature Communications*, 10(1), 5415. <https://doi.org/10.1038/s41467-019-13055-y>

- Beltraminelli, T., & De Palma, M. (2020). Biology and therapeutic targeting of tumour-associated macrophages. *The Journal of Pathology*, 250(5), 573-592. <https://doi.org/10.1002/path.5403>
- Berg, A. C., Chernavsky-Sequeira, C., Lindsey, J., Gomez, R. A., & Sequeira-Lopez, M. L. S. (2013). Pericytes synthesize renin. *World Journal of Nephrology*, 2(1), 11-16. <https://doi.org/10.5527/wjn.v2.i1.11>
- Bernasconi, R., & Nystrom, A. (2018). Balance and circumstance: The renin angiotensin system in wound healing and fibrosis. *Cell Signal*, 51, 34-46. <https://doi.org/10.1016/j.cellsig.2018.07.011>
- Berthel, A., Zoernig, I., Valous, N. A., Kahlert, C., Klupp, F., Ulrich, A., Weitz, J., Jaeger, D., & Halama, N. (2017). Detailed resolution analysis reveals spatial T cell heterogeneity in the invasive margin of colorectal cancer liver metastases associated with improved survival. *Oncoimmunology*, 6(3), e1286436-e1286436. <https://doi.org/10.1080/2162402X.2017.1286436>
- Berx, G., Cleton-Jansen, A. M., Nollet, F., de Leeuw, W. J., van de Vijver, M., Cornelisse, C., & van Roy, F. (1995). E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *Embo j*, 14(24), 6107-6115.
- Beyrend, G., van der Gracht, E., Yilmaz, A., van Duikeren, S., Camps, M., Höllt, T., Vilanova, A., van Unen, V., Koning, F., de Miranda, N. F. C. C., Arens, R., & Ossendorp, F. (2019). PD-L1 blockade engages tumor-infiltrating lymphocytes to co-express targetable activating and inhibitory receptors. *J Immunother Cancer*, 7(1), 217. <https://doi.org/10.1186/s40425-019-0700-3>
- Bhardwaj, G. (2006). How the antihypertensive losartan was discovered. *Expert Opinion on Drug Discovery*, 1(6), 609-618. <https://doi.org/10.1517/17460441.1.6.609>
- Bhargava, R., & Madabhushi, A. (2016). Emerging Themes in Image Informatics and Molecular Analysis for Digital Pathology. *Annual review of biomedical engineering*, 18, 387-412. <https://doi.org/10.1146/annurev-bioeng-112415-114722>
- Bindea, G., Mlecnik, B., Tosolini, M., Kirilovsky, A., Waldner, M., Obenauf, A. C., Angell, H., Fredriksen, T., Lafontaine, L., Berger, A., Bruneval, P., Fridman, W. H., Becker, C., Pages, F., Speicher, M. R., Trajanoski, Z., & Galon, J. (2013). Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity*, 39(4), 782-795. <https://doi.org/10.1016/j.immuni.2013.10.003>
- Blaes, N., & Girolami, J. P. (2013). Targeting the 'Janus face' of the B2-bradykinin receptor. *Expert Opin Ther Targets*, 17(10), 1145-1166. <https://doi.org/10.1517/14728222.2013.827664>

- Blom, S., Paavolainen, L., Bychkov, D., Turkki, R., Mäki-Teeri, P., Hemmes, A., Välimäki, K., Lundin, J., Kallioniemi, O., & Pellinen, T. (2017). Systems pathology by multiplexed immunohistochemistry and whole-slide digital image analysis. *Scientific Reports*, 7(1), 15580. <https://doi.org/10.1038/s41598-017-15798-4>
- Borghaei, H., Paz-Ares, L., Horn, L., Spigel, D. R., Steins, M., Ready, N. E., Chow, L. Q., Vokes, E. E., Felip, E., Holgado, E., Barlesi, F., Kohlhäufel, M., Arrieta, O., Burgio, M. A., Fayette, J., Lena, H., Poddubskaya, E., Gerber, D. E., Gettinger, S. N., Rudin, C. M., Rizvi, N., Crinò, L., Blumenschein, G. R., Antonia, S. J., Dorange, C., Harbison, C. T., Graf Finckenstein, F., & Brahmer, J. R. (2015). Nivolumab versus Docetaxel in Advanced Nonsquamous Non–Small-Cell Lung Cancer. *New England Journal of Medicine*, 373(17), 1627-1639. <https://doi.org/10.1056/NEJMoa1507643>
- Borlado, L. R., & Méndez, J. (2008). CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis*, 29(2), 237-243. <https://doi.org/10.1093/carcin/bgm268>
- Boros, P., Ochando, J., & Zeher, M. (2016). Myeloid derived suppressor cells and autoimmunity. *Hum Immunol*, 77(8), 631-636. <https://doi.org/10.1016/j.humimm.2016.05.024>
- Bras, G., Bochenska, O., Rapala-Kozik, M., Guevara-Lora, I., Faussner, A., & Kozik, A. (2012). Extracellular aspartic protease SAP2 of *Candida albicans* yeast cleaves human kininogens and releases proinflammatory peptides, Met-Lys-bradykinin and des-Arg(9)-Met-Lys-bradykinin. *Biol Chem*, 393(8), 829-839. <https://doi.org/10.1515/hsz-2012-0157>
- Brieher, W. M., & Gumbiner, B. M. (1994). Regulation of C-cadherin function during activin induced morphogenesis of *Xenopus* animal caps. *The Journal of cell biology*, 126(2), 519-527. <https://doi.org/10.1083/jcb.126.2.519>
- Brodt, P. (2016). Role of the Microenvironment in Liver Metastasis: From Pre- to Prometastatic Niches. *Clin Cancer Res*, 22(24), 5971-5982. <https://doi.org/10.1158/1078-0432.Ccr-16-0460>
- Bronte, V., Apolloni, E., Cabrelle, A., Ronca, R., Serafini, P., Zamboni, P., Restifo, N. P., & Zanovello, P. (2000). Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. *Blood*, 96(12), 3838-3846.
- Bronte, V., Brandau, S., Chen, S.-H., Colombo, M. P., Frey, A. B., Greten, T. F., Mandruzzato, S., Murray, P. J., Ochoa, A., Ostrand-Rosenberg, S., Rodriguez, P. C., Sica, A., Umansky, V., Vonderheide, R. H., & Gabrilovich, D. I. (2016). Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards [Review Article]. *Nature Communications*, 7, 12150. <https://doi.org/10.1038/ncomms12150>

- Brudecki, L., Ferguson, D. A., McCall, C. E., & El Gazzar, M. (2012). Myeloid-Derived Suppressor Cells Evolve during Sepsis and Can Enhance or Attenuate the Systemic Inflammatory Response. *Infection and Immunity*, 80(6), 2026-2034. <https://doi.org/10.1128/iai.00239-12>
- Calcinotto, A., Filipazzi, P., Grioni, M., Iero, M., De Mito, A., Ricupito, A., Cova, A., Canese, R., Jachetti, E., Rossetti, M., Huber, V., Parmiani, G., Generoso, L., Santinami, M., Borghi, M., Fais, S., Bellone, M., & Rivoltini, L. (2012). Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes. *Cancer Res*, 72(11), 2746-2756. <https://doi.org/10.1158/0008-5472.Can-11-1272>
- Campbell, D. J. (2014). Clinical Relevance of Local Renin Angiotensin Systems. *Frontiers in Endocrinology*, 5, 113. <https://doi.org/10.3389/fendo.2014.00113>
- Cao, D.-Y., Saito, S., Veiras, L. C., Okwan-Duodu, D., Bernstein, E. A., Giani, J. F., Bernstein, K. E., & Khan, Z. (2020). Role of angiotensin-converting enzyme in myeloid cell immune responses. *Cellular & Molecular Biology Letters*, 25(1), 31. <https://doi.org/10.1186/s11658-020-00225-w>
- Capettini, L. S., Montecucco, F., Mach, F., Stergiopoulos, N., Santos, R. A., & da Silva, R. F. (2012). Role of renin-angiotensin system in inflammation, immunity and aging. *Curr Pharm Des*, 18(7), 963-970. <https://doi.org/10.2174/138161212799436593>
- Capone, I., Marchetti, P., Ascierto, P. A., Malorni, W., & Gabriele, L. (2018). Sexual Dimorphism of Immune Responses: A New Perspective in Cancer Immunotherapy. *Frontiers in Immunology*, 9, 552-552. <https://doi.org/10.3389/fimmu.2018.00552>
- Carbajo-Lozoya, J., Lutz, S., Feng, Y., Kroll, J., Hammes, H. P., & Wieland, T. (2012). Angiotensin II modulates VEGF-driven angiogenesis by opposing effects of type 1 and type 2 receptor stimulation in the microvascular endothelium. *Cell Signal*, 24(6), 1261-1269. <https://doi.org/10.1016/j.cellsig.2012.02.005>
- Carey, R. M., & Siragy, H. M. (2003). Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev*, 24(3), 261-271. <https://doi.org/10.1210/er.2003-0001>
- Chae, Y. K., Chang, S., Ko, T., Anker, J., Agte, S., Iams, W., Choi, W. M., Lee, K., & Cruz, M. (2018). Epithelial-mesenchymal transition (EMT) signature is inversely associated with T-cell infiltration in non-small cell lung cancer (NSCLC). *Scientific Reports*, 8(1), 2918. <https://doi.org/10.1038/s41598-018-21061-1>
- Chae, Y. K., Valsecchi, M. E., Kim, J., Bianchi, A. L., Khemasuwan, D., Desai, A., & Tester, W. (2011). Reduced risk of breast cancer recurrence in patients using

- ACE inhibitors, ARBs, and/or statins. *Cancer Invest*, 29(9), 585-593.
<https://doi.org/10.3109/07357907.2011.616252>
- Chanmee, T., Ontong, P., Konno, K., & Itano, N. (2014). Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment. *Cancers*, 6(3), 1670-1690. <https://doi.org/10.3390/cancers6031670>
- Chauhan, V. P., Chen, I. X., Tong, R., Ng, M. R., Martin, J. D., Naxerova, K., Wu, M. W., Huang, P., Boucher, Y., Kohane, D. S., Langer, R., & Jain, R. K. (2019). Reprogramming the microenvironment with tumor-selective angiotensin blockers enhances cancer immunotherapy. *Proceedings of the National Academy of Sciences*, 116(22), 10674-10680.
<https://doi.org/10.1073/pnas.1819889116>
- Chauhan, V. P., Martin, J. D., Liu, H., Lacorre, D. A., Jain, S. R., Kozin, S. V., Stylianopoulos, T., Mousa, A. S., Han, X., Adstamongkonkul, P., Popovic, Z., Huang, P., Bawendi, M. G., Boucher, Y., & Jain, R. K. (2013). Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels. *Nat Commun*, 4, 2516.
<https://doi.org/10.1038/ncomms3516>
- Chee, J., Singh, J., Naran, A., Misso, N. L., Thompson, P. J., & Bhoola, K. D. (2007). Novel expression of kallikreins, kallikrein-related peptidases and kinin receptors in human pleural mesothelioma. *Biol Chem*, 388(11), 1235-1242.
<https://doi.org/10.1515/bc.2007.139>
- Chen, I. X., Chauhan, V. P., Posada, J., Ng, M. R., Wu, M. W., Adstamongkonkul, P., Huang, P., Lindeman, N., Langer, R., & Jain, R. K. (2019). Blocking CXCR4 alleviates desmoplasia, increases T-lymphocyte infiltration, and improves immunotherapy in metastatic breast cancer. *Proc Natl Acad Sci U S A*, 116(10), 4558-4566. <https://doi.org/10.1073/pnas.1815515116>
- Chen, S., Akbar, S. M., Abe, M., Hiasa, Y., & Onji, M. (2011). Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus. *Clin Exp Immunol*, 166(1), 134-142.
<https://doi.org/10.1111/j.1365-2249.2011.04445.x>
- Chen, X., Howatt, D. A., Balakrishnan, A., Moorlegghen, J. J., Wu, C., Cassis, L. A., Daugherty, A., & Lu, H. (2016). Angiotensin-Converting Enzyme in Smooth Muscle Cells Promotes Atherosclerosis. *Brief Report. Arteriosclerosis, Thrombosis, and Vascular Biology*, 36(6), 1085-1089.
<https://doi.org/doi:10.1161/ATVBAHA.115.307038>
- Chen, Y. J., Li, L. J., Tang, W. L., Song, J. Y., Qiu, R., Li, Q., Xue, H., & Wright, J. M. (2018). First-line drugs inhibiting the renin angiotensin system versus other first-line antihypertensive drug classes for hypertension. *Cochrane Database of Systematic Reviews*(11).
<https://doi.org/10.1002/14651858.CD008170.pub3>

- Cheng, Q., Zhou, L., Zhou, J., Wan, H., Li, Q., & Feng, Y. (2016). ACE2 overexpression inhibits acquired platinum resistance-induced tumor angiogenesis in NSCLC. *Oncol Rep*, 36(3), 1403-1410. <https://doi.org/10.3892/or.2016.4967>
- Chhabra, S. (2017). Novel Proteasome Inhibitors and Histone Deacetylase Inhibitors: Progress in Myeloma Therapeutics. *Pharmaceuticals (Basel)*, 10(2). <https://doi.org/10.3390/ph10020040>
- Chiang, Y. Y., Chen, K. B., Tsai, T. H., & Tsai, W. C. (2014). Lowered cancer risk with ACE inhibitors/ARBs: a population-based cohort study. *J Clin Hypertens (Greenwich)*, 16(1), 27-33. <https://doi.org/10.1111/jch.12228>
- Chiorean, E. G., Nandakumar, G., Fadelu, T., Temin, S., Alarcon-Rozas, A. E., Bejarano, S., Croitoru, A.-E., Grover, S., Lohar, P. V., Odhiambo, A., Park, S. H., Garcia, E. R., Teh, C., Rose, A., Zaki, B., & Chamberlin, M. D. (2020). Treatment of Patients With Late-Stage Colorectal Cancer: ASCO Resource-Stratified Guideline. *JCO Global Oncology*(6), 414-438. <https://doi.org/10.1200/jgo.19.00367>
- Chisi, J. E., Wdzieczak-Bakala, J., Thierry, J., Briscoe, C. V., & Riches, A. C. (1999). Captopril inhibits the proliferation of hematopoietic stem and progenitor cells in murine long-term bone marrow cultures. *Stem Cells*, 17(6), 339-344. <https://doi.org/10.1002/stem.170339>
- Chuah, S., & Chew, V. (2020). High-dimensional immune-profiling in cancer: implications for immunotherapy. *J Immunother Cancer*, 8(1). <https://doi.org/10.1136/jitc-2019-000363>
- Chung, E. Y. M., Ruospo, M., Natale, P., Bolignano, D., Navaneethan, S. D., Palmer, S. C., & Strippoli, G. F. M. (2020). Aldosterone antagonists in addition to renin angiotensin system antagonists for preventing the progression of chronic kidney disease. *Cochrane Database of Systematic Reviews*(10). <https://doi.org/10.1002/14651858.CD007004.pub4>
- Clement, M., Fornasa, G., Guedj, K., Ben Mkaddem, S., Gaston, A.-T., Khallou-Laschet, J., Morvan, M., Nicoletti, A., & Caligiuri, G. (2014). CD31 is a key coinhibitory receptor in the development of immunogenic dendritic cells. *Proc Natl Acad Sci U S A*, 111(12), E1101-E1110. <https://doi.org/10.1073/pnas.1314505111>
- Connolly, M. K., Mallen-St Clair, J., Bedrosian, A. S., Malhotra, A., Vera, V., Ibrahim, J., Henning, J., Pachter, H. L., Bar-Sagi, D., Frey, A. B., & Miller, G. (2010). Distinct populations of metastases-enabling myeloid cells expand in the liver of mice harboring invasive and preinvasive intra-abdominal tumor. *J Leukoc Biol*, 87(4), 713-725. <https://doi.org/10.1189/jlb.0909607>
- Consortium, T. U. (2020). UniProt: the universal protein knowledgebase in 2021. *Nucleic acids research*, 49(D1), D480-D489. <https://doi.org/10.1093/nar/gkaa1100>

- Corbett, T. H., Griswold, D. P., Jr., Roberts, B. J., Peckham, J. C., & Schabel, F. M., Jr. (1975). Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. *Cancer Res*, 35(9), 2434-2439.
- Corgnac, S., Boutet, M., Kfoury, M., Naltet, C., & Mami-Chouaib, F. (2018). The Emerging Role of CD8(+) Tissue Resident Memory T (T(RM)) Cells in Antitumor Immunity: A Unique Functional Contribution of the CD103 Integrin. *Frontiers in Immunology*, 9, 1904-1904.
<https://doi.org/10.3389/fimmu.2018.01904>
- Cortese, N., Soldani, C., Franceschini, B., Barbagallo, M., Marchesi, F., Torzilli, G., & Donadon, M. (2019). Macrophages in Colorectal Cancer Liver Metastases. *Cancers (Basel)*, 11(5). <https://doi.org/10.3390/cancers11050633>
- Cortez-Retamozo, V., Etzrodt, M., Newton, A., Ryan, R., Pucci, F., Sio, Selena W., Kuswanto, W., Rauch, Philipp J., Chudnovskiy, A., Iwamoto, Y., Kohler, R., Marinelli, B., Gorbato, R., Wojtkiewicz, G., Panizzi, P., Mino-Kenudson, M., Forghani, R., Figueiredo, J.-L., Chen, John W., Xavier, R., Swirski, Filip K., Nahrendorf, M., Weissleder, R., & Pittet, Mikael J. (2013). Angiotensin II Drives the Production of Tumor-Promoting Macrophages. *Immunity*, 38(2), 296-308.
<https://doi.org/https://doi.org/10.1016/j.immuni.2012.10.015>
- Costa, A., Kieffer, Y., Scholer-Dahirel, A., Pelon, F., Bourachot, B., Cardon, M., Sirven, P., Magagna, I., Fuhrmann, L., Bernard, C., Bonneau, C., Kondratova, M., Kuperstein, I., Zinovyev, A., Givel, A. M., Parrini, M. C., Soumelis, V., Vincent-Salomon, A., & Mechta-Grigoriou, F. (2018). Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer. *Cancer Cell*, 33(3), 463-479.e410. <https://doi.org/10.1016/j.ccell.2018.01.011>
- Coulson, R., Liew, S. H., Connelly, A. A., Yee, N. S., Deb, S., Kumar, B., Vargas, A. C., O'Toole, S. A., Parslow, A. C., Poh, A., Putoczki, T., Morrow, R. J., Alorro, M., Lazarus, K. A., Yeap, E. F. W., Walton, K. L., Harrison, C. A., Hannan, N. J., George, A. J., Clyne, C. D., Ernst, M., Allen, A. M., & Chand, A. L. (2017). The angiotensin receptor blocker, Losartan, inhibits mammary tumor development and progression to invasive carcinoma. *Oncotarget*, 8(12), 18640-18656.
<https://doi.org/10.18632/oncotarget.15553>
- Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology*, 26(12), 1367-1372.
<https://doi.org/10.1038/nbt.1511>
- Crowley, S. D., & Rudemiller, N. P. (2017). Immunologic Effects of the Renin-Angiotensin System. *J Am Soc Nephrol*, 28(5), 1350-1361.
<https://doi.org/10.1681/asn.2016101066>

- Cui, T., Nakagami, H., Iwai, M., Takeda, Y., Shiuchi, T., Daviet, L., Nahmias, C., & Horiuchi, M. (2001). Pivotal role of tyrosine phosphatase SHP-1 in AT2 receptor-mediated apoptosis in rat fetal vascular smooth muscle cell. *Cardiovasc Res*, 49(4), 863-871.
- da Costa, P. L., Sirois, P., Tannock, I. F., & Chammas, R. (2014). The role of kinin receptors in cancer and therapeutic opportunities. *Cancer Lett*, 345(1), 27-38. <https://doi.org/10.1016/j.canlet.2013.12.009>
- da Costa, P. L. N., Wynne, D., Fifis, T., Nguyen, L., Perini, M., & Christophi, C. (2018). The kallikrein-Kinin system modulates the progression of colorectal liver metastases in a mouse model. *BMC cancer*, 18(1), 382. <https://doi.org/10.1186/s12885-018-4260-6>
- Dai, J., El Gazzar, M., Li, G. Y., Moorman, J. P., & Yao, Z. Q. (2015). Myeloid-derived suppressor cells: paradoxical roles in infection and immunity. *J Innate Immun*, 7(2), 116-126. <https://doi.org/10.1159/000368233>
- Daien, V., Duny, Y., Ribstein, J., du Cailar, G., Mimran, A., Villain, M., Daures, J. P., & Fesler, P. (2012). Treatment of hypertension with renin-angiotensin system inhibitors and renal dysfunction: a systematic review and meta-analysis. *Am J Hypertens*, 25(1), 126-132. <https://doi.org/10.1038/ajh.2011.180>
- Danilov, S. M., Sadovnikova, E., Scharenborg, N., Balyasnikova, I. V., Svinareva, D. A., Semikina, E. L., Parovichnikova, E. N., Savchenko, V. G., & Adema, G. J. (2003). Angiotensin-converting enzyme (CD143) is abundantly expressed by dendritic cells and discriminates human monocyte-derived dendritic cells from acute myeloid leukemia-derived dendritic cells. *Exp Hematol*, 31(12), 1301-1309. <https://doi.org/10.1016/j.exphem.2003.08.018>
- Dayan, F., Mazure, N. M., Brahimi-Horn, M. C., & Pouyssegur, J. (2008). A Dialogue between the Hypoxia-Inducible Factor and the Tumor Microenvironment. *Cancer Microenvironment*, 1(1), 53-68. <https://doi.org/10.1007/s12307-008-0006-3>
- De Tullio, G., Strippoli, S., Angarano, R., De Fazio, V., Sgherza, N., Negri, A., Albano, A., Iacopino, P., Guarini, A., & Guida, M. (2015). $\alpha\beta$ -Double Negative CD4/CD8 (CD56) T cell (DNTs) in metastatic melanoma: basal frequency and behaviour during Ipilimumab treatment. Preliminary evaluations. *Journal of Translational Medicine*, 13(Suppl 1), O10-O10. <https://doi.org/10.1186/1479-5876-13-S1-O10>
- den Haan, J. M., Lehar, S. M., & Bevan, M. J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *The Journal of experimental medicine*, 192(12), 1685-1696. <https://doi.org/10.1084/jem.192.12.1685>
- Dieci, M. V., Tsvetkova, V., Griguolo, G., Miglietta, F., Tasca, G., Giorgi, C. A., Cumerlato, E., Massa, D., Lo Mele, M., Orvieto, E., Guarneri, V., & Conte, P.

- (2020). Integration of tumour infiltrating lymphocytes, programmed cell-death ligand-1, CD8 and FOXP3 in prognostic models for triple-negative breast cancer: Analysis of 244 stage I–III patients treated with standard therapy. *European Journal of Cancer*, 136, 7-15.
<https://doi.org/https://doi.org/10.1016/j.ejca.2020.05.014>
- Diop-Frimpong, B., Chauhan, V. P., Krane, S., Boucher, Y., & Jain, R. K. (2011). Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci U S A*, 108(7), 2909-2914. <https://doi.org/10.1073/pnas.1018892108>
- Djenidi, F., Adam, J., Goubar, A., Durgeau, A., Meurice, G., de Montpréville, V., Validire, P., Besse, B., & Mami-Chouaib, F. (2015). CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol*, 194(7), 3475-3486. <https://doi.org/10.4049/jimmunol.1402711>
- Dlamini, Z., & Bhoola, K. D. (2005). Upregulation of tissue kallikrein, kinin B(1) receptor, and kinin B(2) receptor in mast and giant cells infiltrating oesophageal squamous cell carcinoma. *Journal of Clinical Pathology*, 58(9), 915-922. <https://doi.org/10.1136/jcp.2004.021444>
- Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K., Jeyaseelan, R., Breitbart, R. E., & Acton, S. (2000). A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res*, 87(5), E1-9.
- Dorhoi, A., & Du Plessis, N. (2017). Monocytic Myeloid-Derived Suppressor Cells in Chronic Infections. *Front Immunol*, 8, 1895.
<https://doi.org/10.3389/fimmu.2017.01895>
- Duhen, T., Duhen, R., Montler, R., Moses, J., Moudgil, T., de Miranda, N. F., Goodall, C. P., Blair, T. C., Fox, B. A., McDermott, J. E., Chang, S.-C., Grunkemeier, G., Leidner, R., Bell, R. B., & Weinberg, A. D. (2018). Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nature Communications*, 9(1), 2724. <https://doi.org/10.1038/s41467-018-05072-0>
- Dumauthioz, N., Labiano, S., & Romero, P. (2018). Tumor Resident Memory T Cells: New Players in Immune Surveillance and Therapy. *Frontiers in Immunology*, 9, 2076-2076. <https://doi.org/10.3389/fimmu.2018.02076>
- Ebstein, F., Textoris-Taube, K., Keller, C., Golnik, R., Vigneron, N., Van den Eynde, B. J., Schuler-Thurner, B., Schadendorf, D., Lorenz, F. K., Uckert, W., Urban, S., Lehmann, A., Albrecht-Koepke, N., Janek, K., Henklein, P., Niewianda, A., Kloetzel, P. M., & Mishto, M. (2016). Proteasomes generate spliced epitopes by two different mechanisms and as efficiently as non-spliced epitopes. *Sci Rep*, 6, 24032. <https://doi.org/10.1038/srep24032>

- Eckert, C., Klein, N., Kornek, M., & Lukacs-Kornek, V. (2015). The Complex Myeloid Network of the Liver with Diverse Functional Capacity at Steady State and in Inflammation [Review]. *Frontiers in Immunology*, 6(179). <https://doi.org/10.3389/fimmu.2015.00179>
- Edmondson, R. D., Vondriska, T. M., Biederman, K. J., Zhang, J., Jones, R. C., Zheng, Y., Allen, D. L., Xiu, J. X., Cardwell, E. M., Pisano, M. R., & Ping, P. (2002). Protein Kinase C ϵ Signaling Complexes Include Metabolism- and Transcription/Translation-related Proteins: Complimentary Separation Techniques With LC/MS/MS*, *S. Molecular & Cellular Proteomics*, 1(6), 421-433. <https://doi.org/https://doi.org/10.1074/mcp.M100036-MCP200>
- Edwards, J., Wilmott, J. S., Madore, J., Gide, T. N., Quek, C., Tasker, A., Ferguson, A., Chen, J., Hewavisenti, R., Hersey, P., Gebhardt, T., Weninger, W., Britton, W. J., Saw, R. P. M., Thompson, J. F., Menzies, A. M., Long, G. V., Scolyer, R. A., & Palendira, U. (2018). CD103(+) Tumor-Resident CD8(+) T Cells Are Associated with Improved Survival in Immunotherapy-Naïve Melanoma Patients and Expand Significantly During Anti-PD-1 Treatment. *Clin Cancer Res*, 24(13), 3036-3045. <https://doi.org/10.1158/1078-0432.Ccr-17-2257>
- Eefsen, R. L., Engelholm, L., Alpizar-Alpizar, W., Van den Eynden, G. G. E., Vermeulen, P. B., Christensen, I. J., Laerum, O. D., Rolff, H. C., Høyer-Hansen, G., Vainer, B., Osterlind, K., & Illemann, M. (2015). Inflammation and uPAR-Expression in Colorectal Liver Metastases in Relation to Growth Pattern and Neo-adjuvant Therapy. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society*, 8(2), 93-100. <https://doi.org/10.1007/s12307-015-0172-z>
- Engineer, D. R., Burney, B. O., Hayes, T. G., & Garcia, J. M. (2013). Exposure to ACEI/ARB and β -Blockers Is Associated with Improved Survival and Decreased Tumor Progression and Hospitalizations in Patients with Advanced Colon Cancer. *Translational oncology*, 6(5), 539-545. Retrieved 2013, from <https://doi.org/10.1593/tlo.13346>
- Engstrand, J., Nilsson, H., Strömberg, C., Jonas, E., & Freedman, J. (2018). Colorectal cancer liver metastases - a population-based study on incidence, management and survival. *BMC cancer*, 18(1), 78-78. <https://doi.org/10.1186/s12885-017-3925-x>
- Engstrand, J., Strömberg, C., Nilsson, H., Freedman, J., & Jonas, E. (2019). Synchronous and metachronous liver metastases in patients with colorectal cancer—towards a clinically relevant definition. *World Journal of Surgical Oncology*, 17(1), 228. <https://doi.org/10.1186/s12957-019-1771-9>
- Erdos, E. G. (1976). Conversion of angiotensin I to angiotensin II. *Am J Med*, 60(6), 749-759.

- Erez, N., Truitt, M., Olson, P., & Hanahan, D. (2010). Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF- κ B-Dependent Manner. *Cancer Cell*, 17(2), 135-147. <https://doi.org/https://doi.org/10.1016/j.ccr.2009.12.041>
- Fabregat, A., Jupe, S., Matthews, L., Sidiropoulos, K., Gillespie, M., Garapati, P., Haw, R., Jassal, B., Korninger, F., May, B., Milacic, M., Roca, C. D., Rothfels, K., Sevilla, C., Shamovsky, V., Shorser, S., Varusai, T., Viteri, G., Weiser, J., Wu, G., Stein, L., Hermjakob, H., & D'Eustachio, P. (2017). The Reactome Pathway Knowledgebase. *Nucleic acids research*, 46(D1), D649-D655. <https://doi.org/10.1093/nar/gkx1132>
- Falco, M., Palma, G., Rea, D., De Biase, D., Scala, S., D'Aiuto, M., Facchini, G., Perdonà, S., Barbieri, A., & Arra, C. (2016). Tumour biomarkers: homeostasis as a novel prognostic indicator. *Open biology*, 6(12), 160254. <https://doi.org/10.1098/rsob.160254>
- Fang, L., Ly, D., Wang, S.-S., Lee, J. B., Kang, H., Xu, H., Yao, J., Tsao, M.-S., Liu, W., & Zhang, L. (2019). Targeting late-stage non-small cell lung cancer with a combination of DNT cellular therapy and PD-1 checkpoint blockade. *Journal of experimental & clinical cancer research : CR*, 38(1), 123-123. <https://doi.org/10.1186/s13046-019-1126-y>
- Farsaci, B., Donahue, R. N., Coplin, M. A., Grenga, I., Lepone, L. M., Molinolo, A. A., & Hodge, J. W. (2014). Immune consequences of decreasing tumor vasculature with antiangiogenic tyrosine kinase inhibitors in combination with therapeutic vaccines. *Cancer Immunol Res*, 2(11), 1090-1102. <https://doi.org/10.1158/2326-6066.Cir-14-0076>
- Fearon, D. T. (2017). Immune-Suppressing Cellular Elements of the Tumor Microenvironment. *Annual Review of Cancer Biology*, 1(1), 241-255. <https://doi.org/10.1146/annurev-cancerbio-050216-034359>
- Fehling, H. J., Swat, W., Laplace, C., Kühn, R., Rajewsky, K., Müller, U., & von Boehmer, H. (1994). MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science*, 265(5176), 1234-1237. <https://doi.org/10.1126/science.8066463>
- Feig, C., Jones, J. O., Kraman, M., Wells, R. J., Deonarine, A., Chan, D. S., Connell, C. M., Roberts, E. W., Zhao, Q., Caballero, O. L., Teichmann, S. A., Janowitz, T., Jodrell, D. I., Tuveson, D. A., & Fearon, D. T. (2013). Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci U S A*, 110(50), 20212-20217. <https://doi.org/10.1073/pnas.1320318110>
- Feng, Z., Puri, S., Moudgil, T., Wood, W., Hoyt, C. C., Wang, C., Urba, W. J., Curti, B. D., Bifulco, C. B., & Fox, B. A. (2015). Multispectral imaging of formalin-fixed tissue predicts ability to generate tumor-infiltrating lymphocytes from

melanoma. *J Immunother Cancer*, 3(1), 47. <https://doi.org/10.1186/s40425-015-0091-z>

- Fernández Moro, C., Bozóky, B., & Gerling, M. (2018). Growth patterns of colorectal cancer liver metastases and their impact on prognosis: a systematic review. *BMJ open gastroenterology*, 5(1), e000217-e000217. <https://doi.org/10.1136/bmjgast-2018-000217>
- Ferrero, E., Belloni, D., Contini, P., Foglieni, C., Ferrero, M. E., Fabbri, M., Poggi, A., & Zocchi, M. R. (2003). Transendothelial migration leads to protection from starvation-induced apoptosis in CD34+CD14+ circulating precursors: evidence for PECAM-1 involvement through Akt/PKB activation. *Blood*, 101(1), 186-193. <https://doi.org/10.1182/blood-2002-03-0768>
- Finlay, I. G., Meek, D., Brunton, F., & McArdle, C. S. (1988). Growth rate of hepatic metastases in colorectal carcinoma. *Br J Surg*, 75(7), 641-644. <https://doi.org/10.1002/bjs.1800750707>
- Fischer, K., Voelkl, S., Heymann, J., Przybylski, G. K., Mondal, K., Laumer, M., Kunz-Schughart, L., Schmidt, C. A., Andreesen, R., & Mackensen, A. (2005). Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8-double-negative regulatory T cells. *Blood*, 105(7), 2828-2835. <https://doi.org/10.1182/blood-2004-07-2583>
- Fisher, A. J., Medaglia, J. D., & Jeronimus, B. F. (2018). Lack of group-to-individual generalizability is a threat to human subjects research. *Proc Natl Acad Sci U S A*, 115(27), E6106-e6115. <https://doi.org/10.1073/pnas.1711978115>
- Fleetwood, A. J., Dinh, H., Cook, A. D., Hertzog, P. J., & Hamilton, J. A. (2009). GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J Leukoc Biol*, 86(2), 411-421. <https://doi.org/10.1189/jlb.1108702>
- Fleming, T. J., Fleming, M. L., & Malek, T. R. (1993). Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol*, 151(5), 2399-2408.
- Fleming, V., Hu, X., Weber, R., Nagibin, V., Groth, C., Altevogt, P., Utikal, J., & Umansky, V. (2018). Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. *Front Immunol*, 9, 398. <https://doi.org/10.3389/fimmu.2018.00398>
- Fong, Y., Fortner, J., Sun, R. L., Brennan, M. F., & Blumgart, L. H. (1999). Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. *Ann Surg*, 230(3), 309-318; discussion 318-321.

- Fu, C., & Jiang, A. (2018). Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment. *Frontiers in Immunology*, 9, 3059-3059. <https://doi.org/10.3389/fimmu.2018.03059>
- Gabrilovich, D. I., Ostrand-Rosenberg, S., & Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*, 12(4), 253-268. <https://doi.org/10.1038/nri3175>
- Gabrilovich, D. I., Velders, M. P., Sotomayor, E. M., & Kast, W. M. (2001). Mechanism of Immune Dysfunction in Cancer Mediated by Immature Gr-1⁺ Myeloid Cells. *The Journal of Immunology*, 166(9), 5398-5406. <https://doi.org/10.4049/jimmunol.166.9.5398>
- Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M., Camus, M., Berger, A., Wind, P., Zinzindohoue, F., Bruneval, P., Cugnenc, P. H., Trajanoski, Z., Fridman, W. H., & Pages, F. (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*, 313(5795), 1960-1964. <https://doi.org/10.1126/science.1129139>
- Ganesan, A. P., Clarke, J., Wood, O., Garrido-Martin, E. M., Chee, S. J., Mellows, T., Samaniego-Castruita, D., Singh, D., Seumois, G., Alzetani, A., Woo, E., Friedmann, P. S., King, E. V., Thomas, G. J., Sanchez-Elsner, T., Vijayanand, P., & Ottensmeier, C. H. (2017). Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. *Nat Immunol*, 18(8), 940-950. <https://doi.org/10.1038/ni.3775>
- Gardes, J., Bouhnik, J., Clauser, E., Corvol, P., & Menard, J. (1982). Role of angiotensinogen in blood pressure homeostasis. *Hypertension*, 4(2), 185-189. <https://doi.org/10.1161/01.hyp.4.2.185>
- Garon, E. B., Rizvi, N. A., Hui, R., Leighl, N., Balmanoukian, A. S., Eder, J. P., Patnaik, A., Aggarwal, C., Gubens, M., Horn, L., Carcereny, E., Ahn, M.-J., Felip, E., Lee, J.-S., Hellmann, M. D., Hamid, O., Goldman, J. W., Soria, J.-C., Dolled-Filhart, M., Rutledge, R. Z., Zhang, J., Lunceford, J. K., Rangwala, R., Lubiniecki, G. M., Roach, C., Emancipator, K., & Gandhi, L. (2015). Pembrolizumab for the Treatment of Non-Small-Cell Lung Cancer. *New England Journal of Medicine*, 372(21), 2018-2028. <https://doi.org/10.1056/NEJMoa1501824>
- Germano, G., Lamba, S., Rospo, G., Barault, L., Magrì, A., Maione, F., Russo, M., Crisafulli, G., Bartolini, A., Lerda, G., Siravegna, G., Mussolin, B., Frapolli, R., Montone, M., Morano, F., de Braud, F., Amirouchene-Angelozzi, N., Marsoni, S., D'Incalci, M., Orlandi, A., Giraudo, E., Sartore-Bianchi, A., Siena, S., Pietrantonio, F., Di Nicolantonio, F., & Bardelli, A. (2017). Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. *Nature*, 552(7683), 116-120. <https://doi.org/10.1038/nature24673>

- Gnjatic, S., Bronte, V., Brunet, L. R., Butler, M. O., Disis, M. L., Galon, J., Hakansson, L. G., Hanks, B. A., Karanikas, V., Khleif, S. N., Kirkwood, J. M., Miller, L. D., Schendel, D. J., Tanneau, I., Wigginton, J. M., & Butterfield, L. H. (2017). Identifying baseline immune-related biomarkers to predict clinical outcome of immunotherapy. *J Immunother Cancer*, 5(1), 44. <https://doi.org/10.1186/s40425-017-0243-4>
- Godfrey, D. I., Le Nours, J., Andrews, D. M., Uldrich, A. P., & Rossjohn, J. (2018). Unconventional T Cell Targets for Cancer Immunotherapy. *Immunity*, 48(3), 453-473. <https://doi.org/10.1016/j.immuni.2018.03.009>
- Godfrey, D. I., Uldrich, A. P., McCluskey, J., Rossjohn, J., & Moody, D. B. (2015). The burgeoning family of unconventional T cells. *Nat Immunol*, 16(11), 1114-1123. <https://doi.org/10.1038/ni.3298>
- Goldberg, M. S. (2019). Improving cancer immunotherapy through nanotechnology. *Nat Rev Cancer*, 19(10), 587-602. <https://doi.org/10.1038/s41568-019-0186-9>
- Gonzalez, H., Hagerling, C., & Werb, Z. (2018). Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes & development*, 32(19-20), 1267-1284. <https://doi.org/10.1101/gad.314617.118>
- Greenplate, A. R., McClanahan, D. D., Oberholtzer, B. K., Doxie, D. B., Roe, C. E., Diggins, K. E., Leelatian, N., Rasmussen, M. L., Kelley, M. C., Gama, V., Siska, P. J., Rathmell, J. C., Ferrell, P. B., Johnson, D. B., & Irish, J. M. (2019). Computational Immune Monitoring Reveals Abnormal Double-Negative T Cells Present across Human Tumor Types. *Cancer Immunol Res*, 7(1), 86-99. <https://doi.org/10.1158/2326-6066.Cir-17-0692>
- Groth, C., Hu, X., Weber, R., Fleming, V., Altevogt, P., Utikal, J., & Umansky, V. (2019). Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression. *Br J Cancer*, 120(1), 16-25. <https://doi.org/10.1038/s41416-018-0333-1>
- Guillaume, B., Chapiro, J., Stroobant, V., Colau, D., Van Holle, B., Parvizi, G., Bousquet-Dubouch, M.-P., Théate, I., Parmentier, N., & Van den Eynde, B. J. (2010). Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. *Proceedings of the National Academy of Sciences*, 107(43), 18599-18604. <https://doi.org/10.1073/pnas.1009778107>
- Guimarães, G., Gomes, M. T. R., Campos, P. C., Marinho, F. V., de Assis, N. R. G., Silveira, T. N., & Oliveira, S. C. (2018). Immunoproteasome Subunits Are Required for CD8(+) T Cell Function and Host Resistance to *Brucella abortus* Infection in Mice. *Infection and Immunity*, 86(3), e00615-00617. <https://doi.org/10.1128/IAI.00615-17>

- Gumbiner, B. M., & Kim, N. G. (2014). The Hippo-YAP signaling pathway and contact inhibition of growth. *J Cell Sci*, 127(Pt 4), 709-717. <https://doi.org/10.1242/jcs.140103>
- Halama, N., Michel, S., Kloor, M., Zoernig, I., Benner, A., Spille, A., Pommerencke, T., von Knebel, D. M., Folprecht, G., Lubber, B., Feyen, N., Martens, U. M., Beckhove, P., Gnjjatic, S., Schirmacher, P., Herpel, E., Weitz, J., Grabe, N., & Jaeger, D. (2011). Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer Res*, 71(17), 5670-5677. <https://doi.org/10.1158/0008-5472.Can-11-0268>
- Hall, J. M. (1997). Bradykinin receptors. *General Pharmacology: The Vascular System*, 28(1), 1-6. [https://doi.org/https://doi.org/10.1016/S0306-3623\(96\)00174-7](https://doi.org/https://doi.org/10.1016/S0306-3623(96)00174-7)
- Halse, H., Colebatch, A. J., Petrone, P., Henderson, M. A., Mills, J. K., Snow, H., Westwood, J. A., Sandhu, S., Raleigh, J. M., Behren, A., Cebon, J., Darcy, P. K., Kershaw, M. H., McArthur, G. A., Gyorki, D. E., & Neeson, P. J. (2018). Multiplex immunohistochemistry accurately defines the immune context of metastatic melanoma. *Scientific Reports*, 8(1), 11158. <https://doi.org/10.1038/s41598-018-28944-3>
- Hanahan, D., & Coussens, L. M. (2012). Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*, 21(3), 309-322. <https://doi.org/10.1016/j.ccr.2012.02.022>
- Haug, T., Aigner, M., Peuser, M. M., Strobl, C. D., Hildner, K., Mougiakakos, D., Bruns, H., Mackensen, A., & Völkl, S. (2019). Human Double-Negative Regulatory T-Cells Induce a Metabolic and Functional Switch in Effector T-Cells by Suppressing mTOR Activity. *Frontiers in Immunology*, 10, 883-883. <https://doi.org/10.3389/fimmu.2019.00883>
- Haznedaroglu, I. C., & Beyazit, Y. (2013). Local bone marrow renin-angiotensin system in primitive, definitive and neoplastic haematopoiesis. *Clin Sci (Lond)*, 124(5), 307-323. <https://doi.org/10.1042/cs20120300>
- Hegde, P. S., & Chen, D. S. (2020). Top 10 Challenges in Cancer Immunotherapy. *Immunity*, 52(1), 17-35. <https://doi.org/10.1016/j.immuni.2019.12.011>
- Hegde, P. S., Karanikas, V., & Evers, S. (2016). The Where, the When, and the How of Immune Monitoring for Cancer Immunotherapies in the Era of Checkpoint Inhibition. *Clin Cancer Res*, 22(8), 1865-1874. <https://doi.org/10.1158/1078-0432.Ccr-15-1507>
- Hendry, S., Salgado, R., Gevaert, T., Russell, P. A., John, T., Thapa, B., Christie, M., van de Vijver, K., Estrada, M. V., Gonzalez-Ericsson, P. I., Sanders, M., Solomon, B., Solinas, C., Van den Eynden, G. G. G. M., Allory, Y., Preusser, M., Hainfellner, J., Pruneri, G., Vingiani, A., Demaria, S., Symmans, F., Nuciforo, P., Comerma, L.,

Thompson, E. A., Lakhani, S., Kim, S.-R., Schnitt, S., Colpaert, C., Sotiriou, C., Scherer, S. J., Ignatiadis, M., Badve, S., Pierce, R. H., Viale, G., Sirtaine, N., Penault-Llorca, F., Sugie, T., Fineberg, S., Paik, S., Srinivasan, A., Richardson, A., Wang, Y., Chmielik, E., Brock, J., Johnson, D. B., Balko, J., Wienert, S., Bossuyt, V., Michiels, S., Ternes, N., Burchardi, N., Luen, S. J., Savas, P., Klauschen, F., Watson, P. H., Nelson, B. H., Criscitiello, C., O'Toole, S., Larsimont, D., de Wind, R., Curigliano, G., André, F., Lacroix-Triki, M., van de Vijver, M., Rojo, F., Floris, G., Bedri, S., Sparano, J., Rimm, D., Nielsen, T., Kos, Z., Hewitt, S., Singh, B., Farshid, G., Loibl, S., Allison, K. H., Tung, N., Adams, S., Willard-Gallo, K., Horlings, H. M., Gandhi, L., Moreira, A., Hirsch, F., Dieci, M. V., Urbanowicz, M., Brcic, I., Korski, K., Gaire, F., Koeppen, H., Lo, A., Giltane, J., Rebelatto, M. C., Steele, K. E., Zha, J., Emancipator, K., Juco, J. W., Denkert, C., Reis-Filho, J., Loi, S., & Fox, S. B. (2017). Assessing Tumor-infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method From the International Immunooncology Biomarkers Working Group: Part 1: Assessing the Host Immune Response, TILs in Invasive Breast Carcinoma and Ductal Carcinoma In Situ, Metastatic Tumor Deposits and Areas for Further Research. *Advances in anatomic pathology*, 24(5), 235-251. <https://doi.org/10.1097/PAP.0000000000000162>

Herring, L. E., Grant, K. G., Blackburn, K., Haugh, J. M., & Goshe, M. B. (2015). Development of a tandem affinity phosphoproteomic method with motif selectivity and its application in analysis of signal transduction networks. *J Chromatogr B Analyt Technol Biomed Life Sci*, 988, 166-174. <https://doi.org/10.1016/j.jchromb.2015.02.017>

Hii, S. I., Nicol, D. L., Gotley, D. C., Thompson, L. C., Green, M. K., & Jonsson, J. R. (1998). Captopril inhibits tumour growth in a xenograft model of human renal cell carcinoma. *Br J Cancer*, 77(6), 880-883. <https://doi.org/10.1038/bjc.1998.145>

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., & Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science*, 322(5904), 1097-1100. <https://doi.org/10.1126/science.1164206>

Hillhouse, E. E., & Lesage, S. (2013). A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. *J Autoimmun*, 40, 58-65. <https://doi.org/10.1016/j.jaut.2012.07.010>

Hof, J., Kok, K., Sijmons, R. H., & de Jong, K. P. (2019). Systematic Review of the Prognostic Role of the Immune System After Surgery of Colorectal Liver Metastases. *Frontiers in Oncology*, 9, 148-148. <https://doi.org/10.3389/fonc.2019.00148>

- Hofmann, M., & Pircher, H. (2011). E-cadherin promotes accumulation of a unique memory CD8 T-cell population in murine salivary glands. *Proc Natl Acad Sci U S A*, 108(40), 16741-16746. <https://doi.org/10.1073/pnas.1107200108>
- Höppener, D. J., Nierop, P. M. H., Herpel, E., Rahbari, N. N., Doukas, M., Vermeulen, P. B., Grünhagen, D. J., & Verhoef, C. (2019). Histopathological growth patterns of colorectal liver metastasis exhibit little heterogeneity and can be determined with a high diagnostic accuracy. *Clin Exp Metastasis*, 36(4), 311-319. <https://doi.org/10.1007/s10585-019-09975-0>
- Huang, W., Hennrick, K., & Drew, S. (2013). A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays. *Human Pathology*, 44(1), 29-38. <https://doi.org/https://doi.org/10.1016/j.humpath.2012.05.009>
- Huang, Y.-K., Wang, M., Sun, Y., Di Costanzo, N., Mitchell, C., Achuthan, A., Hamilton, J. A., Busuttil, R. A., & Boussioutas, A. (2019). Macrophage spatial heterogeneity in gastric cancer defined by multiplex immunohistochemistry. *Nature Communications*, 10(1), 3928. <https://doi.org/10.1038/s41467-019-11788-4>
- Husain, K., Hernandez, W., Ansari, R. A., & Ferder, L. (2015). Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J Biol Chem*, 6(3), 209-217. <https://doi.org/10.4331/wjbc.v6.i3.209>
- Idos, G. E., Kwok, J., Bonthala, N., Kysh, L., Gruber, S. B., & Qu, C. (2020). The Prognostic Implications of Tumor Infiltrating Lymphocytes in Colorectal Cancer: A Systematic Review and Meta-Analysis. *Scientific Reports*, 10(1), 3360. <https://doi.org/10.1038/s41598-020-60255-4>
- Ignjatovic, T., Tan, F., Brovkovich, V., Skidgel, R. A., & Erdos, E. G. (2002). Activation of bradykinin B1 receptor by ACE inhibitors. *Int Immunopharmacol*, 2(13-14), 1787-1793.
- Ikeda, Y., Hayashi, I., Kamoshita, E., Yamazaki, A., Endo, H., Ishihara, K., Yamashina, S., Tsutsumi, Y., Matsubara, H., & Majima, M. (2004). Host stromal bradykinin B2 receptor signaling facilitates tumor-associated angiogenesis and tumor growth. *Cancer Res*, 64(15), 5178-5185. <https://doi.org/10.1158/0008-5472.Can-03-3589>
- Ilkovitch, D., & Lopez, D. M. (2009). The liver is a site for tumor-induced myeloid-derived suppressor cell accumulation and immunosuppression. *Cancer Res*, 69(13), 5514-5521. <https://doi.org/10.1158/0008-5472.Can-08-4625>
- Incio, J., Liu, H., Suboj, P., Chin, S. M., Chen, I. X., Pinter, M., Ng, M. R., Nia, H. T., Grahovac, J., Kao, S., Babykutty, S., Huang, Y., Jung, K., Rahbari, N. N., Han, X., Chauhan, V. P., Martin, J. D., Kahn, J., Huang, P., Desphande, V., Michaelson, J., Michelakos, T. P., Ferrone, C. R., Soares, R., Boucher, Y., Fukumura, D., &

- Jain, R. K. (2016). Obesity-Induced Inflammation and Desmoplasia Promote Pancreatic Cancer Progression and Resistance to Chemotherapy. *Cancer Discov*, 6(8), 852-869. <https://doi.org/10.1158/2159-8290.Cd-15-1177>
- Ino, K., Shibata, K., Kajiyama, H., Yamamoto, E., Nagasaka, T., Nawa, A., Nomura, S., & Kikkawa, F. (2006). Angiotensin II type 1 receptor expression in ovarian cancer and its correlation with tumour angiogenesis and patient survival. *Br J Cancer*, 94(4), 552-560. <https://doi.org/10.1038/sj.bjc.6602961>
- Ishihara, K., Kamata, M., Hayashi, I., Yamashina, S., & Majima, M. (2002). Roles of bradykinin in vascular permeability and angiogenesis in solid tumor. *Int Immunopharmacol*, 2(4), 499-509.
- Izzedine, H., Derosa, L., Le Teuff, G., Albiges, L., & Escudier, B. (2015). Hypertension and angiotensin system inhibitors: impact on outcome in sunitinib-treated patients for metastatic renal cell carcinoma. *Ann Oncol*, 26(6), 1128-1133. <https://doi.org/10.1093/annonc/mdv147>
- Jackson, S. H., Devadas, S., Kwon, J., Pinto, L. A., & Williams, M. S. (2004). T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol*, 5(8), 818-827. <https://doi.org/10.1038/ni1096>
- Jain, R. K., Duda, D. G., Willett, C. G., Sahani, D. V., Zhu, A. X., Loeffler, J. S., Batchelor, T. T., & Sorensen, A. G. (2009). Biomarkers of response and resistance to antiangiogenic therapy. *Nature reviews. Clinical oncology*, 6(6), 327-338. <https://doi.org/10.1038/nrclinonc.2009.63>
- Jardim, D. L., Goodman, A., de Melo Gagliato, D., & Kurzrock, R. (2020). The Challenges of Tumor Mutational Burden as an Immunotherapy Biomarker. *Cancer Cell*. <https://doi.org/10.1016/j.ccell.2020.10.001>
- Jenkins, R. W., Barbie, D. A., & Flaherty, K. T. (2018). Mechanisms of resistance to immune checkpoint inhibitors [Review]. *Br J Cancer*, 118, 9. <https://doi.org/10.1038/bjc.2017.434>
- Jhunjhunwala, S., Hammer, C., & Delamarre, L. (2021). Antigen presentation in cancer: insights into tumour immunogenicity and immune evasion. *Nature Reviews Cancer*. <https://doi.org/10.1038/s41568-021-00339-z>
- Jiang, A., Bloom, O., Ono, S., Cui, W., Unternaehrer, J., Jiang, S., Whitney, J. A., Connolly, J., Banchereau, J., & Mellman, I. (2007). Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity*, 27(4), 610-624. <https://doi.org/10.1016/j.immuni.2007.08.015>
- Kalaora, S., Lee, J. S., Barnea, E., Levy, R., Greenberg, P., Alon, M., Yagel, G., Bar Eli, G., Oren, R., Peri, A., Patkar, S., Bitton, L., Rosenberg, S. A., Lotem, M., Levin, Y.,

- Admon, A., Ruppin, E., & Samuels, Y. (2020). Immunoproteasome expression is associated with better prognosis and response to checkpoint therapies in melanoma. *Nature Communications*, 11(1), 896. <https://doi.org/10.1038/s41467-020-14639-9>
- Kamitani, T., Kito, K., Nguyen, H. P., & Yeh, E. T. (1997). Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. *J Biol Chem*, 272(45), 28557-28562. <https://doi.org/10.1074/jbc.272.45.28557>
- Kanas, G. P., Taylor, A., Primrose, J. N., Langeberg, W. J., Kelsh, M. A., Mowat, F. S., Alexander, D. D., Choti, M. A., & Poston, G. (2012). Survival after liver resection in metastatic colorectal cancer: review and meta-analysis of prognostic factors. *Clinical epidemiology*, 4, 283-301. <https://doi.org/10.2147/CLEP.S34285>
- Katz, S. C., Bamboat, Z. M., Maker, A. V., Shia, J., Pillarisetty, V. G., Yopp, A. C., Hedvat, C. V., Gonen, M., Jarnagin, W. R., Fong, Y., D'Angelica, M. I., & DeMatteo, R. P. (2013). Regulatory T Cell Infiltration Predicts Outcome Following Resection of Colorectal Cancer Liver Metastases [journal article]. *Annals of Surgical Oncology*, 20(3), 946-955. <https://doi.org/10.1245/s10434-012-2668-9>
- Keizman, D., Gottfried, M., Ish-Shalom, M., Maimon, N., Peer, A., Neumann, A., Hammers, H., Eisenberger, M. A., Sinibaldi, V., Pili, R., Hayat, H., Kovel, S., Sella, A., Boursi, B., Weitzen, R., Mermershtain, W., Rouvinov, K., Berger, R., & Carducci, M. A. (2014). Active Smoking May Negatively Affect Response Rate, Progression-Free Survival, and Overall Survival of Patients With Metastatic Renal Cell Carcinoma Treated With Sunitinib. *The Oncologist*, 19(1), 51-60. <https://doi.org/10.1634/theoncologist.2012-0335>
- Keizman, D., Huang, P., Eisenberger, M. A., Pili, R., Kim, J. J., Antonarakis, E. S., Hammers, H., & Carducci, M. A. (2011). Angiotensin system inhibitors and outcome of sunitinib treatment in patients with metastatic renal cell carcinoma: a retrospective examination. *Eur J Cancer*, 47(13), 1955-1961. <https://doi.org/10.1016/j.ejca.2011.04.019>
- Kemeny, N. E., Chou, J. F., Capanu, M., Gewirtz, A. N., Cercek, A., Kingham, T. P., Jarnagin, W. R., Fong, Y. C., DeMatteo, R. P., Allen, P. J., Shia, J., Ang, C., Vakiani, E., & D'Angelica, M. I. (2014). KRAS mutation influences recurrence patterns in patients undergoing hepatic resection of colorectal metastases. *Cancer*, 120(24), 3965-3971. <https://doi.org/10.1002/cncr.28954>
- Keum, N., & Giovannucci, E. (2019). Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nat Rev Gastroenterol Hepatol*, 16(12), 713-732. <https://doi.org/10.1038/s41575-019-0189-8>

- Kim, G., Kim, J., Lim, Y. L., Kim, M. Y., & Baik, S. K. (2016). Renin-angiotensin system inhibitors and fibrosis in chronic liver disease: a systematic review. *Hepatol Int*, 10(5), 819-828. <https://doi.org/10.1007/s12072-016-9705-x>
- Kim, N. G., Koh, E., Chen, X., & Gumbiner, B. M. (2011). E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. *Proc Natl Acad Sci U S A*, 108(29), 11930-11935. <https://doi.org/10.1073/pnas.1103345108>
- Kim, O. H., Kang, G. H., Noh, H., Cha, J. Y., Lee, H. J., Yoon, J. H., Mamura, M., Nam, J. S., Lee, D. H., Kim, Y. A., Park, Y. J., Kim, H., & Oh, B. C. (2013). Proangiogenic TIE2(+)/CD31 (+) macrophages are the predominant population of tumor-associated macrophages infiltrating metastatic lymph nodes. *Mol Cells*, 36(5), 432-438. <https://doi.org/10.1007/s10059-013-0194-7>
- Kim, S. A., Inamura, K., Yamauchi, M., Nishihara, R., Mima, K., Sukawa, Y., Li, T., Yasunari, M., Morikawa, T., Fitzgerald, K. C., Fuchs, C. S., Wu, K., Chan, A. T., Zhang, X., Ogino, S., & Qian, Z. R. (2016). Loss of CDH1 (E-cadherin) expression is associated with infiltrative tumour growth and lymph node metastasis. *Br J Cancer*, 114(2), 199-206. <https://doi.org/10.1038/bjc.2015.347>
- Kim, S. T., Park, K. H., Oh, S. C., Seo, J. H., Kim, J. S., Shin, S. W., & Kim, Y. H. (2012). How Does Inhibition of the Renin-Angiotensin System Affect the Prognosis of Advanced Gastric Cancer Patients Receiving Platinum-Based Chemotherapy? *Oncology*, 83(6), 354-360. <https://doi.org/10.1159/000337979>
- Kincaid, E. Z., Che, J. W., York, I., Escobar, H., Reyes-Vargas, E., Delgado, J. C., Welsh, R. M., Karow, M. L., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., & Rock, K. L. (2012). Mice completely lacking immunoproteasomes show major changes in antigen presentation. *Nat Immunol*, 13(2), 129-135. <https://doi.org/10.1038/ni.2203>
- Kiuchi, T., Tomaru, U., Ishizu, A., Imagawa, M., Iwasaki, S., Suzuki, A., Otsuka, N., Ohhara, Y., Kinoshita, I., Matsuno, Y., Dosaka-Akita, H., & Kasahara, M. (2020). Expression of the immunoproteasome subunit $\beta 5i$ in non-small cell lung carcinomas. *Journal of Clinical Pathology*, jclinpath-2020-206618. <https://doi.org/10.1136/jclinpath-2020-206618>
- Koerner, J., Brunner, T., & Groettrup, M. (2017). Inhibition and deficiency of the immunoproteasome subunit LMP7 suppress the development and progression of colorectal carcinoma in mice. *Oncotarget*, 8(31), 50873-50888. <https://doi.org/10.18632/oncotarget.15141>
- Koh, J., Kim, S., Kim, M. Y., Go, H., Jeon, Y. K., & Chung, D. H. (2017). Prognostic implications of intratumoral CD103+ tumor-infiltrating lymphocytes in pulmonary squamous cell carcinoma. *Oncotarget*, 8(8), 13762-13769. <https://doi.org/10.18632/oncotarget.14632>

- Kohlstedt, K., Trouvain, C., Namgaladze, D., & Fleming, I. (2011). Adipocyte-derived lipids increase angiotensin-converting enzyme (ACE) expression and modulate macrophage phenotype. *Basic Res Cardiol*, 106(2), 205-215. <https://doi.org/10.1007/s00395-010-0137-9>
- Komdeur, F. L., Prins, T. M., van de Wall, S., Plat, A., Wisman, G. B. A., Hollema, H., Daemen, T., Church, D. N., de Bruyn, M., & Nijman, H. W. (2017). CD103+ tumor-infiltrating lymphocytes are tumor-reactive intraepithelial CD8+ T cells associated with prognostic benefit and therapy response in cervical cancer. *Oncoimmunology*, 6(9), e1338230. <https://doi.org/10.1080/2162402x.2017.1338230>
- Komura, D., & Ishikawa, S. (2018). Machine Learning Methods for Histopathological Image Analysis. *Comput Struct Biotechnol J*, 16, 34-42. <https://doi.org/10.1016/j.csbj.2018.01.001>
- Kong, J. C., Guerra, G. R., Pham, T., Mitchell, C., Lynch, A. C., Warriar, S. K., Ramsay, R. G., & Heriot, A. G. (2019). Prognostic Impact of Tumor-Infiltrating Lymphocytes in Primary and Metastatic Colorectal Cancer: A Systematic Review and Meta-analysis. *Dis Colon Rectum*, 62(4), 498-508. <https://doi.org/10.1097/dcr.0000000000001332>
- Kong, J. C. H., Guerra, G. R., Millen, R. M., Roth, S., Xu, H., Neeson, P. J., Darcy, P. K., Kershaw, M. H., Sampurno, S., Malaterre, J., Liu, D. S. H., Pham, T. D., Narasimhan, V., Wang, M., Huang, Y.-K., Visvanathan, K., McCormick, J., Lynch, A. C., Warriar, S., Michael, M., Desai, J., Murray, W., Mitchell, C., Ngan, S., Phillips, W. A., Heriot, A. G., & Ramsay, R. G. (2018). Tumor-Infiltrating Lymphocyte Function Predicts Response to Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer. *JCO Precision Oncology*(2), 1-15. <https://doi.org/10.1200/po.18.00075>
- Kraman, M., Bambrough, P. J., Arnold, J. N., Roberts, E. W., Magiera, L., Jones, J. O., Gopinathan, A., Tuveson, D. A., & Fearon, D. T. (2010). Suppression of Antitumor Immunity by Stromal Cells Expressing Fibroblast Activation Protein- α . *Science*, 330(6005), 827-830. <https://doi.org/10.1126/science.1195300>
- Krüger, E., & Kloetzel, P. M. (2012). Immunoproteasomes at the interface of innate and adaptive immune responses: two faces of one enzyme. *Curr Opin Immunol*, 24(1), 77-83. <https://doi.org/10.1016/j.coi.2012.01.005>
- Kuruppu, D., Christophi, C., Bertram, J. F., & Brien, P. E. O. (1996). Characterization of an animal model of hepatic metastasis. *Journal of gastroenterology and hepatology*, 11(1), 26-32. <https://doi.org/10.1111/j.1440-1746.1996.tb00006.x>
- Kusmartsev, S., & Gabrilovich, D. I. (2006). Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid

- cells in cancer. *Cancer and Metastasis Reviews*, 25(3), 323-331.
<https://doi.org/10.1007/s10555-006-9002-6>
- Lanitis, E., Dangaj, D., Irving, M., & Coukos, G. (2017). Mechanisms regulating T-cell infiltration and activity in solid tumors. *Annals of Oncology*, 28, xii18-xii32.
<https://doi.org/https://doi.org/10.1093/annonc/mdx238>
- Laoui, D., Keirsse, J., Morias, Y., Van Overmeire, E., Geeraerts, X., Elkrim, Y., Kiss, M., Bolli, E., Lahmar, Q., Sichien, D., Serneels, J., Scott, C. L., Boon, L., De Baetselier, P., Mazzone, M., Guillems, M., & Van Ginderachter, J. A. (2016). The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nature Communications*, 7(1), 13720. <https://doi.org/10.1038/ncomms13720>
- Larsen, F., Solheim, J., Kristensen, T., Kolstø, A.-B., & Prydz, H. (1993). A tight cluster of five unrelated human genes on chromosome 16q22.1. *Human Molecular Genetics*, 2(10), 1589-1595. <https://doi.org/10.1093/hmg/2.10.1589>
- Lautner, R. Q., Villela, D. C., Fraga-Silva, R. A., Silva, N. C., Verano-Braga, T., Costa-Fraga, F., Jankowski, J., Jankowski, V., De Sousa, F. B., Alzamora, A. C., Soares, E. R., Barbosa, C. M., Kjeldsen, F., Oliveira, A. C., Braga, J. F., Savergnini, S. Q., Etelvino, G. M., Bastos Peluso, A. A., Passos-Silva, D. G., Ferreira, A. J., Alves, F., Martins, A. s., Raizada, M. K., Paula, R. D., Motta-Santos, D., Klempin, F., Pimenta, A. M. C., Alenina, N., Sinisterra, R. D. M., Bader, M., Campagnole-Santos, M. J., & Santos, R. A. S. (2013). Discovery and Characterization of Alamandine, a Novel Component of the Renin-Angiotensin System. *Circ Res*. <https://doi.org/10.1161/circresaha.113.301077>
- Layton, T. B., Williams, L., McCann, F., Zhang, M., Fritzsche, M., Colin-York, H., Cabrita, M., Ng, M. T. H., Feldmann, M., Sansom, S. N., Furniss, D., Xie, W., & Nanchahal, J. (2020). Cellular census of human fibrosis defines functionally distinct stromal cell types and states. *Nature Communications*, 11(1), 2768.
<https://doi.org/10.1038/s41467-020-16264-y>
- Lazarus, J., Maj, T., Smith, J. J., Perusina Lanfranca, M., Rao, A., D'Angelica, M. I., Delrosario, L., Girgis, A., Schukow, C., Shia, J., Kryczek, I., Shi, J., Wasserman, I., Crawford, H., Nathan, H., Pasca Di Magliano, M., Zou, W., & Frankel, T. L. (2018). Spatial and phenotypic immune profiling of metastatic colon cancer. *JCI Insight*, 3(22). <https://doi.org/10.1172/jci.insight.121932>
- Le Floc'h, A., Jalil, A., Vergnon, I., Le Maux Chansac, B., Lazar, V., Bismuth, G., Chouaib, S., & Mami-Chouaib, F. (2007). Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. *The Journal of experimental medicine*, 204(3), 559-570. <https://doi.org/10.1084/jem.20061524>

- Lee, M. Y., Jeon, J. W., Sievers, C., & Allen, C. T. (2020). Antigen processing and presentation in cancer immunotherapy. *J Immunother Cancer*, 8(2), e001111. <https://doi.org/10.1136/jitc-2020-001111>
- Leeb-Lundberg, L. M., Marceau, F., Muller-Esterl, W., Pettibone, D. J., & Zuraw, B. L. (2005). International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev*, 57(1), 27-77. <https://doi.org/10.1124/pr.57.1.2>
- Leithäuser, F., Meinhardt-Krajina, T., Fink, K., Wotschke, B., Möller, P., & Reimann, J. (2006). Foxp3-expressing CD103+ regulatory T cells accumulate in dendritic cell aggregates of the colonic mucosa in murine transfer colitis. *The American journal of pathology*, 168(6), 1898-1909. <https://doi.org/10.2353/ajpath.2006.050228>
- Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., Fong, S. F. T., Csiszar, K., Giaccia, A., Weninger, W., Yamauchi, M., Gasser, D. L., & Weaver, V. M. (2009). Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell*, 139(5), 891-906. <https://doi.org/https://doi.org/10.1016/j.cell.2009.10.027>
- Levin, V. A., Chan, J., Datta, M., Yee, J. L., & Jain, R. K. (2017). Effect of angiotensin system inhibitors on survival in newly diagnosed glioma patients and recurrent glioblastoma patients receiving chemotherapy and/or bevacizumab [journal article]. *Journal of Neuro-Oncology*, 134(2), 325-330. <https://doi.org/10.1007/s11060-017-2528-3>
- Levine, J. H., Simonds, E. F., Bendall, S. C., Davis, K. L., Amir el, A. D., Tadmor, M. D., Litvin, O., Fienberg, H. G., Jager, A., Zunder, E. R., Finck, R., Gedman, A. L., Radtke, I., Downing, J. R., Pe'er, D., & Nolan, G. P. (2015). Data-Driven Phenotypic Dissection of AML Reveals Progenitor-like Cells that Correlate with Prognosis. *Cell*, 162(1), 184-197. <https://doi.org/10.1016/j.cell.2015.05.047>
- Li, H., & Auwerx, J. (2020). Mouse Systems Genetics as a Prelude to Precision Medicine. *Trends Genet*, 36(4), 259-272. <https://doi.org/10.1016/j.tig.2020.01.004>
- Li, H., Han, Y., Guo, Q., Zhang, M., & Cao, X. (2009). Cancer-Expanded Myeloid-Derived Suppressor Cells Induce Anergy of NK Cells through Membrane-Bound TGF- β 1. *The Journal of Immunology*, 182(1), 240-249. <https://doi.org/10.4049/jimmunol.182.1.240>
- Lin, C., Datta, V., Okwan-Duodu, D., Chen, X., Fuchs, S., Alsabeh, R., Billet, S., Bernstein, K. E., & Shen, X. Z. (2011). Angiotensin-converting enzyme is required for normal myelopoiesis. *Faseb j*, 25(4), 1145-1155. <https://doi.org/10.1096/fj.10-169433>

- Ling, V., Luxenberg, D., Wang, J., Nickbarg, E., Leenen, P. J., Neben, S., & Kobayashi, M. (1997). Structural identification of the hematopoietic progenitor antigen ER-MP12 as the vascular endothelial adhesion molecule PECAM-1 (CD31). *Eur J Immunol*, 27(2), 509-514. <https://doi.org/10.1002/eji.1830270223>
- Listovsky, T., Oren, Y. S., Yudkovsky, Y., Mahbubani, H. M., Weiss, A. M., Lebendiker, M., & Brandeis, M. (2004). Mammalian Cdh1/Fzr mediates its own degradation. *Embo j*, 23(7), 1619-1626. <https://doi.org/10.1038/sj.emboj.7600149>
- Liu, H., Naxerova, K., Pinter, M., Incio, J., Lee, H., Shigeta, K., Ho, W. W., Crain, J. A., Jacobson, A., Michelakos, T., Dias-Santos, D., Zanconato, A., Hong, T. S., Clark, J. W., Murphy, J. E., Ryan, D. P., Deshpande, V., Lillemoe, K. D., Fernandez-Del Castillo, C., Downes, M., Evans, R. M., Michaelson, J., Ferrone, C. R., Boucher, Y., & Jain, R. K. (2017). Use of Angiotensin System Inhibitors Is Associated with Immune Activation and Longer Survival in Nonmetastatic Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res*, 23(19), 5959-5969. <https://doi.org/10.1158/1078-0432.Ccr-17-0256>
- Lo, A., Wang, L. S., Scholler, J., Monslow, J., Avery, D., Newick, K., O'Brien, S., Evans, R. A., Bajor, D. J., Clendenin, C., Durham, A. C., Buza, E. L., Vonderheide, R. H., June, C. H., Albelda, S. M., & Puré, E. (2015). Tumor-Promoting Desmoplasia Is Disrupted by Depleting FAP-Expressing Stromal Cells. *Cancer Res*, 75(14), 2800-2810. <https://doi.org/10.1158/0008-5472.Can-14-3041>
- Lynch, R. W., Hawley, C. A., Pellicoro, A., Bain, C. C., Iredale, J. P., & Jenkins, S. J. (2018). An efficient method to isolate Kupffer cells eliminating endothelial cell contamination and selective bias. *J Leukoc Biol*, 104(3), 579-586. <https://doi.org/10.1002/JLB.1TA0517-169R>
- Ma, C., Kapanadze, T., Gamrekelashvili, J., Manns, M. P., Korangy, F., & Greten, T. F. (2012). Anti-Gr-1 antibody depletion fails to eliminate hepatic myeloid-derived suppressor cells in tumor-bearing mice. *J Leukoc Biol*, 92(6), 1199-1206. <https://doi.org/10.1189/jlb.0212059>
- Mandilaras, V., Bouganim, N., Yin, H., Asselah, J., & Azoulay, L. (2017). The use of drugs acting on the renin-angiotensin system and the incidence of pancreatic cancer. *Br J Cancer*, 116(1), 103-108. <https://doi.org/10.1038/bjc.2016.375>
- Mantovani, A., Sozzani, S., Locati, M., Allavena, P., & Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*, 23(11), 549-555. [https://doi.org/10.1016/s1471-4906\(02\)02302-5](https://doi.org/10.1016/s1471-4906(02)02302-5)
- Maria, A. G., Dillenburg-Pilla, P., Reis, R. I., Floriano, E. M., Tefé-Silva, C., Ramos, S. G., Pesquero, J. B., Nahmias, C., & Costa-Neto, C. M. (2016). Host kinin B1

- receptor plays a protective role against melanoma progression [Article]. *Scientific Reports*, 6, 22078. <https://doi.org/10.1038/srep22078>
<https://www.nature.com/articles/srep22078#supplementary-information>
- Marquardt, N., Kekäläinen, E., Chen, P., Lourda, M., Wilson, J. N., Scharenberg, M., Bergman, P., Al-Ameri, M., Hård, J., Mold, J. E., Ljunggren, H.-G., & Michaëlsson, J. (2019). Unique transcriptional and protein-expression signature in human lung tissue-resident NK cells. *Nature Communications*, 10(1), 3841. <https://doi.org/10.1038/s41467-019-11632-9>
- Marshall, H. T., & Djamgoz, M. B. A. (2018). Immuno-Oncology: Emerging Targets and Combination Therapies [Review]. *Frontiers in Oncology*, 8(315). <https://doi.org/10.3389/fonc.2018.00315>
- Martin, J. D., Cabral, H., Stylianopoulos, T., & Jain, R. K. (2020). Improving cancer immunotherapy using nanomedicines: progress, opportunities and challenges. *Nat Rev Clin Oncol*, 17(4), 251-266. <https://doi.org/10.1038/s41571-019-0308-z>
- Martin, N., Manoharan, K., Thomas, J., Davies, C., & Lumbers, R. T. (2018). Beta-blockers and inhibitors of the renin-angiotensin aldosterone system for chronic heart failure with preserved ejection fraction. *Cochrane Database of Systematic Reviews*(6). <https://doi.org/10.1002/14651858.CD012721.pub2>
- Martina, M. N., Noel, S., Saxena, A., Bandapalle, S., Majithia, R., Jie, C., Arend, L. J., Allaf, M. E., Rabb, H., & Hamad, A. R. (2016). Double-Negative alphabeta T Cells Are Early Responders to AKI and Are Found in Human Kidney. *J Am Soc Nephrol*, 27(4), 1113-1123. <https://doi.org/10.1681/asn.2014121214>
- Masamune, A., Hamada, S., Kikuta, K., Takikawa, T., Miura, S., Nakano, E., & Shimosegawa, T. (2013). The angiotensin II type I receptor blocker olmesartan inhibits the growth of pancreatic cancer by targeting stellate cell activities in mice. *Scand J Gastroenterol*, 48(5), 602-609. <https://doi.org/10.3109/00365521.2013.777776>
- Masucci, G. V., Cesano, A., Hawtin, R., Janetzki, S., Zhang, J., Kirsch, I., Dobbin, K. K., Alvarez, J., Robbins, P. B., Selvan, S. R., Streicher, H. Z., Butterfield, L. H., & Thurin, M. (2016). Validation of biomarkers to predict response to immunotherapy in cancer: Volume I – pre-analytical and analytical validation. *J Immunother Cancer*, 4(1), 76. <https://doi.org/10.1186/s40425-016-0178-1>
- McKay, R. R., Rodriguez, G. E., Lin, X., Kaymakcalan, M. D., Hamnvik, O.-P. R., Sabbisetti, V. S., Bhatt, R. S., Simantov, R., & Choueiri, T. K. (2015). Angiotensin System Inhibitors and Survival Outcomes in Patients with Metastatic Renal Cell Carcinoma. *Clinical Cancer Research*, 21(11), 2471-2479. <https://doi.org/10.1158/1078-0432.Ccr-14-2332>

- McKenney, J. K., Weiss, S. W., & Folpe, A. L. (2001). CD31 expression in intratumoral macrophages: a potential diagnostic pitfall. *Am J Surg Pathol*, 25(9), 1167-1173. <https://doi.org/10.1097/00000478-200109000-00007>
- Medina-Echeverez, J., Eggert, T., Han, M., & Greten, T. F. (2015). Hepatic myeloid-derived suppressor cells in cancer. *Cancer Immunol Immunother*, 64(8), 931-940. <https://doi.org/10.1007/s00262-015-1736-y>
- Medjebar, S., Truntzer, C., Perrichet, A., Limagne, E., Fumet, J.-D., Richard, C., Elkrief, A., Routy, B., Rébé, C., & Ghiringhelli, F. (2020). Angiotensin-converting enzyme (ACE) inhibitor prescription affects non-small-cell lung cancer (NSCLC) patients response to PD-1/PD-L1 immune checkpoint blockers. *Oncoimmunology*, 9(1), 1836766. <https://doi.org/10.1080/2162402X.2020.1836766>
- Mendonsa, A. M., Na, T.-Y., & Gumbiner, B. M. (2018). E-cadherin in contact inhibition and cancer. *Oncogene*, 37(35), 4769-4780. <https://doi.org/10.1038/s41388-018-0304-2>
- Meng, H., Zhao, H., Cao, X., Hao, J., Zhang, H., Liu, Y., Zhu, M.-s., Fan, L., Weng, L., Qian, L., Wang, X., & Xu, Y. (2019). Double-negative T cells remarkably promote neuroinflammation after ischemic stroke. *Proceedings of the National Academy of Sciences*, 116(12), 5558-5563. <https://doi.org/10.1073/pnas.1814394116>
- Meng, Y., Chen, C., Liu, Y., Tian, C., & Li, H. H. (2017). Angiotensin II Regulates Dendritic Cells through Activation of NF- κ B /p65, ERK1/2 and STAT1 Pathways. *Cellular Physiology and Biochemistry*, 42(4), 1550-1558. <https://doi.org/10.1159/000479272>
- Milette, S., Sicklick, J. K., Lowy, A. M., & Brodt, P. (2017). Molecular Pathways: Targeting the Microenvironment of Liver Metastases. *Clin Cancer Res*, 23(21), 6390-6399. <https://doi.org/10.1158/1078-0432.Ccr-15-1636>
- Millen, R., Hendry, S., Narasimhan, V., Abbott, R., Croxford, M., Gibbs, P., Tie, J., Wong, H.-L., Jones, I., Kosmider, S., Byrne, D., Zalcberg, J., Fox, S., Desai, J., Visvanathan, K., Ramsay, R. G., & Tran, B. (2020). CD8+ tumor-infiltrating lymphocytes within the primary tumor of patients with synchronous de novo metastatic colorectal carcinoma do not track with survival. *Clinical & Translational Immunology*, 9(7), e1155. <https://doi.org/https://doi.org/10.1002/cti2.1155>
- Min, L. J., Cui, T. X., Yahata, Y., Yamasaki, K., Shiuchi, T., Liu, H. W., Chen, R., Li, J. M., Okumura, M., Jinno, T., Wu, L., Iwai, M., Nahmias, C., Hashimoto, K., & Horiuchi, M. (2004). Regulation of collagen synthesis in mouse skin fibroblasts by distinct angiotensin II receptor subtypes. *Endocrinology*, 145(1), 253-260. <https://doi.org/10.1210/en.2003-0673>

- Mittal, K., Ebos, J., & Rini, B. (2014). Angiogenesis and the Tumor Microenvironment: Vascular Endothelial Growth Factor and Beyond. *Seminars in Oncology*, 41(2), 235-251. <https://doi.org/https://doi.org/10.1053/j.seminoncol.2014.02.007>
- Mlecnik, B., Bindea, G., Angell, H. K., Maby, P., Angelova, M., Tougeron, D., Church, S. E., Lafontaine, L., Fischer, M., Fredriksen, T., Sasso, M., Bilocq, A. M., Kirilovsky, A., Obenauf, A. C., Hamieh, M., Berger, A., Bruneval, P., Tuech, J. J., Sabourin, J. C., Le Pessot, F., Maussion, J., Rafii, A., Laurent-Puig, P., Speicher, M. R., Trajanoski, Z., Michel, P., Sesboue, R., Frebourg, T., Pages, F., Valge-Archer, V., Latouche, J. B., & Galon, J. (2016). Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity*, 44(3), 698-711. <https://doi.org/10.1016/j.immuni.2016.02.025>
- Moeller, I., Allen, A. M., Chai, S. Y., Zhuo, J., & Mendelsohn, F. A. (1998). Bioactive angiotensin peptides. *J Hum Hypertens*, 12(5), 289-293.
- Molina, L., Matus, C. E., Astroza, A., Pavicic, F., Tapia, E., Toledo, C., Perez, J. A., Nualart, F., Gonzalez, C. B., Burgos, R. A., Figueroa, C. D., Ehrenfeld, P., & Poblete, M. T. (2009). Stimulation of the bradykinin B(1) receptor induces the proliferation of estrogen-sensitive breast cancer cells and activates the ERK1/2 signaling pathway. *Breast Cancer Res Treat*, 118(3), 499-510. <https://doi.org/10.1007/s10549-009-0314-4>
- Molteni, A., Ward, W. F., Ts'ao, C. H., Taylor, J., Small, W., Jr., Brizio-Molteni, L., & Veno, P. A. (2003). Cytostatic properties of some angiotensin I converting enzyme inhibitors and of angiotensin II type I receptor antagonists. *Curr Pharm Des*, 9(9), 751-761. <https://doi.org/10.2174/1381612033455396>
- Moodley, R., Snyman, C., Odhav, B., & Bhoola, K. D. (2005). Visualisation of transforming growth factor-beta 1, tissue kallikrein, and kinin and transforming growth factor-beta receptors on human clear-cell renal carcinoma cells. *Biol Chem*, 386(4), 375-382. <https://doi.org/10.1515/bc.2005.045>
- Moreau, M. E., Garbacki, N., Molinaro, G., Brown, N. J., Marceau, F., & Adam, A. (2005). The kallikrein-kinin system: current and future pharmacological targets. *J Pharmacol Sci*, 99(1), 6-38.
- Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K., & McDonald, D. M. (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *The American journal of pathology*, 160(3), 985-1000. [https://doi.org/10.1016/S0002-9440\(10\)64920-6](https://doi.org/10.1016/S0002-9440(10)64920-6)
- Mosely, S. I., Prime, J. E., Sainson, R. C., Koopmann, J. O., Wang, D. Y., Greenawalt, D. M., Ahdesmaki, M. J., Leyland, R., Mullins, S., Pacelli, L., Marcus, D., Anderton, J., Watkins, A., Coates Ulrichsen, J., Brohawn, P., Higgs, B. W., McCourt, M., Jones, H., Harper, J. A., Morrow, M., Valge-Archer, V., Stewart, R., Dovedi, S. J.,

- & Wilkinson, R. W. (2017). Rational Selection of Syngeneic Preclinical Tumor Models for Immunotherapeutic Drug Discovery. *Cancer Immunol Res*, 5(1), 29-41. <https://doi.org/10.1158/2326-6066.Cir-16-0114>
- Mpekris, F., Voutouri, C., Baish, J. W., Duda, D. G., Munn, L. L., Stylianopoulos, T., & Jain, R. K. (2020). Combining microenvironment normalization strategies to improve cancer immunotherapy. *Proceedings of the National Academy of Sciences*, 117(7), 3728-3737. <https://doi.org/10.1073/pnas.1919764117>
- Mrass, P., Kinjyo, I., Ng, L. G., Reiner, S. L., Pure, E., & Weninger, W. (2008). CD44 mediates successful interstitial navigation by killer T cells and enables efficient antitumor immunity. *Immunity*, 29(6), 971-985. <https://doi.org/10.1016/j.immuni.2008.10.015>
- Mueller, L., Goumas, F. A., Affeldt, M., Sandtner, S., Gehling, U. M., Brilloff, S., Walter, J., Karnatz, N., Lamszus, K., Rogiers, X., & Broering, D. C. (2007). Stromal fibroblasts in colorectal liver metastases originate from resident fibroblasts and generate an inflammatory microenvironment. *The American journal of pathology*, 171(5), 1608-1618. <https://doi.org/10.2353/ajpath.2007.060661>
- Müller, D. N., & Luft, F. C. (2006). Direct Renin Inhibition with Aliskiren in Hypertension and Target Organ Damage. *Clinical Journal of the American Society of Nephrology*, 1(2), 221-228. <https://doi.org/10.2215/cjn.01201005>
- Murata, S., Udono, H., Tanahashi, N., Hamada, N., Watanabe, K., Adachi, K., Yamano, T., Yui, K., Kobayashi, N., Kasahara, M., Tanaka, K., & Chiba, T. (2001). Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. *Embo j*, 20(21), 5898-5907. <https://doi.org/10.1093/emboj/20.21.5898>
- Murdoch, C., Giannoudis, A., & Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood*, 104(8), 2224-2234. <https://doi.org/10.1182/blood-2004-03-1109>
- Mustafa, T., Lee, J. H., Chai, S. Y., Albiston, A. L., McDowall, S. G., & Mendelsohn, F. A. (2001). Bioactive angiotensin peptides: focus on angiotensin IV. *Journal of the Renin-Angiotensin-Aldosterone System*, 2(4), 205-210. <https://doi.org/10.3317/jraas.2001.032>
- Na, T. Y., Schecterson, L., Mendonsa, A. M., & Gumbiner, B. M. (2020). The functional activity of E-cadherin controls tumor cell metastasis at multiple steps. *Proc Natl Acad Sci U S A*, 117(11), 5931-5937. <https://doi.org/10.1073/pnas.1918167117>
- Nakamura, K., Yaguchi, T., Ohmura, G., Kobayashi, A., Kawamura, N., Iwata, T., Kiniwa, Y., Okuyama, R., & Kawakami, Y. (2018). Involvement of local renin-

- angiotensin system in immunosuppression of tumor microenvironment. *Cancer Sci*, 109(1), 54-64. <https://doi.org/10.1111/cas.13423>
- Neefjes, J., & Ovaa, H. (2013). A peptide's perspective on antigen presentation to the immune system. *Nature Chemical Biology*, 9(12), 769-775. <https://doi.org/10.1038/nchembio.1391>
- Nelson, W. J., & Nusse, R. (2004). Convergence of Wnt, β -Catenin, and Cadherin Pathways. *Science*, 303(5663), 1483-1487. <https://doi.org/10.1126/science.1094291>
- Neo, J. H., Ager, E. I., Angus, P. W., Zhu, J., Herath, C. B., & Christophi, C. (2010). Changes in the renin angiotensin system during the development of colorectal cancer liver metastases. *BMC cancer*, 10, 134-134. <https://doi.org/10.1186/1471-2407-10-134>
- Neo, J. H., Malcontenti-Wilson, C., Muralidharan, V., & Christophi, C. (2007). Effect of ACE inhibitors and angiotensin II receptor antagonists in a mouse model of colorectal cancer liver metastases. *J Gastroenterol Hepatol*, 22(4), 577-584. <https://doi.org/10.1111/j.1440-1746.2006.04797.x>
- Newell, L. E., & Heddle, J. A. (2004). The potent colon carcinogen, 1,2-dimethylhydrazine induces mutations primarily in the colon. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 564(1), 1-7. <https://doi.org/https://doi.org/10.1016/j.mrgentox.2004.06.005>
- Nguyen, L., Ager, E. I., Neo, J., & Christophi, C. (2016). Regulation of colorectal cancer cell epithelial to mesenchymal transition by the renin angiotensin system. *J Gastroenterol Hepatol*, 31(10), 1773-1782. <https://doi.org/10.1111/jgh.13307>
- Nielsen, K., Rolff, H. C., Eefsen, R. L., & Vainer, B. (2014). The morphological growth patterns of colorectal liver metastases are prognostic for overall survival. *Mod Pathol*, 27(12), 1641-1648. <https://doi.org/10.1038/modpathol.2014.4>
- Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., & Bernards, R. (2005). A genomic and functional inventory of deubiquitinating enzymes. *Cell*, 123(5), 773-786. <https://doi.org/10.1016/j.cell.2005.11.007>
- Noble, F. A. L., Schreurs, N. H., Straaten, H. W. v., Slaaf, D. W., Smits, J. F., Rogg, H., & Struijker-Boudier, H. A. (1993). Evidence for a novel angiotensin II receptor involved in angiogenesis in chick embryo chorioallantoic membrane. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 264(2), R460-R465. <https://doi.org/10.1152/ajpregu.1993.264.2.R460>
- Norman, J., Badie-Dezfooly, B., Nord, E. P., Kurtz, I., Schlosser, J., Chaudhari, A., & Fine, L. G. (1987). EGF-induced mitogenesis in proximal tubular cells:

potentiation by angiotensin II. *American Journal of Physiology-Renal Physiology*, 253(2), F299-F309.

<https://doi.org/10.1152/ajprenal.1987.253.2.F299>

Nowak-Sliwinska, P., Scapozza, L., & Ruiz i Altaba, A. (2019). Drug repurposing in oncology: Compounds, pathways, phenotypes and computational approaches for colorectal cancer. *Biochimica et biophysica acta. Reviews on cancer*, 1871(2), 434-454. <https://doi.org/10.1016/j.bbcan.2019.04.005>

Ochando, J., Conde, P., Utrero-Rico, A., & Paz-Artal, E. (2019). Tolerogenic Role of Myeloid Suppressor Cells in Organ Transplantation. *Front Immunol*, 10, 374. <https://doi.org/10.3389/fimmu.2019.00374>

Okazaki, M., Fushida, S., Harada, S., Tsukada, T., Kinoshita, J., Oyama, K., Tajima, H., Ninomiya, I., Fujimura, T., & Ohta, T. (2014). The Angiotensin II type 1 receptor blocker candesartan suppresses proliferation and fibrosis in gastric cancer. *Cancer Lett*, 355(1), 46-53. <https://doi.org/https://doi.org/10.1016/j.canlet.2014.09.019>

Okura, H., Kataoka, T., & Yoshida, K. (2016). Renin-angiotensin system inhibitors in patients with myocardial infarction and secondary mitral regurgitation. *Heart*, 102(9), 694-700. <https://doi.org/10.1136/heartjnl-2015-308536>

Okwan-Duodu, D., Landry, J., Shen, X. Z., & Diaz, R. (2013). Angiotensin-converting enzyme and the tumor microenvironment: mechanisms beyond angiogenesis. *Am J Physiol Regul Integr Comp Physiol*, 305(3), R205-215. <https://doi.org/10.1152/ajpregu.00544.2012>

Osumi, H., Matsusaka, S., Wakatsuki, T., Suenaga, M., Shinozaki, E., & Mizunuma, N. (2015). Angiotensin II type-1 receptor blockers enhance the effects of bevacizumab-based chemotherapy in metastatic colorectal cancer patients. *Molecular and Clinical Oncology*, 3(6), 1295-1300. <https://doi.org/10.3892/mco.2015.630>

Ozawa, T., Hashiguchi, Y., Yagi, T., Fukushima, Y., Shimada, R., Hayama, T., Tsuchiya, T., Nozawa, K., Iinuma, H., Ishihara, S., & Matsuda, K. (2019). Angiotensin I-converting enzyme inhibitors/angiotensin II receptor blockers may reduce tumor recurrence in left-sided and early colorectal cancers. *International journal of colorectal disease*, 34(10), 1731-1739. <https://doi.org/10.1007/s00384-019-03379-y>

Özdemir, B. C., Pentcheva-Hoang, T., Carstens, J. L., Zheng, X., Wu, C. C., Simpson, T. R., Laklai, H., Sugimoto, H., Kahlert, C., Novitskiy, S. V., De Jesus-Acosta, A., Sharma, P., Heidari, P., Mahmood, U., Chin, L., Moses, H. L., Weaver, V. M., Maitra, A., Allison, J. P., LeBleu, V. S., & Kalluri, R. (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell*, 25(6), 719-734. <https://doi.org/10.1016/j.ccr.2014.04.005>

- Pacurari, M., Kafoury, R., Tchounwou, P. B., & Ndebele, K. (2014). The Renin-Angiotensin-Aldosterone System in Vascular Inflammation and Remodeling. *International Journal of Inflammation*, 2014, 689360. <https://doi.org/10.1155/2014/689360>
- Padmanaban, V., Krol, I., Suhail, Y., Szczerba, B. M., Aceto, N., Bader, J. S., & Ewald, A. J. (2019). E-cadherin is required for metastasis in multiple models of breast cancer. *Nature*, 573(7774), 439-444. <https://doi.org/10.1038/s41586-019-1526-3>
- Pang, K. C., Sanders, M. T., Monaco, J. J., Doherty, P. C., Turner, S. J., & Chen, W. (2006). Immunoproteasome subunit deficiencies impact differentially on two immunodominant influenza virus-specific CD8+ T cell responses. *J Immunol*, 177(11), 7680-7688. <https://doi.org/10.4049/jimmunol.177.11.7680>
- Parra, E. R., Behrens, C., Rodriguez-Canales, J., Lin, H., Mino, B., Blando, J., Zhang, J., Gibbons, D. L., Heymach, J. V., Sepesi, B., Swisher, S. G., Weissferdt, A., Kalhor, N., Izzo, J., Kadara, H., Moran, C., Lee, J. J., & Wistuba, II. (2016). Image Analysis-based Assessment of PD-L1 and Tumor-Associated Immune Cells Density Supports Distinct Intratumoral Microenvironment Groups in Non-small Cell Lung Carcinoma Patients. *Clin Cancer Res*, 22(24), 6278-6289. <https://doi.org/10.1158/1078-0432.Ccr-15-2443>
- Parra, E. R., Uraoka, N., Jiang, M., Cook, P., Gibbons, D., Forget, M.-A., Bernatchez, C., Haymaker, C., Wistuba, I. I., & Rodriguez-Canales, J. (2017). Validation of multiplex immunofluorescence panels using multispectral microscopy for immune-profiling of formalin-fixed and paraffin-embedded human tumor tissues. *Scientific Reports*, 7(1), 13380. <https://doi.org/10.1038/s41598-017-13942-8>
- Patel, V. B., Zhong, J.-C., Grant, M. B., & Oudit, G. Y. (2016). Role of the ACE2/Angiotensin 1–7 axis of the Renin-Angiotensin System in Heart Failure. *Circ Res*, 118(8), 1313-1326. <https://doi.org/10.1161/CIRCRESAHA.116.307708>
- Pathan, M., Keerthikumar, S., Ang, C.-S., Gangoda, L., Quek, C. Y. J., Williamson, N. A., Mouradov, D., Sieber, O. M., Simpson, R. J., Salim, A., Bacic, A., Hill, A. F., Stroud, D. A., Ryan, M. T., Agbinya, J. I., Mariadason, J. M., Burgess, A. W., & Mathivanan, S. (2015). FunRich: An open access standalone functional enrichment and interaction network analysis tool. *PROTEOMICS*, 15(15), 2597-2601. <https://doi.org/10.1002/pmic.201400515>
- Pei, N., Wan, R., Chen, X., Li, A., Zhang, Y., Li, J., Du, H., Chen, B., Wei, W., Qi, Y., Zhang, Y., Katovich, M. J., Sumners, C., Zheng, H., & Li, H. (2016). Angiotensin-(1-7) Decreases Cell Growth and Angiogenesis of Human Nasopharyngeal Carcinoma Xenografts. *Mol Cancer Ther*, 15(1), 37-47. <https://doi.org/10.1158/1535-7163.Mct-14-0981>

- Peng, J., Wang, Y., Zhang, R., Deng, Y., Xiao, B., Ou, Q., Sui, Q., Xu, J., Qin, J., Lin, J., & Pan, Z. (2019). Immune Cell Infiltration in the Microenvironment of Liver Oligometastasis from Colorectal Cancer: Intratumoural CD8/CD3 Ratio Is a Valuable Prognostic Index for Patients Undergoing Liver Metastasectomy. *Cancers*, 11(12), 1922. <https://doi.org/10.3390/cancers11121922>
- Pereira, C., Gimenez-Xavier, P., Pros, E., Pajares, M. J., Moro, M., Gomez, A., Navarro, A., Condom, E., Moran, S., Gomez-Lopez, G., Graña, O., Rubio-Camarillo, M., Martinez-Martí, A., Yokota, J., Carretero, J., Galbis, J. M., Nadal, E., Pisano, D., Sozzi, G., Felip, E., Montuenga, L. M., Roz, L., Villanueva, A., & Sanchez-Cespedes, M. (2017). Genomic Profiling of Patient-Derived Xenografts for Lung Cancer Identifies B2M Inactivation Impairing Immunorecognition. *Clin Cancer Res*, 23(12), 3203-3213. <https://doi.org/10.1158/1078-0432.Ccr-16-1946>
- Pereira, L. X., Alves da Silva, L. C., de Oliveira Feitosa, A., Santos Ferreira, R. J., Fernandes Duarte, A. K., da Conceição, V., de Sales Marques, C., Barros Ferreira Rodrigues, A. K., Del Vechio Koike, B., Cavalcante de Queiroz, A., Guimaraes, T. A., Freire de Souza, C. D., & Alberto de Carvalho Fraga, C. (2019). Correlation between renin-angiotensin system (RAS) related genes, type 2 diabetes, and cancer: Insights from metanalysis of transcriptomics data. *Mol Cell Endocrinol*, 493, 110455. <https://doi.org/10.1016/j.mce.2019.110455>
- Perini, M. V., Dmello, R. S., Nero, T. L., & Chand, A. L. (2020). Evaluating the benefits of renin-angiotensin system inhibitors as cancer treatments. *Pharmacology & Therapeutics*, 211, 107527. <https://doi.org/https://doi.org/10.1016/j.pharmthera.2020.107527>
- Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., & Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature*, 392(6672), 190-193. <https://doi.org/10.1038/32433>
- Petrowsky, H., Fritsch, R., Guckenberger, M., De Oliveira, M. L., Dutkowski, P., & Clavien, P.-A. (2020). Modern therapeutic approaches for the treatment of malignant liver tumours. *Nature Reviews Gastroenterology & Hepatology*, 17(12), 755-772. <https://doi.org/10.1038/s41575-020-0314-8>
- Pinheiro, R. S., Herman, P., Lupinacci, R. M., Lai, Q., Mello, E. S., Coelho, F. F., Perini, M. V., Pugliese, V., Andraus, W., Cecconello, I., & D'Albuquerque, L. C. (2014). Tumor growth pattern as predictor of colorectal liver metastasis recurrence. *Am J Surg*, 207(4), 493-498. <https://doi.org/10.1016/j.amjsurg.2013.05.015>
- Pinter, M., & Jain, R. K. (2017). Targeting the renin-angiotensin system to improve cancer treatment: Implications for immunotherapy. *Science Translational Medicine*, 9(410). <https://doi.org/10.1126/scitranslmed.aan5616>

- Pinter, M., Kwanten, W. J., & Jain, R. K. (2018). Renin-Angiotensin System Inhibitors to Mitigate Cancer Treatment-Related Adverse Events. *Clin Cancer Res*, 24(16), 3803-3812. <https://doi.org/10.1158/1078-0432.CCR-18-0236>
- Pinter, M., Weinmann, A., Wörns, M.-A., Hucke, F., Bota, S., Marquardt, J. U., Duda, D. G., Jain, R. K., Galle, P. R., Trauner, M., Peck-Radosavljevic, M., & Sieghart, W. (2017). Use of inhibitors of the renin–angiotensin system is associated with longer survival in patients with hepatocellular carcinoma. *United European Gastroenterology Journal*, 5(7), 987-996. <https://doi.org/10.1177/2050640617695698>
- Pyzer, A. R., Cole, L., Rosenblatt, J., & Avigan, D. E. (2016). Myeloid-derived suppressor cells as effectors of immune suppression in cancer. *Int J Cancer*, 139(9), 1915-1926. <https://doi.org/10.1002/ijc.30232>
- Qin, X. Y., Zhang, Y. L., Chi, Y. F., Yan, B., Zeng, X. J., Li, H. H., & Liu, Y. (2018). Angiotensin II Regulates Th1 T Cell Differentiation Through Angiotensin II Type 1 Receptor-PKA-Mediated Activation of Proteasome. *Cellular Physiology and Biochemistry*, 45(4), 1366-1376. <https://doi.org/10.1159/000487562>
- Quinn, E., Hawkins, N., Yip, Y. L., Suter, C., & Ward, R. (2003). CD103+ intraepithelial lymphocytes—a unique population in microsatellite unstable sporadic colorectal cancer. *Eur J Cancer*, 39(4), 469-475. [https://doi.org/10.1016/s0959-8049\(02\)00633-0](https://doi.org/10.1016/s0959-8049(02)00633-0)
- Ramachandran, P., Pellicoro, A., Vernon, M. A., Boulter, L., Aucott, R. L., Ali, A., Hartland, S. N., Snowden, V. K., Cappon, A., Gordon-Walker, T. T., Williams, M. J., Dunbar, D. R., Manning, J. R., van Rooijen, N., Fallowfield, J. A., Forbes, S. J., & Iredale, J. P. (2012). Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proceedings of the National Academy of Sciences*, 109(46), E3186-E3195. <https://doi.org/10.1073/pnas.1119964109>
- Rees, M., Tekkis, P. P., Welsh, F. K., O'Rourke, T., & John, T. G. (2008). Evaluation of long-term survival after hepatic resection for metastatic colorectal cancer: a multifactorial model of 929 patients. *Ann Surg*, 247(1), 125-135. <https://doi.org/10.1097/SLA.0b013e31815aa2c2>
- Regulska, K., Regulski, M., Karolak, B., Murias, M., & Stanisiz, B. (2019). Can cardiovascular drugs support cancer treatment? The rationale for drug repurposing. *Drug Discov Today*, 24(4), 1059-1065. <https://doi.org/10.1016/j.drudis.2019.03.010>
- Remark, R., Merghoub, T., Grabe, N., Litjens, G., Damotte, D., Wolchok, J. D., Merad, M., & Gnjjatic, S. (2016). In-depth tissue profiling using multiplexed immunohistochemical consecutive staining on single slide. *Sci Immunol*, 1(1), aaf6925. <https://doi.org/10.1126/sciimmunol.aaf6925>

- Reyes-Turcu, F. E., & Wilkinson, K. D. (2009). Polyubiquitin binding and disassembly by deubiquitinating enzymes. *Chem Rev*, 109(4), 1495-1508.
<https://doi.org/10.1021/cr800470j>
- Rhodes, D. R., Ateeq, B., Cao, Q., Tomlins, S. A., Mehra, R., Laxman, B., Kalyana-Sundaram, S., Lonigro, R. J., Helgeson, B. E., Bhojani, M. S., Rehemtulla, A., Kleer, C. G., Hayes, D. F., Lucas, P. C., Varambally, S., & Chinnaiyan, A. M. (2009). AGTR1 overexpression defines a subset of breast cancer and confers sensitivity to losartan, an AGTR1 antagonist. *Proc Natl Acad Sci U S A*, 106(25), 10284-10289. <https://doi.org/10.1073/pnas.0900351106>
- Riedl, E., Stöckl, J., Majdic, O., Scheinecker, C., Knapp, W., & Strobl, H. (2000). Ligation of E-cadherin on in vitro-generated immature Langerhans-type dendritic cells inhibits their maturation. *Blood*, 96(13), 4276-4284.
- Riemann, A., Reime, S., & Thews, O. (2017). Tumor Acidosis and Hypoxia Differently Modulate the Inflammatory Program: Measurements In Vitro and In Vivo. *Neoplasia (New York, N.Y.)*, 19(12), 1033-1042.
<https://doi.org/10.1016/j.neo.2017.09.005>
- Riley, R. S., June, C. H., Langer, R., & Mitchell, M. J. (2019). Delivery technologies for cancer immunotherapy. *Nat Rev Drug Discov*, 18(3), 175-196.
<https://doi.org/10.1038/s41573-018-0006-z>
- Rivera, L. B., & Bergers, G. (2015). Intertwined regulation of angiogenesis and immunity by myeloid cells. *Trends Immunol*, 36(4), 240-249.
<https://doi.org/10.1016/j.it.2015.02.005>
- Robert, C., Thomas, L., Bondarenko, I., O'Day, S., Weber, J., Garbe, C., Lebbe, C., Baurain, J.-F., Testori, A., Grob, J.-J., Davidson, N., Richards, J., Maio, M., Hauschild, A., Miller, W. H., Gascon, P., Lotem, M., Harmankaya, K., Ibrahim, R., Francis, S., Chen, T.-T., Humphrey, R., Hoos, A., & Wolchok, J. D. (2011). Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. *New England Journal of Medicine*, 364(26), 2517-2526.
<https://doi.org/10.1056/NEJMoa1104621>
- Rosano, G. M. C., Tamargo, J., Kjeldsen, K. P., Lainscak, M., Agewall, S., Anker, S. D., Ceconi, C., Coats, A. J. S., Drexel, H., Filippatos, G., Kaski, J. C., Lund, L., Niessner, A., Ponikowski, P., Savarese, G., Schmidt, T. A., Seferovic, P., Wassmann, S., Walther, T., & Lewis, B. S. (2018). Expert consensus document on the management of hyperkalaemia in patients with cardiovascular disease treated with renin angiotensin aldosterone system inhibitors: coordinated by the Working Group on Cardiovascular Pharmacotherapy of the European Society of Cardiology. *Eur Heart J Cardiovasc Pharmacother*, 4(3), 180-188.
<https://doi.org/10.1093/ehjcvp/pyy015>
- Rouette, A., Trofimov, A., Haberl, D., Boucher, G., Lavallée, V.-P., D'Angelo, G., Hébert, J., Sauvageau, G., Lemieux, S., & Perreault, C. (2016). Expression of

immunoproteasome genes is regulated by cell-intrinsic and –extrinsic factors in human cancers. *Scientific Reports*, 6(1), 34019.

<https://doi.org/10.1038/srep34019>

Saber, S., Mahmoud, A. A. A., Helal, N. S., El-Ahwany, E., & Abdelghany, R. H. (2018). Renin-angiotensin system inhibition ameliorates CCl₄-induced liver fibrosis in mice through the inactivation of nuclear transcription factor kappa B. *Can J Physiol Pharmacol*, 1-8. <https://doi.org/10.1139/cjpp-2017-0728>

Sahai, E., Astsaturov, I., Cukierman, E., DeNardo, D. G., Egeblad, M., Evans, R. M., Fearon, D., Greten, F. R., Hingorani, S. R., Hunter, T., Hynes, R. O., Jain, R. K., Janowitz, T., Jorgensen, C., Kimmelman, A. C., Kolonin, M. G., Maki, R. G., Powers, R. S., Puré, E., Ramirez, D. C., Scherz-Shouval, R., Sherman, M. H., Stewart, S., Tlsty, T. D., Tuveson, D. A., Watt, F. M., Weaver, V., Weeraratna, A. T., & Werb, Z. (2020). A framework for advancing our understanding of cancer-associated fibroblasts. *Nature Reviews Cancer*, 20(3), 174-186.

<https://doi.org/10.1038/s41568-019-0238-1>

Salmon, H., Franciszkiewicz, K., Damotte, D., Dieu-Nosjean, M.-C., Validire, P., Trautmann, A., Mami-Chouaib, F., & Donnadieu, E. (2012). Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. *The Journal of Clinical Investigation*, 122(3), 899-910.

<https://doi.org/10.1172/JCI45817>

Salmon, H., Idoyaga, J., Rahman, A., Leboeuf, M., Remark, R., Jordan, S., Casanova-Acebes, M., Khudoynazarova, M., Agudo, J., Tung, N., Chakarov, S., Rivera, C., Hogstad, B., Bosenberg, M., Hashimoto, D., Gnjjatic, S., Bhardwaj, N., Palucka, A. K., Brown, B. D., Brody, J., Ginhoux, F., & Merad, M. (2016). Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity*, 44(4), 924-938. <https://doi.org/10.1016/j.immuni.2016.03.012>

Sanders, P. M., Russell, S. T., & Tisdale, M. J. (2005). Angiotensin II directly induces muscle protein catabolism through the ubiquitin-proteasome proteolytic pathway and may play a role in cancer cachexia. *Br J Cancer*, 93(4), 425-434.

<https://doi.org/10.1038/sj.bjc.6602725>

Sasson, S. C., Gordon, C. L., Christo, S. N., Klenerman, P., & Mackay, L. K. (2020). Local heroes or villains: tissue-resident memory T cells in human health and disease. *Cellular & molecular immunology*, 17(2), 113-122.

<https://doi.org/10.1038/s41423-019-0359-1>

Satoh, T., Takeuchi, O., Vandenbon, A., Yasuda, K., Tanaka, Y., Kumagai, Y., Miyake, T., Matsushita, K., Okazaki, T., Saitoh, T., Honma, K., Matsuyama, T., Yui, K., Tsujimura, T., Standley, D. M., Nakanishi, K., Nakai, K., & Akira, S. (2010). The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol*, 11(10), 936-944.

<https://doi.org/10.1038/ni.1920>

- Schaaf, M. B., Garg, A. D., & Agostinis, P. (2018). Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death & Disease*, 9(2), 115. <https://doi.org/10.1038/s41419-017-0061-0>
- Schmid, P., Adams, S., Rugo, H. S., Schneeweiss, A., Barrios, C. H., Iwata, H., Diéras, V., Hegg, R., Im, S. A., Shaw Wright, G., Henschel, V., Molinero, L., Chui, S. Y., Funke, R., Husain, A., Winer, E. P., Loi, S., & Emens, L. A. (2018). Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. *N Engl J Med*, 379(22), 2108-2121. <https://doi.org/10.1056/NEJMoa1809615>
- Schnittert, J., Bansal, R., Storm, G., & Prakash, J. (2018). Integrins in wound healing, fibrosis and tumor stroma: High potential targets for therapeutics and drug delivery. *Advanced Drug Delivery Reviews*, 129, 37-53. <https://doi.org/https://doi.org/10.1016/j.addr.2018.01.020>
- Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R., & Villadangos, J. A. (2006). The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci U S A*, 103(28), 10729-10734. <https://doi.org/10.1073/pnas.0601956103>
- Scott, C. L., Zheng, F., De Baetselier, P., Martens, L., Saeys, Y., De Prijck, S., Lippens, S., Abels, C., Schoonoghe, S., Raes, G., Devoogdt, N., Lambrecht, B. N., Beschin, A., & Guilliams, M. (2016). Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun*, 7, 10321. <https://doi.org/10.1038/ncomms10321>
- Sedgwick, P. (2014). Retrospective cohort studies: advantages and disadvantages. *BMJ : British Medical Journal*, 348, g1072. <https://doi.org/10.1136/bmj.g1072>
- Sendo, S., Saegusa, J., & Morinobu, A. (2018). Myeloid-derived suppressor cells in non-neoplastic inflamed organs. *Inflammation and Regeneration*, 38(1), 19. <https://doi.org/10.1186/s41232-018-0076-7>
- Senger, D. R., & Davis, G. E. (2011). Angiogenesis. *Cold Spring Harbor Perspectives in Biology*, 3(8), a005090. <https://doi.org/10.1101/cshperspect.a005090>
- Serrablo, A., Paliogiannis, P., Pulighe, F., Moro, S. S., Borrego-Estella, V., Attene, F., Scognamillo, F., & Horndler, C. (2016). Impact of novel histopathological factors on the outcomes of liver surgery for colorectal cancer metastases. *Eur J Surg Oncol*, 42(9), 1268-1277. <https://doi.org/10.1016/j.ejso.2016.02.013>
- Sharman, J. L., Harding, S. D., Southan, C., Faccenda, E., Pawson, A. J., Davies, J. A., & NC-IUPHAR. (2018). Accessing Expert-Curated Pharmacological Data in the IUPHAR/BPS Guide to PHARMACOLOGY. *Current Protocols in Bioinformatics*, 61(1), 1.34.31-31.34.46. <https://doi.org/https://doi.org/10.1002/cpbi.46>

- Shen, X. Z., Billet, S., Lin, C., Okwan-Duodu, D., Chen, X., Lukacher, A. E., & Bernstein, K. E. (2011). The carboxypeptidase ACE shapes the MHC class I peptide repertoire. *Nat Immunol*, 12(11), 1078-1085. <https://doi.org/10.1038/ni.2107>
- Shen, X. Z., Okwan-Duodu, D., Blackwell, W. L., Ong, F. S., Janjulia, T., Bernstein, E. A., Fuchs, S., Alkan, S., & Bernstein, K. E. (2014). Myeloid expression of angiotensin-converting enzyme facilitates myeloid maturation and inhibits the development of myeloid-derived suppressor cells. *Lab Invest*, 94(5), 536-544. <https://doi.org/10.1038/labinvest.2014.41>
- Shen, Y., Wang, X., Lu, J., Salfenmoser, M., Wirsik, N. M., Schleussner, N., Imle, A., Freire Valls, A., Radhakrishnan, P., Liang, J., Wang, G., Muley, T., Schneider, M., Ruiz de Almodovar, C., Diz-Muñoz, A., & Schmidt, T. (2020). Reduction of Liver Metastasis Stiffness Improves Response to Bevacizumab in Metastatic Colorectal Cancer. *Cancer Cell*, 37(6), 800-817.e807. <https://doi.org/10.1016/j.ccell.2020.05.005>
- Shields, B. D., Koss, B., Taylor, E. M., Storey, A. J., West, K. L., Byrum, S. D., Mackintosh, S. G., Edmondson, R., Mahmoud, F., Shalin, S. C., & Tackett, A. J. (2019). Loss of E-Cadherin Inhibits CD103 Antitumor Activity and Reduces Checkpoint Blockade Responsiveness in Melanoma. *Cancer Res*, 79(6), 1113-1123. <https://doi.org/10.1158/0008-5472.Can-18-1722>
- Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H., & Takeyama, H. (2015). Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers*, 7(4), 2443-2458. <https://doi.org/10.3390/cancers7040902>
- Shirotake, S., Miyajima, A., Kosaka, T., Tanaka, N., Maeda, T., Kikuchi, E., & Oya, M. (2011). Angiotensin II type 1 receptor expression and microvessel density in human bladder cancer. *Urology*, 77(4), 1009.e1019-1025. <https://doi.org/10.1016/j.urology.2010.11.002>
- Shurin, G. V., Ma, Y., & Shurin, M. R. (2013). Immunosuppressive Mechanisms of Regulatory Dendritic Cells in Cancer. *Cancer Microenvironment*, 6(2), 159-167. <https://doi.org/10.1007/s12307-013-0133-3>
- Siegel, D. S., Martin, T., Wang, M., Viji, R., Jakubowiak, A. J., Lonial, S., Trudel, S., Kukreti, V., Bahlis, N., Alsina, M., Chanan-Khan, A., Buadi, F., Reu, F. J., Somlo, G., Zonder, J., Song, K., Stewart, A. K., Stadtmauer, E., Kunkel, L., Wear, S., Wong, A. F., Orlowski, R. Z., & Jagannath, S. (2012). A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. *Blood*, 120(14), 2817-2825. <https://doi.org/10.1182/blood-2012-05-425934>
- Small, W., Jr., Molteni, A., Kim, Y. T., Taylor, J. M., Ts'ao, C. H., & Ward, W. F. (1999). Mechanism of captopril toxicity to a human mammary ductal carcinoma cell

- line in the presence of copper. *Breast Cancer Res Treat*, 55(3), 223-229.
<https://doi.org/10.1023/a:1006233521325>
- Smith, H. A., & Kang, Y. (2013). The metastasis-promoting roles of tumor-associated immune cells. *Journal of molecular medicine (Berlin, Germany)*, 91(4), 411-429.
<https://doi.org/10.1007/s00109-013-1021-5>
- Sorrelle, N., Ganguly, D., Dominguez, A. T. A., Zhang, Y., Huang, H., Dahal, L. N., Burton, N., Ziemys, A., & Brekken, R. A. (2019). Improved Multiplex Immunohistochemistry for Immune Microenvironment Evaluation of Mouse Formalin-Fixed, Paraffin-Embedded Tissues. *J Immunol*, 202(1), 292-299.
<https://doi.org/10.4049/jimmunol.1800878>
- Soucy, T. A., Dick, L. R., Smith, P. G., Milhollen, M. A., & Brownell, J. E. (2010). The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy. *Genes Cancer*, 1(7), 708-716.
<https://doi.org/10.1177/1947601910382898>
- Stack, E. C., Wang, C., Roman, K. A., & Hoyt, C. C. (2014). Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods*, 70(1), 46-58.
<https://doi.org/https://doi.org/10.1016/j.ymeth.2014.08.016>
- Stefanska, A., Kenyon, C., Christian, H. C., Buckley, C., Shaw, I., Mullins, J. J., & Péault, B. (2016). Human kidney pericytes produce renin. *Kidney International*, 90(6), 1251-1261. <https://doi.org/10.1016/j.kint.2016.07.035>
- Sugimoto, M., Yamaoka, Y., Shirai, N., & Furuta, T. (2012). Role of renin-angiotensin system in gastric oncogenesis. *Journal of gastroenterology and hepatology*, 27(3), 442-451. <https://doi.org/10.1111/j.1440-1746.2011.06964.x>
- Sullivan, J. C. (2008). Sex and the renin-angiotensin system: inequality between the sexes in response to RAS stimulation and inhibition. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 294(4), R1220-R1226. <https://doi.org/10.1152/ajpregu.00864.2007>
- Sun, H., Li, T., Zhuang, R., Cai, W., & Zheng, Y. (2017). Do renin–angiotensin system inhibitors influence the recurrence, metastasis, and survival in cancer patients?: Evidence from a meta-analysis including 55 studies. *Medicine*, 96(13), e6394. <https://doi.org/10.1097/MD.0000000000006394>
- Szabo, P. A., Miron, M., & Farber, D. L. (2019). Location, location, location: Tissue resident memory T cells in mice and humans. *Science immunology*, 4(34), eaas9673. <https://doi.org/10.1126/sciimmunol.aas9673>
- Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N. T., Morris, J. H., Bork, P., Jensen, L. J., & Mering, C. v. (2019).

STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic acids research*, 47(D1), D607-D613. <https://doi.org/10.1093/nar/gky1131>

Tannir, N. M., Schwab, G., & Grünwald, V. (2017). Cabozantinib: an Active Novel Multikinase Inhibitor in Renal Cell Carcinoma. *Current Oncology Reports*, 19(2), 14. <https://doi.org/10.1007/s11912-017-0566-9>

Tao, L., Huang, G., Song, H., Chen, Y., & Chen, L. (2017). Cancer associated fibroblasts: An essential role in the tumor microenvironment. *Oncology Letters*, 14(3), 2611-2620. <https://doi.org/10.3892/ol.2017.6497>

Tardif, J. C., & Gregoire, J. (2013). Renin-angiotensin system inhibition and secondary cardiovascular prevention. *Jama*, 310(11), 1130-1131. <https://doi.org/10.1001/jama.2013.277170>

Taube, J. M., Akturk, G., Angelo, M., Engle, E. L., Gnjjatic, S., Greenbaum, S., Greenwald, N. F., Hedvat, C. V., Hollmann, T. J., Juco, J., Parra, E. R., Rebelatto, M. C., Rimm, D. L., Rodriguez-Canales, J., Schalper, K. A., Stack, E. C., Ferreira, C. S., Korski, K., Lako, A., Rodig, S. J., Schenck, E., Steele, K. E., Surace, M. J., Tetzlaff, M. T., von Loga, K., Wistuba, I. I., & Bifulco, C. B. (2020). The Society for Immunotherapy of Cancer statement on best practices for multiplex immunohistochemistry (IHC) and immunofluorescence (IF) staining and validation. *J Immunother Cancer*, 8(1), e000155. <https://doi.org/10.1136/jitc-2019-000155>

Tcyganov, E., Mastio, J., Chen, E., & Gabrilovich, D. I. (2018). Plasticity of myeloid-derived suppressor cells in cancer. *Curr Opin Immunol*, 51, 76-82. <https://doi.org/10.1016/j.coi.2018.03.009>

Teo, K., Sleight, P., Gao, P., Yusuf, S., Connolly, S., Yusuf, S., Swedberg, K., Pfeffer, M., Granger, C., McMurray, J., Sjoelie, A., Massie, B., Carson, P., Lewis, J., Wachtell, K., Dahlof, B., Devereux, R., Kjeldsen, S., Julius, S., Ibsen, H., Lindholm, L., Olsen, M., Okin, P., Califf, R., Holman, R., Haffner, S., Dagenais, G., Probstfield, J., Anderson, C., Diaz, R., Dans, A., Levine, M., Unger, T., Fagard, R., Diener, H.-C., Sacco, R., Zanchetti, A., Cohn, J., Weber, M., & Collaboration, A. (2011). Effects of telmisartan, irbesartan, valsartan, candesartan, and losartan on cancers in 15 trials enrolling 138 769 individuals The ARB Trialists Collaboration. *JOURNAL OF HYPERTENSION*, 29(4), 623-635.

Terry, S., Savagner, P., Ortiz-Cuaran, S., Mahjoubi, L., Saintigny, P., Thiery, J. P., & Chouaib, S. (2017). New insights into the role of EMT in tumor immune escape. *Molecular Oncology*, 11(7), 824-846. <https://doi.org/10.1002/1878-0261.12093>

Thews, O., Kelleher, D. K., & Vaupel, P. (2000). Disparate responses of tumour vessels to angiotensin II: tumour volume-dependent effects on perfusion and

- oxygenation. *Br J Cancer*, 83(2), 225-231.
<https://doi.org/10.1054/bjoc.2000.1229>
- Thibaut, T. A., Anderson, R. T., & Smith, D. M. (2018). A common mechanism of proteasome impairment by neurodegenerative disease-associated oligomers. *Nature Communications*, 9(1), 1097. <https://doi.org/10.1038/s41467-018-03509-0>
- Thompson, J. C., Davis, C., Deshpande, C., Hwang, W.-T., Jeffries, S., Huang, A., Mitchell, T. C., Langer, C. J., & Albelda, S. M. (2020). Gene signature of antigen processing and presentation machinery predicts response to checkpoint blockade in non-small cell lung cancer (NSCLC) and melanoma. *J Immunother Cancer*, 8(2), e000974. <https://doi.org/10.1136/jitc-2020-000974>
- Toghi Eshghi, S., Au-Yeung, A., Takahashi, C., Bolen, C. R., Nyachienga, M. N., Lear, S. P., Green, C., Mathews, W. R., & O'Gorman, W. E. (2019). Quantitative Comparison of Conventional and t-SNE-guided Gating Analyses [Technology Report]. *Frontiers in Immunology*, 10(1194).
<https://doi.org/10.3389/fimmu.2019.01194>
- Tomlinson, J. S., Jarnagin, W. R., DeMatteo, R. P., Fong, Y., Kornprat, P., Gonen, M., Kemeny, N., Brennan, M. F., Blumgart, L. H., & D'Angelica, M. (2007). Actual 10-year survival after resection of colorectal liver metastases defines cure. *J Clin Oncol*, 25(29), 4575-4580. <https://doi.org/10.1200/jco.2007.11.0833>
- Topham, D. J., & Reilly, E. C. (2018). Tissue-Resident Memory CD8(+) T Cells: From Phenotype to Function. *Frontiers in Immunology*, 9, 515-515.
<https://doi.org/10.3389/fimmu.2018.00515>
- Traver, D., Akashi, K., Manz, M., Merad, M., Miyamoto, T., Engleman, E. G., & Weissman, I. L. (2000). Development of CD8 α -Positive Dendritic Cells from a Common Myeloid Progenitor. *Science*, 290(5499), 2152-2154.
<https://doi.org/10.1126/science.290.5499.2152>
- Tripathi, S. C., Peters, H. L., Taguchi, A., Katayama, H., Wang, H., Momin, A., Jolly, M. K., Celiktas, M., Rodriguez-Canales, J., Liu, H., Behrens, C., Wistuba, I. I., Ben-Jacob, E., Levine, H., Molldrem, J. J., Hanash, S. M., & Ostrin, E. J. (2016). Immunoproteasome deficiency is a feature of non-small cell lung cancer with a mesenchymal phenotype and is associated with a poor outcome. *Proceedings of the National Academy of Sciences*, 113(11), E1555-E1564.
<https://doi.org/10.1073/pnas.1521812113>
- Tseng, Y. H., Ho, H. L., Lai, C. R., Luo, Y. H., Tseng, Y. C., Whang-Peng, J., Lin, Y. H., Chou, T. Y., & Chen, Y. M. (2018). PD-L1 Expression of Tumor Cells, Macrophages, and Immune Cells in Non-Small Cell Lung Cancer Patients with Malignant Pleural Effusion. *J Thorac Oncol*, 13(3), 447-453.
<https://doi.org/10.1016/j.jtho.2017.10.034>

- Tsujikawa, T., Kumar, S., Borkar, R. N., Azimi, V., Thibault, G., Chang, Y. H., Balter, A., Kawashima, R., Choe, G., Sauer, D., El Rassi, E., Clayburgh, D. R., Kulesz-Martin, M. F., Lutz, E. R., Zheng, L., Jaffee, E. M., Leyshock, P., Margolin, A. A., Mori, M., Gray, J. W., Flint, P. W., & Coussens, L. M. (2017). Quantitative Multiplex Immunohistochemistry Reveals Myeloid-Inflamed Tumor-Immune Complexity Associated with Poor Prognosis. *Cell Reports*, 19(1), 203-217.
<https://doi.org/https://doi.org/10.1016/j.celrep.2017.03.037>
- Tsvetkov, P., Reuven, N., & Shaul, Y. (2010). Ubiquitin-independent p53 proteasomal degradation. *Cell Death & Differentiation*, 17(1), 103-108.
<https://doi.org/10.1038/cdd.2009.67>
- Tyanova, S., Temu, T., Carlson, A., Sinitcyn, P., Mann, M., & Cox, J. (2015). Visualization of LC-MS/MS proteomics data in MaxQuant. *PROTEOMICS*, 15(8), 1453-1456. <https://doi.org/10.1002/pmic.201400449>
- Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., & Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods*, 13(9), 731-740.
<https://doi.org/10.1038/nmeth.3901>
- Uemura, H., Ishiguro, H., Ishiguro, Y., Hoshino, K., Takahashi, S., & Kubota, Y. (2008). Angiotensin II induces oxidative stress in prostate cancer. *Mol Cancer Res*, 6(2), 250-258. <https://doi.org/10.1158/1541-7786.Mcr-07-0289>
- Usui, T., Sugisaki, K., Iriyama, A., Yokoo, S., Yamagami, S., Nagai, N., Ishida, S., & Amano, S. (2008). Inhibition of corneal neovascularization by blocking the angiotensin II type 1 receptor. *Invest Ophthalmol Vis Sci*, 49(10), 4370-4376.
<https://doi.org/10.1167/iovs.07-0964>
- Vallejo-Ardila, D. L., Fifis, T., Burrell, L. M., Walsh, K., & Christophi, C. (2018). Renin-angiotensin inhibitors reprogram tumor immune microenvironment: A comprehensive view of the influences on anti-tumor immunity. *Oncotarget*, 9(84), 35500-35511. <https://doi.org/10.18632/oncotarget.26174>
- Vallejo Ardila, D. L., Tamayo Cáceres, Y. R., & Munive, A. A. (2020). Uso terapéutico de los inhibidores de la enzima convertidora de angiotensina en pacientes con COVID-19: las «dos caras de la moneda» Therapeutic use of angiotensin converting enzyme inhibitors in patients with COVID-19: the “two sides of the coin”. *Revista Colombiana De Cardiología*, 27(4), 212-222.
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7365126/pdf/?tool=EBI>
<https://europepmc.org/articles/PMC7365126>
- Vallejo Ardila, D. L., Walsh, K. A., Fifis, T., Paolini, R., Kastrappis, G., Christophi, C., & Perini, M. V. (2020). Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases. *J Immunother Cancer*, 8(1). <https://doi.org/10.1136/jitc-2019-000487>

- Van Cutsem, E., Cervantes, A., Nordlinger, B., Arnold, D., & Group, o. b. o. t. E. G. W. (2014). Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. *Annals of Oncology*, 25(suppl_3), iii1-iii9. <https://doi.org/10.1093/annonc/mdu260>
- van Dam, P. J., van der Stok, E. P., Teuwen, L. A., Van den Eynden, G. G., Illemann, M., Frentzas, S., Majeed, A. W., Eefsen, R. L., Coebergh van den Braak, R. R. J., Lazaris, A., Fernandez, M. C., Galjart, B., Laerum, O. D., Rayes, R., Grunhagen, D. J., Van de Paer, M., Sucaet, Y., Mudhar, H. S., Schvimer, M., Nystrom, H., Kockx, M., Bird, N. C., Vidal-Vanaclocha, F., Metrakos, P., Simoneau, E., Verhoef, C., Dirix, L. Y., Van Laere, S., Gao, Z. H., Brodt, P., Reynolds, A. R., & Vermeulen, P. B. (2017). International consensus guidelines for scoring the histopathological growth patterns of liver metastasis. *Br J Cancer*, 117(10), 1427-1441. <https://doi.org/10.1038/bjc.2017.334>
- Van den Eynden, G. G., Bird, N. C., Majeed, A. W., Van Laere, S., Dirix, L. Y., & Vermeulen, P. B. (2012). The histological growth pattern of colorectal cancer liver metastases has prognostic value. *Clin Exp Metastasis*, 29(6), 541-549. <https://doi.org/10.1007/s10585-012-9469-1>
- Vasudev, N. S., & Reynolds, A. R. (2014). Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions [journal article]. *Angiogenesis*, 17(3), 471-494. <https://doi.org/10.1007/s10456-014-9420-y>
- Vera, R., González-Flores, E., Rubio, C., Urbano, J., Valero Camps, M., Ciampi-Dopazo, J. J., Orcajo Rincón, J., Morillo Macías, V., Gomez Braco, M. A., & Suarez-Artacho, G. (2019). Multidisciplinary management of liver metastases in patients with colorectal cancer: a consensus of SEOM, AEC, SEOR, SERVEI, and SEMNIM. *Clinical and Translational Oncology*. <https://doi.org/10.1007/s12094-019-02182-z>
- Vinay, D. S., Ryan, E. P., Pawelec, G., Talib, W. H., Stagg, J., Elkord, E., Lichtor, T., Decker, W. K., Whelan, R. L., Kumara, H. M. C. S., Signori, E., Honoki, K., Georgakilas, A. G., Amin, A., Helferich, W. G., Boosani, C. S., Guha, G., Ciriolo, M. R., Chen, S., Mohammed, S. I., Azmi, A. S., Keith, W. N., Bilsland, A., Bhakta, D., Halicka, D., Fujii, H., Aquilano, K., Ashraf, S. S., Newsheem, S., Yang, X., Choi, B. K., & Kwon, B. S. (2015). Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in Cancer Biology*, 35, S185-S198. <https://doi.org/https://doi.org/10.1016/j.semcancer.2015.03.004>
- Vinh, A., Chen, W., Blinder, Y., Weiss, D., Taylor, W. R., Goronzy, J. J., Weyand, C. M., Harrison, D. G., & Guzik, T. J. (2010). Inhibition and genetic ablation of the B7/CD28 T-cell costimulation axis prevents experimental hypertension. *Circulation*, 122(24), 2529-2537. <https://doi.org/10.1161/circulationaha.109.930446>

- Vleminckx, K., Vakaet, L., Jr., Mareel, M., Fiers, W., & van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*, 66(1), 107-119. [https://doi.org/10.1016/0092-8674\(91\)90143-m](https://doi.org/10.1016/0092-8674(91)90143-m)
- Voutsadakis, I. A. (2008). The ubiquitin-proteasome system in colorectal cancer. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1782(12), 800-808. <https://doi.org/https://doi.org/10.1016/j.bbadis.2008.06.007>
- Walmor De, M. (2014). Beyond the conventional Renin Angiotensin System. <http://www.doabooks.org/doab?func=fulltext&rid=18666>
- Walther, T., Menrad, A., Orzechowski, H. D., Siemeister, G., Paul, M., & Schirner, M. (2003). Differential regulation of in vivo angiogenesis by angiotensin II receptors. *Faseb j*, 17(14), 2061-2067. <https://doi.org/10.1096/fj.03-0129com>
- Wang, B., Wu, S., Zeng, H., Liu, Z., Dong, W., He, W., Chen, X., Dong, X., Zheng, L., Lin, T., & Huang, J. (2015). CD103+ Tumor Infiltrating Lymphocytes Predict a Favorable Prognosis in Urothelial Cell Carcinoma of the Bladder. *J Urol*, 194(2), 556-562. <https://doi.org/10.1016/j.juro.2015.02.2941>
- Wang, G., Lu, X., Dey, P., Deng, P., Wu, C. C., Jiang, S., Fang, Z., Zhao, K., Konaparthi, R., Hua, S., Zhang, J., Li-Ning-Tapia, E. M., Kapoor, A., Wu, C. J., Patel, N. B., Guo, Z., Ramamoorthy, V., Tieu, T. N., Heffernan, T., Zhao, D., Shang, X., Khadka, S., Hou, P., Hu, B., Jin, E. J., Yao, W., Pan, X., Ding, Z., Shi, Y., Li, L., Chang, Q., Troncoso, P., Logothetis, C. J., McArthur, M. J., Chin, L., Wang, Y. A., & DePinho, R. A. (2016). Targeting YAP-Dependent MDSC Infiltration Impairs Tumor Progression. *Cancer Discov*, 6(1), 80-95. <https://doi.org/10.1158/2159-8290.Cd-15-0224>
- Wang, M., Huang, Y. K., Kong, J. C., Sun, Y., Tantalos, D. G., Yeang, H. X. A., Ying, L., Yan, F., Xu, D., Halse, H., Di Costanzo, N., Gordon, I. R., Mitchell, C., Mackay, L. K., Busuttil, R. A., Neeson, P. J., & Boussioutas, A. (2020). High-dimensional analyses reveal a distinct role of T-cell subsets in the immune microenvironment of gastric cancer. *Clin Transl Immunology*, 9(5), e1127. <https://doi.org/10.1002/cti2.1127>
- Wang, M., Sun, Y. & Huang, Y. K. (2018). ISAT: extract cell density and nearest distance based on 'PerkinElmer InForm' software output. <https://doi.org/https://cran.r-project.org/web/packages/ISAT/index.html>
- Wang, Y., Ding, Y., Deng, Y., Zheng, Y., & Wang, S. (2020). Role of myeloid-derived suppressor cells in the promotion and immunotherapy of colitis-associated cancer. *J Immunother Cancer*, 8(2), e000609. <https://doi.org/10.1136/jitc-2020-000609>
- Wang, Y., Lin, H. C., Huang, M. Y., Shao, Q., Wang, Z. Q., Wang, F. H., Yuan, Y. F., Li, B. K., Wang, D. S., Ding, P. R., Chen, G., Wu, X. J., Lu, Z. H., Li, L. R., Pan, Z. Z., Sun,

- P., Yan, S. M., Wan, D. S., Xu, R. H., & Li, Y. H. (2018). The Immunoscore system predicts prognosis after liver metastasectomy in colorectal cancer liver metastases. *Cancer Immunol Immunother*, 67(3), 435-444. <https://doi.org/10.1007/s00262-017-2094-8>
- Wang, Z.-Q., Milne, K., Derocher, H., Webb, J. R., Nelson, B. H., & Watson, P. H. (2016). CD103 and Intratumoral Immune Response in Breast Cancer. *Clinical Cancer Research*, 22(24), 6290-6297. <https://doi.org/10.1158/1078-0432.Ccr-16-0732>
- Watt, J., & Kocher, H. M. (2013). The desmoplastic stroma of pancreatic cancer is a barrier to immune cell infiltration. *Oncoimmunology*, 2(12), e26788. <https://doi.org/10.4161/onci.26788>
- Webb, J. R., Milne, K., Watson, P., Deleeuw, R. J., & Nelson, B. H. (2014). Tumor-infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer. *Clin Cancer Res*, 20(2), 434-444. <https://doi.org/10.1158/1078-0432.Ccr-13-1877>
- Wegman-Ostrosky, T., Soto-Reyes, E., Vidal-Millan, S., & Sanchez-Corona, J. (2015). The renin-angiotensin system meets the hallmarks of cancer. *J Renin Angiotensin Aldosterone Syst*, 16(2), 227-233. <https://doi.org/10.1177/1470320313496858>
- Wen, Ager, E. I., Neo, J., & Christophi, C. (2013). The renin angiotensin system regulates Kupffer cells in colorectal liver metastases. *Cancer Biol Ther*, 14(8), 720-727. <https://doi.org/10.4161/cbt.25092>
- Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. <https://ggplot2.tidyverse.org>
- Wiggins, M. G., Shahtahmassebi, G., Malcolm, P., McCormick, F., Aroori, S., Bowles, M. J., & Stell, D. A. (2013). Extended pathology reporting of resection specimens of colorectal liver metastases: the significance of a tumour pseudocapsule. *HPB : the official journal of the International Hepato Pancreato Biliary Association*, 15(9), 687-694. <https://doi.org/10.1111/hpb.12028>
- Williams, B. (2016). Drug discovery in renin-angiotensin system intervention: past and future. *Therapeutic advances in cardiovascular disease*, 10(3), 118-125. <https://doi.org/10.1177/1753944716642680>
- Williford, J. M., Ishihara, J., Ishihara, A., Mansurov, A., Hosseini, P., Marchell, T. M., Potin, L., Swartz, M. A., & Hubbell, J. A. (2019). Recruitment of CD103(+) dendritic cells via tumor-targeted chemokine delivery enhances efficacy of checkpoint inhibitor immunotherapy. *Sci Adv*, 5(12), eaay1357. <https://doi.org/10.1126/sciadv.aay1357>

- Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., & Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science*, 290(5500), 2309-2312. <https://doi.org/10.1126/science.290.5500.2309>
- Wolf, G., & Neilson, E. G. (1990). Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. *American Journal of Physiology-Renal Physiology*, 259(5), F768-F777. <https://doi.org/10.1152/ajprenal.1990.259.5.F768>
- Wong, M. T., Ong, D. E., Lim, F. S., Teng, K. W., McGovern, N., Narayanan, S., Ho, W. Q., Cerny, D., Tan, H. K., Anicete, R., Tan, B. K., Lim, T. K., Chan, C. Y., Cheow, P. C., Lee, S. Y., Takano, A., Tan, E. H., Tam, J. K., Tan, E. Y., Chan, J. K., Fink, K., Bertoletti, A., Ginhoux, F., Curotto de Lafaille, M. A., & Newell, E. W. (2016). A High-Dimensional Atlas of Human T Cell Diversity Reveals Tissue-Specific Trafficking and Cytokine Signatures. *Immunity*, 45(2), 442-456. <https://doi.org/10.1016/j.immuni.2016.07.007>
- Workel, H. H., Komdeur, F. L., Wouters, M. C. A., Plat, A., Klip, H. G., Eggink, F. A., Wisman, G. B. A., Arts, H. J. G., Oonk, M. H. M., Mourits, M. J. E., Yigit, R., Versluis, M., Duiker, E. W., Hollema, H., de Bruyn, M., & Nijman, H. W. (2016). CD103 defines intraepithelial CD8+ PD1+ tumour-infiltrating lymphocytes of prognostic significance in endometrial adenocarcinoma. *European Journal of Cancer*, 60, 1-11. <https://doi.org/10.1016/j.ejca.2016.02.026>
- Wu, J., Akaike, T., Hayashida, K., Miyamoto, Y., Nakagawa, T., Miyakawa, K., Muller-Esterl, W., & Maeda, H. (2002). Identification of bradykinin receptors in clinical cancer specimens and murine tumor tissues. *Int J Cancer*, 98(1), 29-35.
- Wysocki, P. J., Kwiatkowska, E. P., Kazimierczak, U., Suchorska, W., Kowalczyk, D. W., & Mackiewicz, A. (2006). Captopril, an angiotensin-converting enzyme inhibitor, promotes growth of immunogenic tumors in mice. *Clin Cancer Res*, 12(13), 4095-4102. <https://doi.org/10.1158/1078-0432.Ccr-05-2489>
- Xia, A., Zhang, Y., Xu, J., Yin, T., & Lu, X.-J. (2019). T Cell Dysfunction in Cancer Immunity and Immunotherapy [Review]. *Frontiers in Immunology*, 10(1719). <https://doi.org/10.3389/fimmu.2019.01719>
- Xie, G., Liu, Y., Yao, Q., Zheng, R., Zhang, L., Lin, J., Guo, Z., Du, S., Ren, C., Yuan, Q., & Yuan, Y. (2017). Hypoxia-induced angiotensin II by the lactate-chymase-dependent mechanism mediates radioresistance of hypoxic tumor cells [Article]. *Scientific Reports*, 7, 42396. <https://doi.org/10.1038/srep42396> <https://www.nature.com/articles/srep42396#supplementary-information>
- Xing, F., Saidou, J., & Watabe, K. (2010). Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Frontiers in bioscience : a journal and virtual library*, 15, 166-179. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2905156/>

- Xu, J., Fan, J., Wu, F., Huang, Q., Guo, M., Lv, Z., Han, J., Duan, L., Hu, G., Chen, L., Liao, T., Ma, W., Tao, X., & Jin, Y. (2017). The ACE2/Angiotensin-(1–7)/Mas Receptor Axis: Pleiotropic Roles in Cancer. *Frontiers in Physiology*, 8, 276. <https://doi.org/10.3389/fphys.2017.00276>
- Yang, L., DeBusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., Matrisian, L. M., Carbone, D. P., & Lin, P. C. (2004). Expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*, 6(4), 409-421. <https://doi.org/10.1016/j.ccr.2004.08.031>
- Yao, L., Abe, M., Kawasaki, K., Akbar, S. M. F., Matsuura, B., Onji, M., & Hiasa, Y. (2016). Characterization of Liver Monocytic Myeloid-Derived Suppressor Cells and Their Role in a Murine Model of Non-Alcoholic Fatty Liver Disease. *PloS one*, 11(2), e0149948-e0149948. <https://doi.org/10.1371/journal.pone.0149948>
- Yap, A. S., & Kovacs, E. M. (2003). Direct cadherin-activated cell signaling: a view from the plasma membrane. *The Journal of cell biology*, 160(1), 11-16. <https://doi.org/10.1083/jcb.200208156>
- Yona, S., Kim, K. W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guillemins, M., Misharin, A., Hume, D. A., Perlman, H., Malissen, B., Zelzer, E., & Jung, S. (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*, 38(1), 79-91. <https://doi.org/10.1016/j.immuni.2012.12.001>
- Yoshiji, H., Kuriyama, S., Kawata, M., Yoshii, J., Ikenaka, Y., Noguchi, R., Nakatani, T., Tsujinoue, H., & Fukui, H. (2001). The angiotensin-I-converting enzyme inhibitor perindopril suppresses tumor growth and angiogenesis: possible role of the vascular endothelial growth factor. *Clin Cancer Res*, 7(4), 1073-1078.
- Yoshiji, H., Yoshii, J., Ikenaka, Y., Noguchi, R., Yanase, K., Tsujinoue, H., Imazu, H., & Fukui, H. (2002). Suppression of the renin-angiotensin system attenuates vascular endothelial growth factor-mediated tumor development and angiogenesis in murine hepatocellular carcinoma cells. *Int J Oncol*, 20(6), 1227-1231.
- Youn, J.-I., Nagaraj, S., Collazo, M., & Gabrilovich, D. I. (2008). Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*, 181(8), 5791-5802. <https://doi.org/10.4049/jimmunol.181.8.5791>
- Youn, J. I., & Gabrilovich, D. I. (2010). The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *Eur J Immunol*, 40(11), 2969-2975. <https://doi.org/10.1002/eji.201040895>

- Yu, C., Tang, W., Wang, Y., Shen, Q., Wang, B., Cai, C., Meng, X., & Zou, F. (2016). Downregulation of ACE2/Ang-(1-7)/Mas axis promotes breast cancer metastasis by enhancing store-operated calcium entry. *Cancer Lett*, 376(2), 268-277. <https://doi.org/10.1016/j.canlet.2016.04.006>
- Yu, Y.-R. A., O'Koren, E. G., Hotten, D. F., Kan, M. J., Kopin, D., Nelson, E. R., Que, L., & Gunn, M. D. (2016). A Protocol for the Comprehensive Flow Cytometric Analysis of Immune Cells in Normal and Inflamed Murine Non-Lymphoid Tissues. *PloS one*, 11(3), e0150606. <https://doi.org/10.1371/journal.pone.0150606>
- Yusuf, S., Pitt, B., Davis, C. E., Hood, W. B., & Cohn, J. N. (1991). Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *N Engl J Med*, 325(5), 293-302. <https://doi.org/10.1056/nejm199108013250501>
- Zeisberg, M., & Neilson, E. G. (2009). Biomarkers for epithelial-mesenchymal transitions. *The Journal of Clinical Investigation*, 119(6), 1429-1437. <https://doi.org/10.1172/JCI36183>
- Zelenay, S., van der Veen, A. G., Bottcher, J. P., Snelgrove, K. J., Rogers, N., Acton, S. E., Chakravarty, P., Girotti, M. R., Marais, R., Quezada, S. A., Sahai, E., & Reis e Sousa, C. (2015). Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. *Cell*, 162(6), 1257-1270. <https://doi.org/10.1016/j.cell.2015.08.015>
- Zhang, B. Y., Jones, J. C., Briggler, A. M., Hubbard, J. M., Kipp, B. R., Sargent, D. J., Dixon, J. G., & Grothey, A. (2017). Lack of Caudal-Type Homeobox Transcription Factor 2 Expression as a Prognostic Biomarker in Metastatic Colorectal Cancer. *Clin Colorectal Cancer*, 16(2), 124-128. <https://doi.org/10.1016/j.clcc.2016.09.003>
- Zhang, H., Ye, J., Shi, Z., Bu, C., & Bao, F. (2017). Quantitative analyses of the global proteome and phosphoproteome reveal the different impacts of propofol and dexmedetomidine on HT22 cells. *Scientific Reports*, 7(1), 46455. <https://doi.org/10.1038/srep46455>
- Zhang, Z., Procissi, D., Li, W., Kim, D.-H., Li, K., Han, G., Huan, Y., & Larson, A. C. (2013). High resolution MRI for non-invasive mouse lymph node mapping. *Journal of immunological methods*, 400-401, 23-29. <https://doi.org/10.1016/j.jim.2013.06.013>
- Zhao, Y., Chen, X., Cai, L., Yang, Y., Sui, G., & Fu, S. (2010). Angiotensin II/angiotensin II type I receptor (AT1R) signaling promotes MCF-7 breast cancer cells survival via PI3-kinase/Akt pathway. *J Cell Physiol*, 225(1), 168-173. <https://doi.org/10.1002/jcp.22209>

- Zhao, Y., Wu, T., Shao, S., Shi, B., & Zhao, Y. (2016). Phenotype, development, and biological function of myeloid-derived suppressor cells. *Oncoimmunology*, 5(2), e1004983. <https://doi.org/10.1080/2162402x.2015.1004983>
- Zheng, M., Yu, J., & Tian, Z. (2013). Characterization of the liver-draining lymph nodes in mice and their role in mounting regional immunity to HBV. *Cellular & molecular immunology*, 10(2), 143-150. <https://doi.org/10.1038/cmi.2012.59>
- Zheng, S., Yang, Y., Song, R., Yang, X., Liu, H., Ma, Q., Yang, L., Meng, R., Tao, T., Wang, S., & He, J. (2015). Ang-(1-7) promotes the migration and invasion of human renal cell carcinoma cells via Mas-mediated AKT signaling pathway. *Biochem Biophys Res Commun*, 460(2), 333-340. <https://doi.org/10.1016/j.bbrc.2015.03.035>
- Zhou, L., Luo, Y., Sato, S., Tanabe, E., Kitayoshi, M., Fujiwara, R., Sasaki, T., Fujii, K., Ohmori, H., & Kuniyasu, H. (2014). Role of Two Types of Angiotensin II Receptors in Colorectal Carcinoma Progression. *Pathobiology*, 81(4), 169-175. <https://doi.org/10.1159/000362092>
- Zhou, L., Zhang, W., Sun, Y., & Jia, L. (2018). Protein neddylation and its alterations in human cancers for targeted therapy. *Cell Signal*, 44, 92-102. <https://doi.org/10.1016/j.cellsig.2018.01.009>
- Zhu, Q., Li, N., Li, F., Zhou, Z., Han, Q., Lv, Y., Sang, J., & Liu, Z. (2016). Therapeutic effect of renin angiotensin system inhibitors on liver fibrosis. *J Renin Angiotensin Aldosterone Syst*, 17(1), 1470320316628717. <https://doi.org/10.1177/1470320316628717>
- Zou, W. (2006). Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol*, 6(4), 295-307. <https://doi.org/10.1038/nri1806>
- Zuccala, E. (2016). Chemotherapy: Clocking up resistance. *Nat Rev Cancer*, 16(6), 343. <https://doi.org/10.1038/nrc.2016.50>

Supplementary Figures and Tables

Supplementary figures- Dora et. al (2020) (Chapter 3): **Fig.S1** Histological quantitation strategy CD3⁺ and CD4⁺. **Fig.S2** Gating strategy for analysis of lymphocyte subpopulations. **Fig.S3** Captopril treatment effect the number and size of metastatic foci. **Fig.S4** Correlation matrix using Pearson r to determine the distribution of tumour infiltrating. **Supplementary Table 1.** FACS panel used in this study.

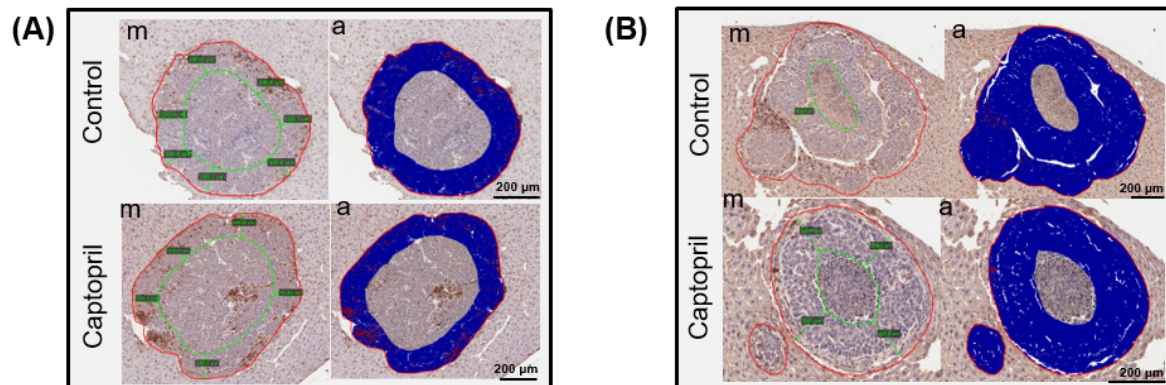


Fig. S1 Histological quantitation strategy. (A) representative microphotographs of IHC CD3 staining; (B) representative microphotographs of IHC CD4 staining; marked-up (m) and annotated (a) tumour margin (red line) and intratumoral (green line) distribution at day 15 post-tumour induction (Scale bar = 200 µm).

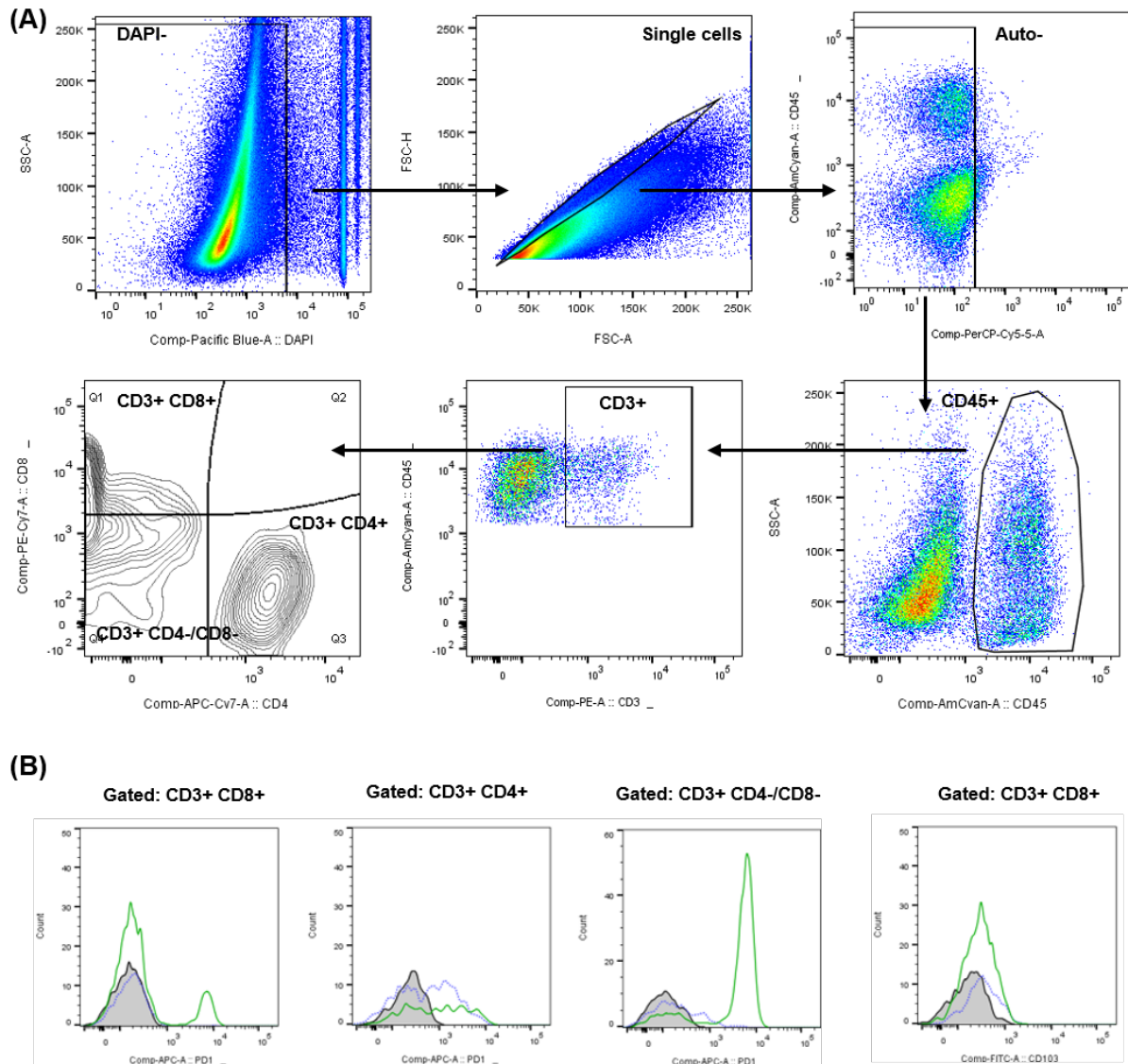


Fig. S2 Gating strategy for analysis of lymphocyte subpopulations. (A) Viability, single cells and autofluorescence were used as optimising strategies to analyse the CD3+ lymphocyte subpopulations within the total CD45+ leukocyte population in the liver (example given) and tumour. (B) The expression of PD1 and CD103 on gated lymphocyte subpopulations on tissues from captopril treated (green line), saline-treated (blue dash) and unstained for PD1 and CD103 controls (grey fill).

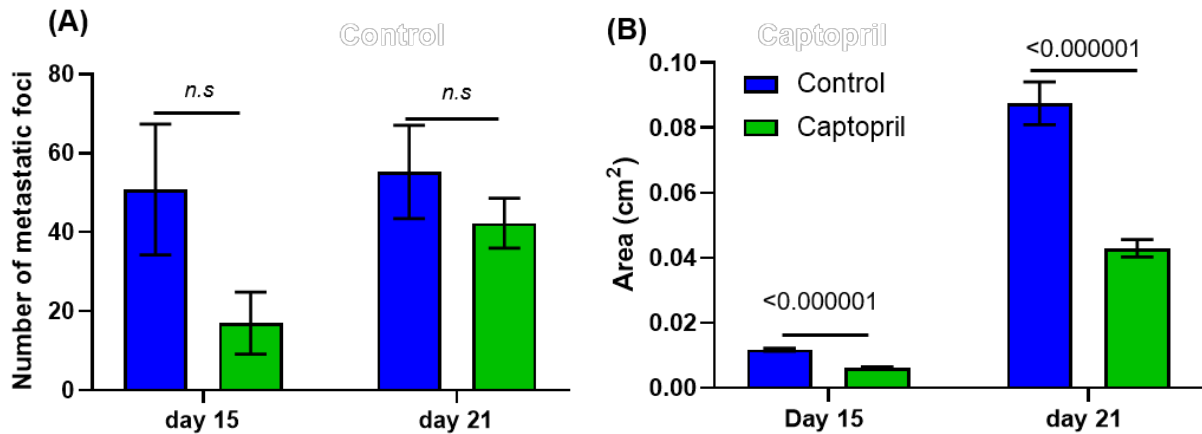


Fig.S3 Captopril treatment effect the number and size of metastatic foci. (A) Number of metastatic foci in the control and captopril treatment group at day 15 and day 21. (B) Tumour region area (cm) of control and captopril treated group at day 15 and day 21. Datasets expressed as mean $\pm$ SEM with $n \geq 5$ mice for each group.

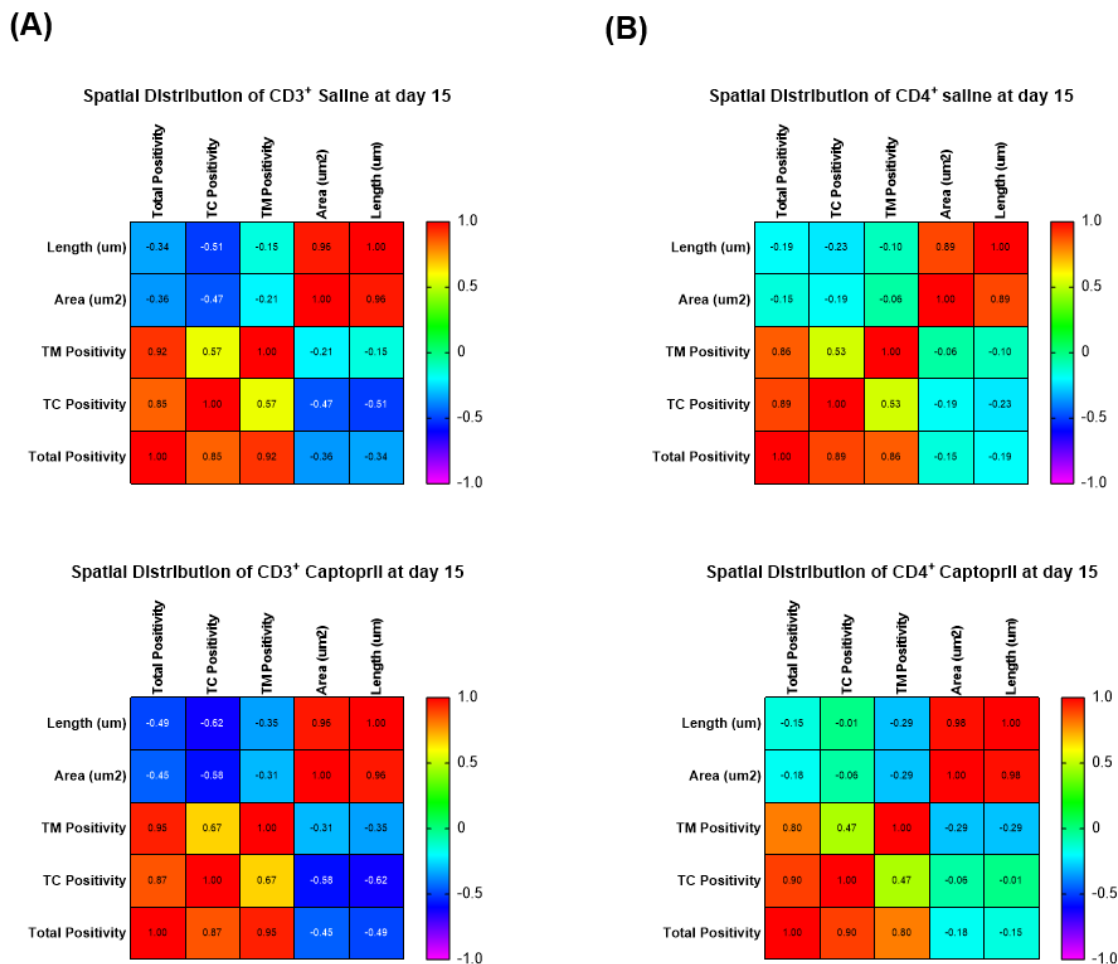


Fig. S4 Correlation matrix using Pearson r to determine the distribution of tumour infiltrating. (A) CD3⁺ and, (B) CD4⁺ lymphocytes within inner tumour invasive margin and intratumorally. Coefficient r (or rs) is shown for each pair of variables: length (μ m), area (μ m²), tumour margin (TM) positivity, tumour core (TC) positivity and total positivity. A heat map of R2 calculating P values (two-tail) was generated. Datasets expressed as mean $\pm$ SEM with $n \geq 5$ mice for each group (equivalent >100 tumour regions in each group) excluding tumour region values outliers ROUT (Q=1%). Significantly different data is represented by * ($p < 0.05$)

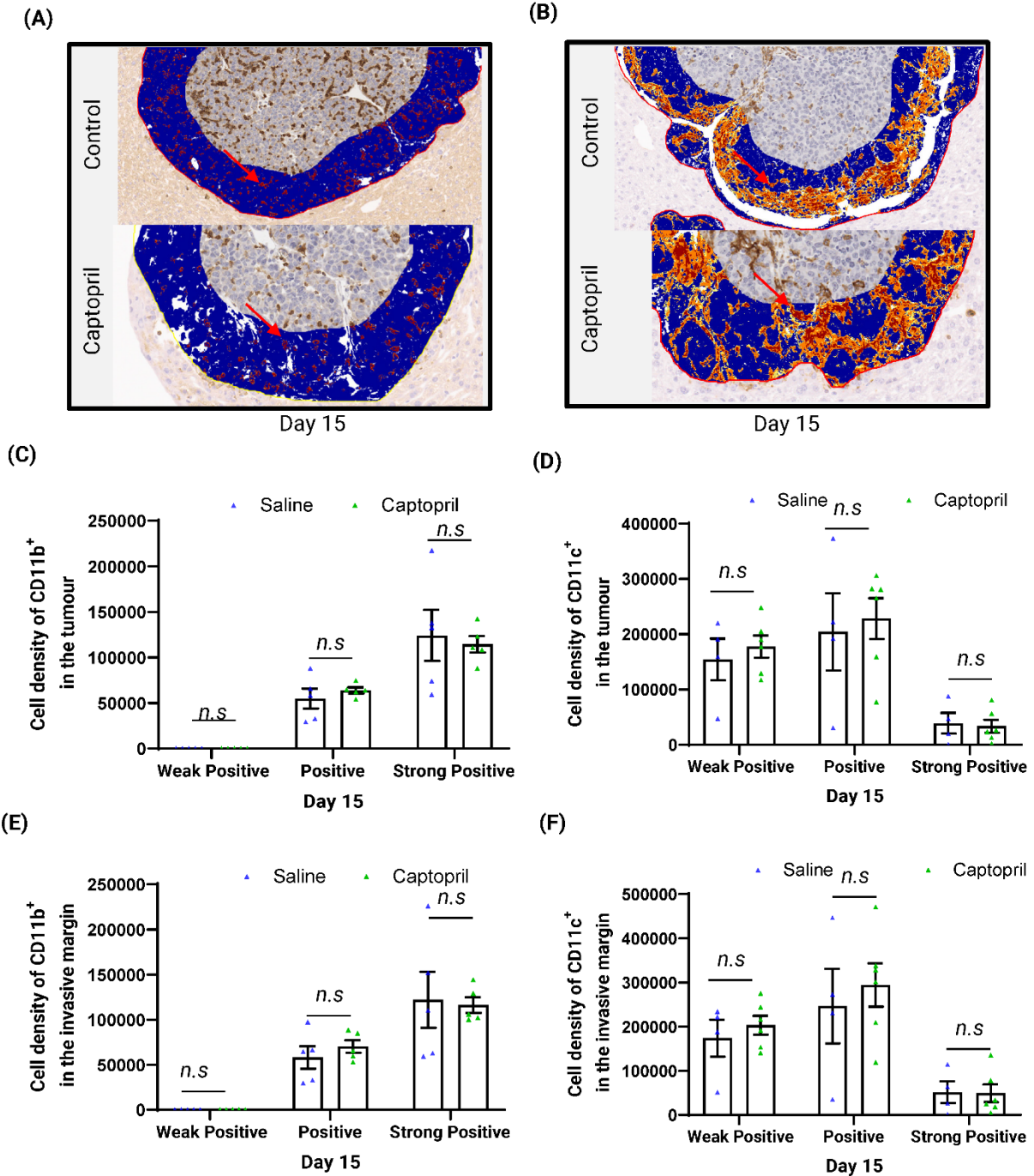


Fig.S5 Histological quantitation of CD11b⁺ and CD11c⁺. Representative microphotographs of IHC annotated of the tumour margin compared to the total area of the tumour of (A) CD11b⁺ and (B) CD11c⁺ at day 15 post-tumour induction (Scale bar = 100 μ m measured from the edge of the tumour in direction to the core). The cell density of CD11b⁺ and CD11c⁺ measured by the intensity of expression as follows: weak positive (in yellow), positive (in orange), and strong positive (in red) in (C-D) the total area of the tumour and (E-F) in the invasive tumour margin.

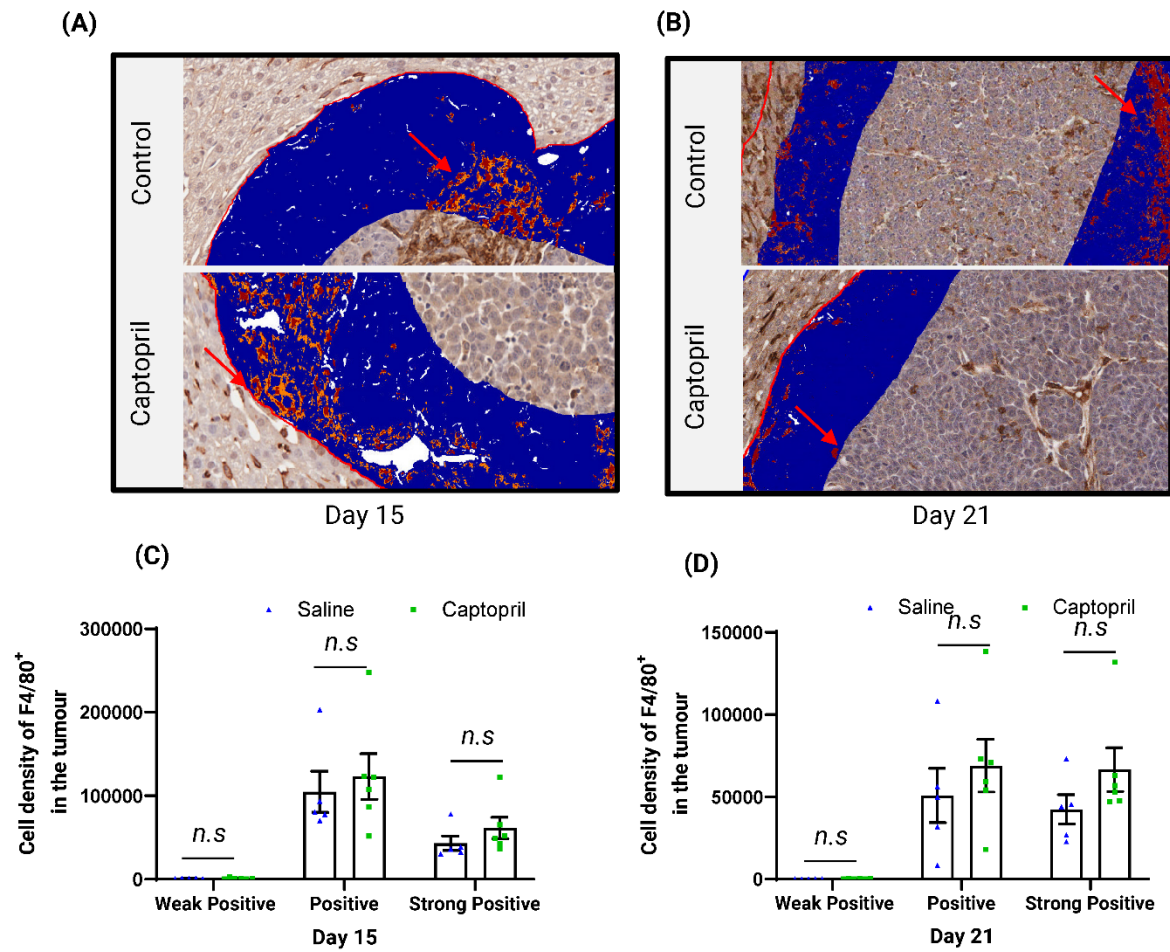


Fig.S6 Histological quantitation of F4/80⁺. Representative microphotographs of IHC annotated of the tumour margin compared to the total area of the tumour of F4/80⁺ (A) at day 15 and (B) day 21 post-tumour induction (Scale bar = 100 μ m measured from the edge of the tumour in direction to the core). The cell density of F4/80⁺ measured by the intensity of expression as follows: weak positive (in yellow), positive (in orange), and strongly positive (in red) in the total area of the tumour (C) at day 15 and (D) day 21 post-tumour induction.

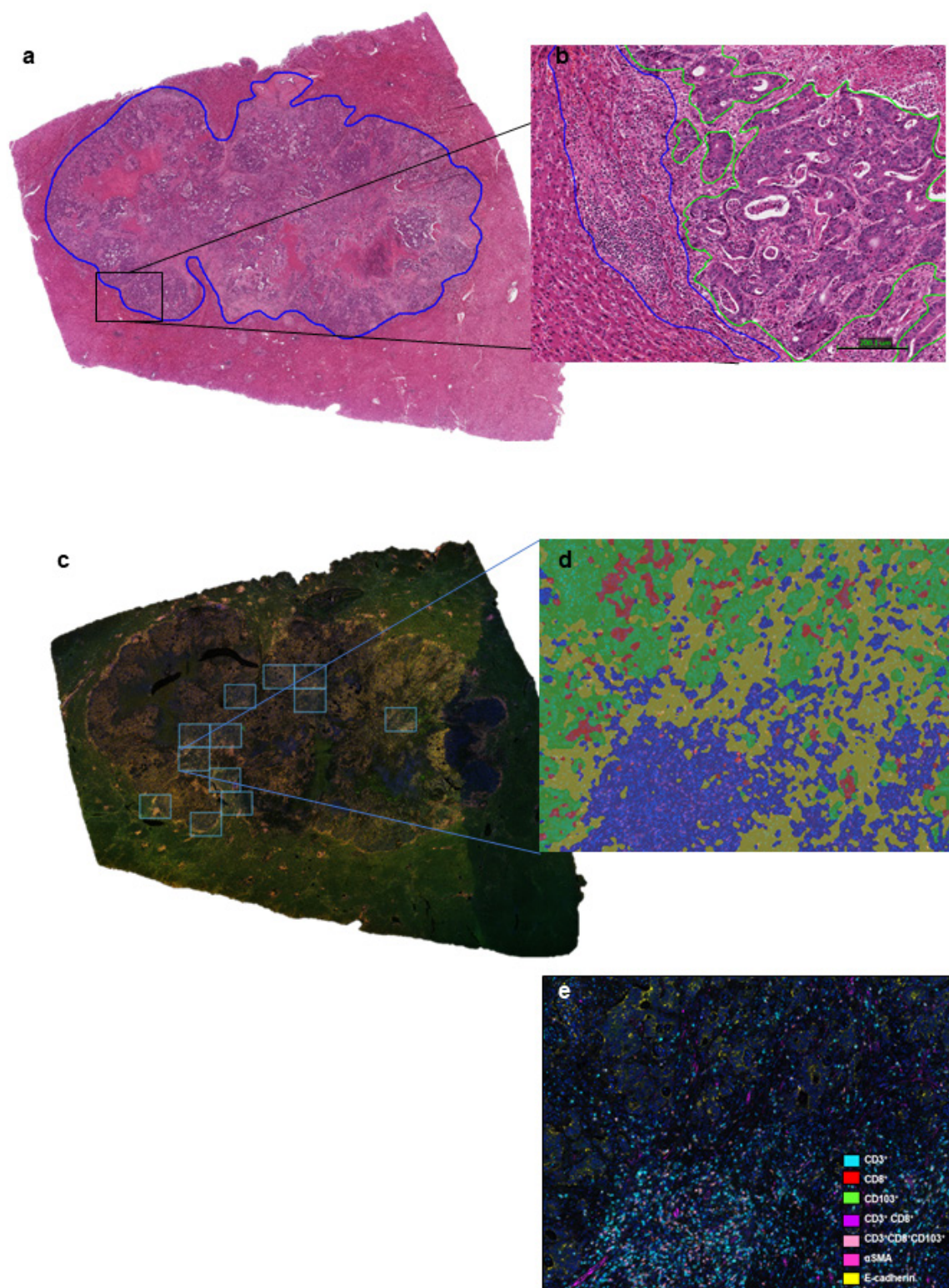


Fig.S7 Methodology for the spatial delineation of tumour regions and spatial immune profiling in CLM. H&E staining was used to (A) identify the tumour position within the liver (B) the tumour invasive margin, and to select areas of high T lymphocyte cell density for image acquisition. (C) OPAL-stained FFPE whole tissue sections (Phenochart) were used to characterize the distinct immune profile of CLM. (D) Using the low power image (10X), an average of 10 high-powered fields (HPF) images were selected from each CLM (HPF is equivalent to fixed-stamp size $3 \times 3 = 2046 \times 1530 \mu\text{m}$, resolution = $0.5 \mu\text{m}$ equivalent to 1 pixel, x 20 magnification). (E) A panel of immune cell and EMT markers were optimized for this study including T Lymphocytes (CD3^+), CD8^+ T cells ($\text{CD3}^+\text{CD8}^+$), T_{RM} ($\text{CD3}^+\text{CD8}^+\text{CD103}^+$), and CD8^+ and CD103^+ populations that did not co-express CD3^+ , $\alpha\text{-SMA}$ and E-cadherin.

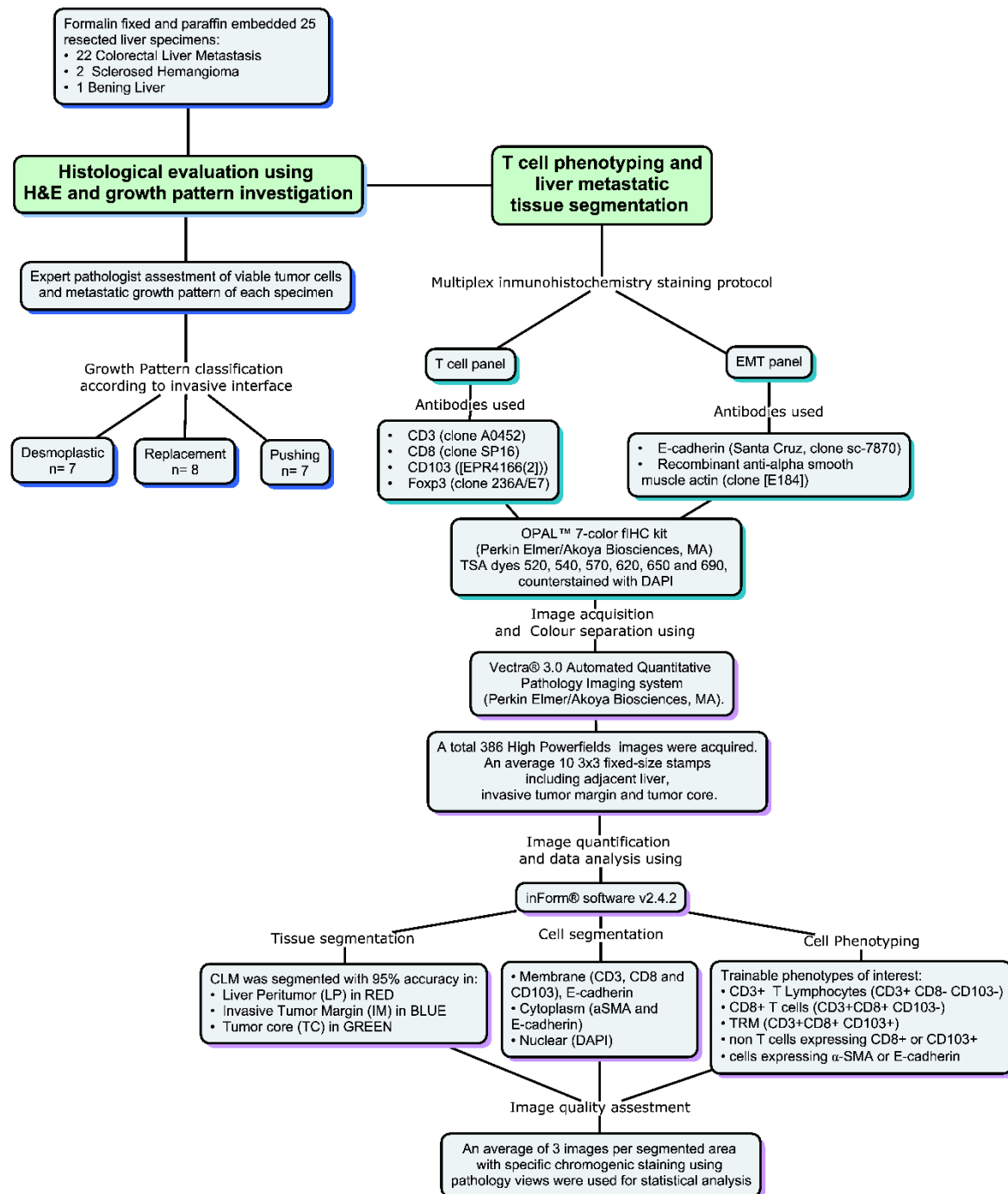


Fig.S8 Study flow diagram for analysis of the tumour microenvironment in Colorectal Liver Metastasis using automated Vectra® imaging and inForm® tissue segmentation and cell phenotyping to determine the immune infiltrate profiles using multiplex immunohistochemistry.

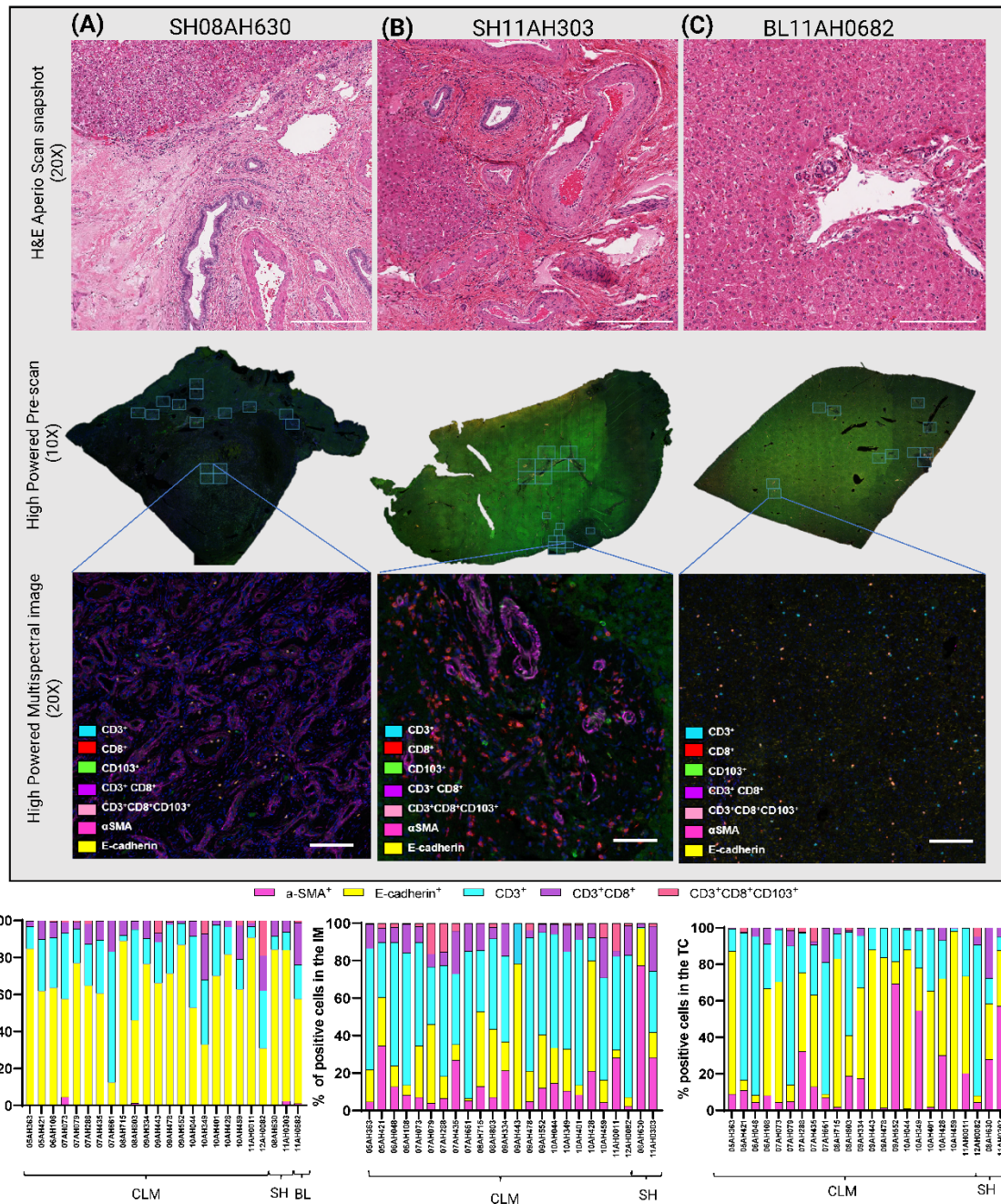


Fig.S9 Individual patient's immune profiles were compared with sclerosed hemangioma and normal liver. To optimize the antibody detection of CD3, CD8, CD103, αSMA, and E-cadherin using the trainable tissue segmentation we compared the percentage of individual immune profiles using a single marker and combination of them to characterize T lymphocyte subtypes in the liver peritumoral (LP), invasive margin (IM) and tumour core (TC). Representative microphotographs of the tissue sections stained with H&E (scale bar: 200μm) and OPAL-stained (scale bar: 100μm) of (A-B) sclerosed hemangioma (SH) and (C) benign liver (BL) were used as technical and biological control. (D) Individual immune profiles for each CLM patient (n= 22).

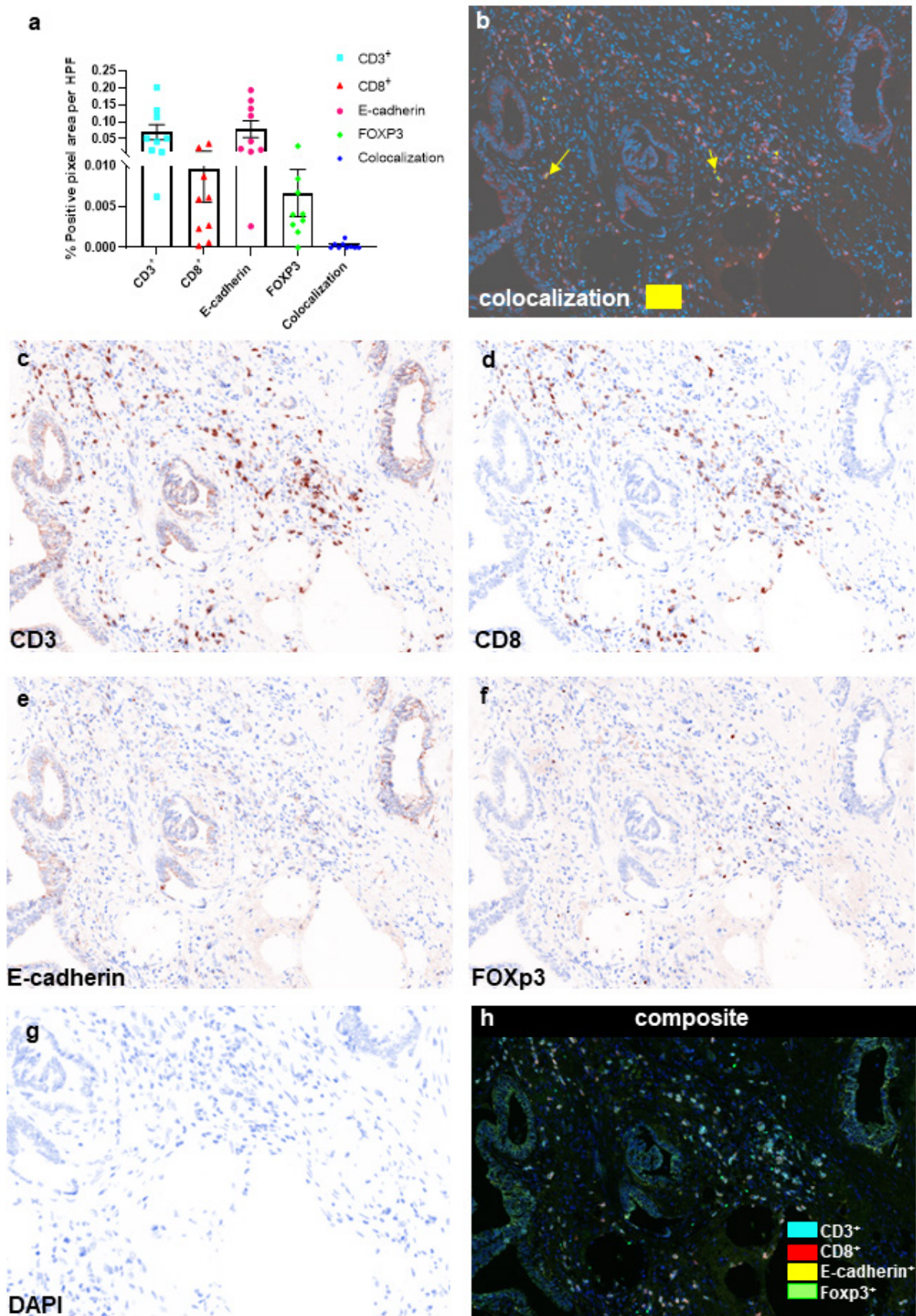


Fig.S10 Colocalization analysis of CD3⁺, CD8⁺, E-cadherin and FOXP3⁺. (A) inForm® was used to calculate the percentage of co-expression of all four markers in a subset of CLM (n=6). (B) The colocalization map indicates in yellow the position of the co-expression of all four markers. Pathology views analysis of (C) CD3, (D) CD8, (E) E-cadherin (F) FOXP3⁺ and (G) DAPI counterstained.

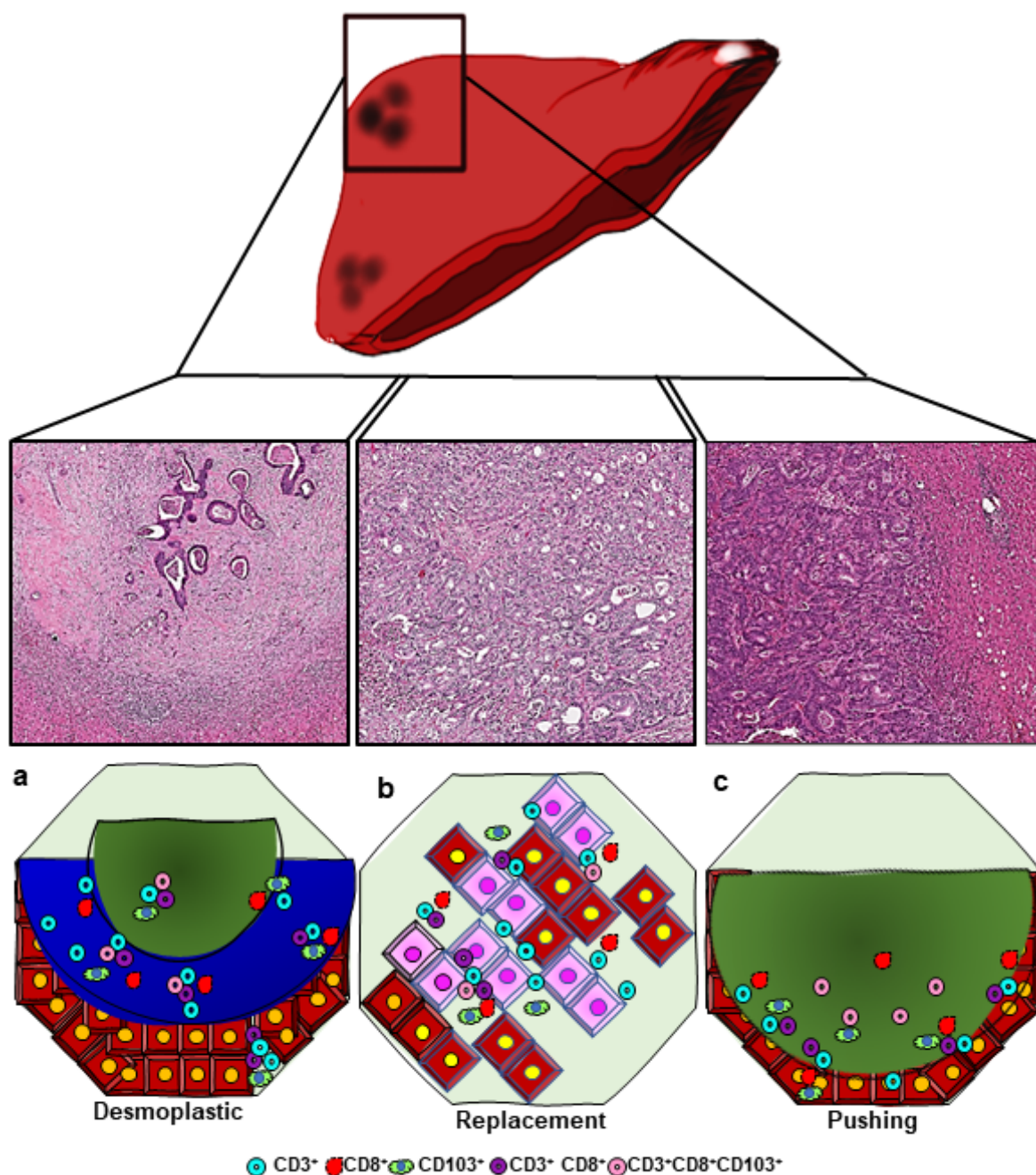


Fig.S11 Schematic representation of the 3 dominant histologic growth pattern of Colorectal Liver Metastases (CLM) and the spatial distribution of distinct immune cells. The histo-morphological characteristics of growth pattern and the proposed location of tumour infiltrating population. Adjacent liver parenchyma in red (LP), invasive tumour margin in blue (IM) and tumour core (TC) in green. Representative microphotographs of (A) desmoplastic, (B) replacement, and (C) pushing growth pattern are shown.

Supplementary Table 1. FACS panel for mouse studies.

CD marker	fluorophore	clone	Brand	cat #
		145-		
CD3e	PE	2c11	BD pharmingen™	553064
CD4	APC cy7	GK1.5	BD pharmingen™	561830
CD8a	PE cy7	53-67	BD Biosciences	561097
CD103	FITC	2 E 7	Miltenyi Biotec	130-118-681
CD279 (PD1)	APC	REA802	Miltenyi Biotec	130-111-801
CD45	BV510	30-F11	BD Horizon	563891
CD16/32	pure	clone93	Miltenyi Biotec	130-092-574

Supplementary Table 2. Descriptive statistics of T Lymphocytes (CD3⁺T-cells), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), T_{RM} (CD3⁺CD8⁺ CD103⁺), and non-lymphocytes expressing CD8⁺ or CD103⁺ in the CLM cohort (cell count per HPF).

Cell phenotype	CD3 ⁺ T-cells		CD8 ⁺ (CD3 ⁻)		CD8 ⁺ T-cells		T _{RM}		CD103 ⁺ (CD3 ⁻)	
Tissue Segmentation	IM	TC	IM	TC	IM	TC	IM	TC	IM	TC
Number of values	22	22	22	22	22	22	22	22	22	22
Minimum	74.33	31.33	5	0	0	0.3333	0	0	0	0
25% Percentile	879.5	185.5	126	25.75	124.5	5.917	4.833	0	2.583	1.75
Median	1149	379.8	176.8	143.5	241.2	15.17	20.67	1	44	76.67
75% Percentile	2008	679.8	315.3	305.3	395.3	75.25	122.9	5.25	194.7	235.4
Maximum	2888	3730	691.7	966	628.3	969.3	600.3	158.3	688.7	603
Range	2814	3699	686.7	966	628.3	969	600.3	158.3	688.7	603
Mean	1348	669	252.6	221.3	261.9	80.7	82.81	11.65	120.4	142.8
Std. Error of Mean	169	191.7	42.41	58.01	37.99	43.66	30.04	7.343	37.48	39.21

Supplementary Table 3. Survival information of the CLM study cohort.

Vital Status						Follow-up (years)		
Specimen ID	Vital Status	Date of Death	Last up-date	Date of Surgery	Cause of Death	years	month	days
05AH363	D	26/08/2010		27/10/2005	Cancer Rectum, Nos	4	57	1764
05AH421	A	-	1/09/2019	1/12/2005	-	13	165	5022
06AH048	D	19/03/2007		15/02/2006	Cancer Rectosigmoid Junction	1	13	397
06AH108	A	-	1/09/2019	5/04/2006	-	13	160	4897
07AH073	D	27/08/2013		1/03/2007	Cancer Rectum, Nos	6	77	2371
07AH079	A	-	1/09/2019	2/03/2007	-	12	149	4566
07AH288	A	-	1/09/2019	27/06/2007	-	12	146	4449
07AH435	A	-	1/09/2019	28/08/2007	-	12	144	4387
07AH661	A	-	1/09/2019	17/12/2007	-	11	140	4276
08AH715	D	7/02/2014		13/11/2008	Cancer Rectum, Nos	5	62	1912
08AH803	D	19/11/2012		10/12/2008	Cancer Rectum, Nos	3	47	1440
09AH334	D	5/08/2012		3/06/2009	Cancer Rectosigmoid Junction	3	38	1159
09AH443	D	9/01/2010		22/07/2009	Cancer Sigmoid Colon	0	5	171
09AH478	D	22/06/2012		4/08/2009	Cancer Colon, Nos	2	34	1053
09AH552	D	2/08/2011		1/09/2009	Cancer Splenic Flexure Colon	1	23	700
10AH044	D	7/10/2011		29/01/2010	Cancer Transverse Colon	1	20	616
10AH349	A	-	1/09/2019	1/06/2010	-	9	111	3379
10AH401	A	-	1/09/2019	12/11/2009	-	9	117	3580
10AH428	D	21/11/2013		1/07/2010	Cancer Rectum, Nos	3	40	1239
10AH459	D	26/11/2011		1/07/2010	Cancer Transverse Colon	1	16	513
11AH0011	D	13/03/2012		1/06/2009	Cancer Ascending Colon	2	33	1016
12AH0082	A	-	1/09/2019	14/03/2012	-	7	89	2727

Supplementary Table 4. Survival information of the control and RASi CLM study cohort.

Group	Specimen ID	Vital Status	Date of Death	Last up-date	Date of Surgery	Cause of Death	Month of follow-up
Control	05AH421	A	-	1/09/2019	1/12/2005	n/a	165
	06AH048	D	19/03/2007		15/02/2006	Cancer Rectosigmoid Junction	13
	06AH108	A	-	1/09/2019	5/04/2006	n/a	160
	07AH079	A	-	1/09/2019	2/03/2007	n/a	149
	07AH435	A	-	1/09/2019	28/08/2007	n/a	144
	07AH661	A	-	1/09/2019	17/12/2007	n/a	140
	08AH803	D	19/11/2012		10/12/2008	Cancer Rectum, Nos	47
	09AH443	D	9/01/2010		22/07/2009	Cancer Sigmoid Colon	5
	09AH478	D	22/06/2012		4/08/2009	Cancer Colon, Nos	34
	09AH552	D	2/08/2011		1/09/2009	Cancer Splenic Flexure Colon	23
	10AH044	D	7/10/2011		29/01/2010	Cancer Transverse Colon	20
	10AH349	A	-	1/09/2019	1/06/2010	n/a	111
	10AH401	A	-	1/09/2019	12/11/2009	n/a	117
	10AH428	D	21/11/2013		1/07/2010	Cancer Rectum, Nos	40
	10AH459	D	26/11/2011		1/07/2010	Cancer Transverse Colon	16
	11AH0011	D	13/03/2012		1/06/2009	Cancer Ascending Colon	33
	12AH0082	A	-	1/09/2019	14/03/2012	n/a	89
RASi	05AH0363	D	1/08/2010		1/10/2005	Neoplasm of rectum	58
	08AH0715	D	1/01/14		1/11/2008	Neoplasm of rectum	62
	09AH0688	D	1/01/2012		1/10/2009	Neoplasm of rectum	27
	13AH0103	D	1/09/2015		1/03/2013	Neoplasm of Ascending Colon	30
	07AH0073	D	1/08/2013		1/03/2007	Neoplasm of rectum	77
	09AH0312	D	1/03/2010		1/05/2009	Neoplasm of Sigmoid colon	10
	07AH0288	A	n/a	1/09/2019	1/10/2007	n/a	143
	09AH0334	D	1/08/2012		1/06/2009	Neoplasm of rectosigmoid junction	38
	10AH0121	D	1/04/2017		1/02/2010	Neoplasm of Colon	86
	12EH0060	A	n/a	1/09/2019	1/05/2012	n/a	88
	09WH0105	D	1/01/2010		1/06/2009	Neoplasm of Sigmoid colon	7
	11MH0108	A	n/a	1/09/2019	1/02/2011	n/a	103
	11WH0134	D	1/07/2015		1/05/2011	Neoplasm of Sigmoid colon	50
	09WH077	D	1/04/2014		1/05/2009	Neoplasm of Sigmoid colon	59
	11MH0179	A	n/a	1/09/2019	1/03/2011	n/a	102
	12MH0607	A	n/a	1/09/2019	1/06/2012	n/a	87
	10PM0199	A	n/a	1/09/2019	1/02/2010	n/a	115

	11CH061	D	1/09/2014		1/03/2011	Neoplasm of Transverse colon	42
	09CH0160	A	n/a	1/09/2019	1/10/2009	n/a	119
	09CH227	A	n/a	1/09/2019	1/10/2009	n/a	119
	11CH111	A	n/a	1/09/2019	1/05/2011	n/a	100

APPENDIX 1

Vallejo-Ardila, D. L., Fifi, T., Burrell, L. M., Walsh, K., & Christophi, C. (2018). Renin-angiotensin inhibitors reprogram tumour immune microenvironment: A comprehensive view of the influences on anti-tumour immunity. *Oncotarget*, 9(84), 35500-35511.

Due to copyright obligations, the author accepted version of this manuscript is presented in Appendix 1.

The published version of this manuscript can be found at <https://doi.org/10.18632/oncotarget.26174>

APPENDIX 2

Vallejo Ardila, D. L., Walsh, K. A., Fifis, T., Paolini, R., Kastrappis, G., Christophi, C., & Perini, M. V. (2020, May). Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases. *J Immunother Cancer*, 8(1).

Due to copyright obligations, the author accepted version of this manuscript is presented in Appendix 2.

The published version of this manuscript can be found at <https://doi.org/10.1136/jitc-2019-000487>

APPENDIX 3

Multiplex Immunohistochemistry protocol in formalin-fixed, paraffin-embedded Colorectal Liver Metastases patient tissues and Machine Learning- based Tissue Segmentation and Cell Phenotyping analysis

Method Article

APPENDIX 4

R code: MapPhenotypes

```
# Package Install/Load
#####

setwd("Dropbox/Dora_Vallejo/Project_2020_1")
library(spatstat)
library(tiff)
library(ISAT)
library(parallel)

#####
# Custom Functions
#####
#####

# idCell -- identifies unique cells by xy coordinates
# ARGUMENTS -- xy_data (data.frame)
#####

idCell <- function(xy_data) {
  location_split <- split(x = xy_data, f = xy_data$location_id)
  for(cell in 1:length(location_split)) {
    cellname <- paste("cell", cell, sep = "")
    location_split[[cell]] <- data.frame("cellid" = cellname,
                                         location_split[[cell]],
                                         stringsAsFactors = F)
  }
  xy_data <- do.call("rbind", location_split)
  row.names(xy_data) <- NULL
  return(xy_data)
}
#####
```



```

# mapLabel -- maps labels to sets of markers
# ARGUMENTS -- xy_data (data.frame); labelList (named list)
# - labelList: named list of named logical vectors. The name of each list element is
#   the label to assign. The names of the logical elements are the marker sets that
#   describe
#   the label.
#####
mapLabel <- function(xy_data, labelList) {
  markers_list <- split(x = xy_data, f = xy_data$Region.Type)
  names(markers_list) <- make.names(names(markers_list))
  location_ids <- sapply(markers_list, function(x) x$location_id)
  location_ids <- unlist(location_ids)
  location_ids <- unique(location_ids)
  locationMatrix <- matrix(data = FALSE, nrow = length(location_ids), ncol =
length(markers_list),
                        dimnames = list("row" = location_ids,
                        "col" = names(markers_list)))
  locationMatrix <- data.frame(locationMatrix, stringsAsFactors = F)
  for(r in row.names(locationMatrix)) {
    for(c in names(markers_list)) {
      pos_eval <- any(markers_list[[c]]$location_id %in% r)
      if(pos_eval) {
        pos_int <- which(markers_list[[c]]$location_id %in% r)
        locationMatrix[r,c] <- TRUE
        locationMatrix[r,"cellid"] <- markers_list[[c]][pos_int,"cellid"]
      }
    }
  }
  locationMatrix_logic <- locationMatrix[,-ncol(locationMatrix)]
  locationMatrix$label <- NA
  for(label in 1:length(labelList)) {
    for(r in row.names(locationMatrix)) {

```

```

        markers <- labelList[[label]]
        markers_ind <- which(names(locationMatrix_logic) %in%
names(markers))
        if(!is.empty(markers_ind)) {
            rowlocation_info <- unlist(locationMatrix_logic[r,])
            ev1 <- any(rowlocation_info[-markers_ind])
            if(!ev1) {
                ev2 <- all(rowlocation_info[markers_ind])
                if(ev2){
                    locationMatrix[r,"label"] <- names(labelList)[label]
                }
            }
        }
    }
}
xy_labeltype <- merge(xy_data, locationMatrix)
return(xy_labeltype)
}

```

```
#####
```

```
# reduceToUniqueCell – reduce coordinate data to unique cell id
```

```
# ARGUMENTS -- xy_data (data.frame)
```

```
#####
```

```

reduceToUniqueCell <- function(xy_data) {
    id_split <- split(x = xy_data, f = xy_data$cellid)
    cells_to_reduce <- which(sapply(id_split, nrow) > 1)
    for(cell in cells_to_reduce) {
        new_elements <- sapply(id_split[[cell]][,c("cellmarkerid", "Region.Type")],
function(c) {
            col_element <- unique(c)
            if(length(c) > 1) {
                paste(col_element, collapse = "|")
            }
        })
    }
}

```

```

    }
  })
  nr <- nrow(id_split[[cell]][,c("cellmarkerid", "Region.Type")])
  for(r in 1:nr) {
    id_split[[cell]][r,c("cellmarkerid", "Region.Type")] <- new_elements
  }
  id_split[[cell]] <- unique(id_split[[cell]])
}
xy_reduced <- do.call("rbind", id_split)
return(xy_reduced)
}

#####
# Load Data
#####
## file paths
xy_folder <- paste(getwd(), "Dataset1_Coloc_XY", sep = "/")
xy_files_paths <- paste(xy_folder, dir(xy_folder), sep = "/")
names(xy_files_paths) <- gsub(pattern = "_quant_data.txt", replacement = "", x =
basename(xy_files_paths))
## read data and subset
xy_files <- mclapply(xy_files_paths, read.delim2, header = T, sep = "\t",
stringsAsFactors = F, mc.cores = 4)
xy_filesList <- mclapply(xy_files, function(x) {
  x <- x[,c("Sample.Name",
            "Region.Type",
            "Region.X.Position",
            "Region.Y.Position")]
  unique(x)
}, mc.cores = 4)
xy_filesList <- mclapply(xy_filesList, function(x) {
  marker <- strsplit(x = x$Region.Type, split = " ")

```

```

    marker <- sapply(marker, "[", 1)
    x$Region.Type <- marker
    return(x)
})
## split by marker
split_data <- mclapply(xy_filesList, function(x) {
    split(x = x, f = x$"Region.Type")
}, mc.cores = 4)
split_data <- mclapply(split_data, function(x) {
    ev <- sapply(x, nrow) > 1
    x[ev]
})
split_data <- mclapply(split_data, function(x) {
    lapply(x, function(y) {
        location_id <- apply(y[,c("Region.X.Position", "Region.Y.Position")], 1, paste,
collapse = "_")
        data.frame("location_id" = location_id, y, stringsAsFactors = F)
    })
}, mc.cores = 4)
split_data <- mclapply(split_data, function(x) {
    lapply(x, function(y) {
        cellid <- paste(paste("cell", 1:nrow(y), sep = ""), y$Region.Type, sep = "_")
        data.frame("cellmarkerid" = cellid, y, stringsAsFactors = F)
    })
}, mc.cores = 4)

#####
# Add Unique CellId
#####
xyData <- mclapply(split_data, function(x) {
    do.call("rbind", x)
}, mc.cores = 4)

```

```

xyData <- mclapply(xyData, idCell, mc.cores = 4)

#####
# Add Cell Phenotype
#####
phenotypeList <- list("Total_T_Lymphocytes" = c("CD3" = T),
  "Cytotoxic_T_Cells" = c("CD3" = T, "CD8" = T),
  "Tissue_Resident_Cells" = c("CD3" = T, "CD8" = T, "CD103" = T),
  "Plus_alpha_sma" = c("aSMA" = T),
  "Cell.to.cell_junction" = c("E.cadherine" = T),
  "Total_CD8" = c("CD8" = T),
  "Total_CD103" = c("CD103" = T),
  "E.cadherine_cytotoxic" = c("CD3" = T, "CD8" = T, "E.cadherine" = T))
xyData <- mclapply(xyData, mapLabel, labelList = phenotypeList, mc.cores = 4)
xyDataReduced <- mclapply(xyData, reduceToUniqueCell, mc.cores = 4)

#####
# Save Mapped Tables
#####
ndir <- paste (getwd(), "MappedTables", sep = "/")
if (!dir.exists(ndir)) {
  dir.create(ndir)
}
for (file in names (xyDataReduced)) {
  fname <- paste(ndir, "/", file, ".txt", sep = "")
  write.table(x = xyDataReduced[[file]], file = fname, sep = "\t", row.names = F)
}

```

R code: Nearest Neighbor

```
#####  
# Package Install/Load  
#####  
setwd("Dropbox/Dora_Vallejo/Project_2020_1")  
library(spatstat)  
library(tiff)  
library(ISAT)  
library(parallel)  
library(reshape2)  
library(ggplot2)  
  
#####  
# Custom Functions  
#####  
#####  
# cell.to.groupDistances -- calculates distances of cells to groups of other cells  
# ARGUMENTS -- xy_data (data.frame); labelCol (character); xpositionCol (character);  
# ypositionCol (character); cellidCol (character); dist_threshold (numeric)  
#####  
cell.to.groupDistances <- function(xy_data,  
                                   labelCol,  
                                   xpositionCol,  
                                   ypositionCol,  
                                   cellidCol,  
                                   dist_threshold = 30  
) {  
  markers_list <- split(x = xy_data, f = xy_data[,labelCol])  
  marker_comb <- NULL  
  for(m in names(markers_list)) {
```

```

marker_comb      <-      rbind(marker_comb,      expand.grid(m,
names(markers_list)[!names(markers_list)%in%m]))
}
marker_comb <- as.matrix(marker_comb)

distance_MatrixList <- list()
for(i in 1:nrow(marker_comb)) {
  pair <- marker_comb[i,]
  marker_1 <- markers_list[[pair[1]]]
  marker_2 <- markers_list[[pair[2]]]
  distance_measure <- NULL
  for(p1 in 1:nrow(marker_1)) {
    distance_measure <- rbind(distance_measure,
                              findNN(cell = marker_1[p1,],
                                      cellList = marker_2,
                                      XY_LABELS = c(xpositionCol,
                                                    ypositionCol)))
  }
  distance_measure <- distance_measure[,c(cellidCol, "distance")]
  distance_measure <- data.frame(marker_1[,cellidCol],
                                distance_measure[,c(cellidCol)],
                                distance_measure[,c("distance")],
                                stringsAsFactors = F)
  names(distance_measure) <- c(paste(cellidCol, pair[1], sep = "_"),
                              paste(cellidCol, pair[2], sep = "_"),
                              "distance")

  pair_id <- paste(pair, collapse = "_to_")
  distance_MatrixList[[pair_id]] <- distance_measure
}
pair_id <- names(distance_MatrixList)
distance_Matrix <- lapply(pair_id, function(x, mtx) {

```

```

        data.frame("pair_distance_label" = x, "distance" = mtx[[x]][,"distance"],
stringsAsFactors = F)
    }, mtx = distance_MatrixList)
    distance_Matrix <- do.call("rbind", distance_Matrix)

    distances_summarystats <- lapply(distance_MatrixList, function(x,
dist_threshold) {
        procDist(distResult = x, thred = dist_threshold)
    }, dist_threshold = dist_threshold)
    distances_summarystats <- do.call("rbind", distances_summarystats)
    distances_summarystats <- data.frame("cell.to.group_distancepair" =
row.names(distances_summarystats),
        distances_summarystats,
        stringsAsFactors = F)
    distances_summarystats$groupSize <- sapply(distance_MatrixList, nrow)

    distanceResultsList <- list("distances_MatrixList" = distance_MatrixList,
        "distance_Matrix" = distance_Matrix,
        "distances_SummaryTable" = distances_summarystats)
    return(distanceResultsList)
}

#####
# mergeSummaryVar -- returns a matrix of merged summary values from all samples
# obtained from 'distances_SummaryTable'
# ARGUMENTS -- summary_list (list); summary_variable (character)
#####
mergeSummaryVar <- function(summary_list, summary_variable = "Median") {
    summary_variable <- match.arg(arg = summary_variable, choices =
c("Percentage", "Mean", "Std.Dev", "Median", "groupSize"),
        several.ok = F)
    var_list <- lapply(summary_list, function(x, variable) {

```



```

        v <- x[,variable]
        names(v) <- x[,"cell.to.group_distancepair"]
        return(v)
    }, variable = summary_variable)
    p <- lapply(summary_list, function(x) x[,"cell.to.group_distancepair"])
    p <- do.call("c", p)
    p <- unique(p)
    var_matrix <- matrix(data = NA, nrow = length(summary_list), ncol = length(p),
                        dimnames = list("row" = names(summary_list), "col" = p))
    for(i in names(summary_list)) {
        var_matrix[i, names(var_list[[i]])] <- var_list[[i]]
    }
    return(var_matrix)
}

#####

# Load Data

#####

## file paths
xy_folder <- paste(getwd(), "MappedTables", sep = "/")
xy_files_paths <- paste(xy_folder, dir(xy_folder), sep = "/")
names(xy_files_paths) <- gsub(pattern = ".txt", replacement = "", x =
basename(xy_files_paths))
## read data and subset
xy_files <- mclapply(xy_files_paths, read.delim2, header = T, sep = "\t",
stringsAsFactors = F, mc.cores = 4)
xy_filesList <- mclapply(xy_files, function(x) {
    na.omit(x)
}, mc.cores = 4)

#####

# Compute Distances

```

```
#####

d_cutoff <- c(10, 20, 30, 60) ## in pixels
names(d_cutoff) <- d_cutoff*0.5 ## in micrometers (1 pixel = 0.5 micrometers)
fileDistances <- list()
for(d in 1:length(d_cutoff)){
  fileDistances[[names(d_cutoff)[d]]] <- mclapply(xy_filesList,
cell.to.groupDistances,
                                labelCol = "label", xpositionCol = "Region.X.Position",
                                ypositionCol = "Region.Y.Position",
                                cellidCol = "cellid",
                                dist_threshold = d_cutoff[d],
                                mc.cores = 4)
}
fname <- paste(getwd(), dir()[grep(pattern = "Dataset1", x = dir())],
"fileDistances_obj.RData", sep = "/")
save(list = "fileDistances", file = fname)
# fileDistances_MatrixLists <- lapply(fileDistances, function(x) {
#   lapply(x, function(x) x$distances_MatrixList)
# })
# fileDistances_Matrix <- lapply(fileDistances, function(x) {
#   lapply(x, function(x) x$distances_Matrix)
# })
fileDistances_SummaryTables <- lapply(fileDistances, function(x) {
  lapply(x, function(x) x$distances_SummaryTable)
})

#####
# Transform distances
#####
## 1 pixel = 0.5 micrometer
fileDistances_SummaryTables_MICROMETER <-
lapply(fileDistances_SummaryTables, function(x) {
```

```

    lapply(x, function(x) {
      x$Mean <- x$Mean*0.5
      return(x)
    })
  }) ## adjust mean
fileDistances_SummaryTables_MICROMETER <-
lapply(fileDistances_SummaryTables_MICROMETER, function(x) {
  lapply(x, function(x) {
    x$Median <- x$Median*0.5
    return(x)
  })
  }) ## adjust median
## z-score normalization
fileDistances_SummaryTables_SCALED <-
lapply(fileDistances_SummaryTables_MICROMETER, function(x) {
  lapply(x, function(x) {
    x$Mean <- scale(x = x$Mean)
    return(x)
  })
  }) ## adjust mean
fileDistances_SummaryTables_SCALED <-
lapply(fileDistances_SummaryTables_SCALED, function(x) {
  lapply(x, function(x) {
    x$Median <- scale(x = x$Median)
    return(x)
  })
  }) ## adjust median

#####
# Merge Distance Tables
#####
## merge summary tables - MICROMETER

```

```

merge_SummaryTables_MICROMETER                                     <-
lapply(names(fileDistances_SummaryTables_MICROMETER), function(i, x) {
  lapply(names(x[[i]]), function(ii, i, y) {
    data.frame("sample" = ii, "dist_cutoff" = i, y[[i]][[ii]], stringsAsFactors = F)
  }, i = i, y = x)
}, x = fileDistances_SummaryTables_MICROMETER)
merge_SummaryTables_MICROMETER                                     <-
lapply(merge_SummaryTables_MICROMETER, function(x) {
  do.call("rbind", x)
})
merge_SummaryTables_MICROMETER                                     <-      do.call("rbind",
merge_SummaryTables_MICROMETER)
row.names(merge_SummaryTables_MICROMETER) <- NULL
## merge summary tables - SCALED
merge_SummaryTables_SCALED                                         <-
lapply(names(fileDistances_SummaryTables_SCALED), function(i, x) {
  lapply(names(x[[i]]), function(ii, i, y) {
    data.frame("sample" = ii, "dist_cutoff" = i, y[[i]][[ii]], stringsAsFactors = F)
  }, i = i, y = x)
}, x = fileDistances_SummaryTables_SCALED)
merge_SummaryTables_SCALED    <-      lapply(merge_SummaryTables_SCALED,
function(x) {
  do.call("rbind", x)
})
merge_SummaryTables_SCALED <- do.call("rbind", merge_SummaryTables_SCALED)
row.names(merge_SummaryTables_SCALED) <- NULL

#####
# Summary Metrics
#####
## MICROMETER
summaryVariables <- c("Percentage", "Mean", "Median")

```

```

names(summaryVariables) <- summaryVariables
distanceSumVars_MICROMETER <- lapply(summaryVariables, function(sumvar,
sumlist) {
  lapply(sumlist, function (sumlist, sumvar) {
    r <- mergeSummaryVar(summary_list = sumlist, summary_variable =
sumvar)
    r <- data.frame("samples" = row.names(r), "SummaryVariable" = sumvar, r,
stringsAsFactors = F)
    return(r)
  }, sumvar = sumvar)
}, sumlist = fileDistances_SummaryTables_MICROMETER)
summaryMetrics_MICROMETER <- lapply(distanceSumVars_MICROMETER,
function(x) {
  lapply(x, function(y) {do.call("cbind", y)})
})
## SCALED
summaryVariables <- c("Percentage", "Mean", "Median")
names(summaryVariables) <- summaryVariables
distanceSumVars_SCALED <- lapply(summaryVariables, function(sumvar, sumlist) {
  lapply(sumlist, function (sumlist, sumvar) {
    r <- mergeSummaryVar(summary_list = sumlist, summary_variable =
sumvar)
    r <- data.frame("samples" = row.names(r), "SummaryVariable" = sumvar, r,
stringsAsFactors = F)
    return(r)
  }, sumvar = sumvar)
}, sumlist = fileDistances_SummaryTables_SCALED)
summaryMetrics_SCALED <- lapply(distanceSumVars_SCALED, function(x) {
  lapply(x, function(y) {do.call("cbind", y)})
})

#####

```

```

# Melt Summary Metrics
#####

## MICROMETER
summaryMetrics_MICROMETER      <-      lapply(summaryMetrics_MICROMETER,
function(x) {
  lnm <- names(x)
  lapply(lnm, function(n, x) {
    data.frame(x[[n]], "dist_cutoff" = n, stringsAsFactors = F)
  }, x = x)
})
summaryMelt_MICROMETER <- melt(summaryMetrics_MICROMETER, id.vars =
c("samples", "SummaryVariable", "dist_cutoff"))
summaryMelt_MICROMETER$variable                                <-
as.character(summaryMelt_MICROMETER$variable)
summaryMelt_MICROMETER$value                                  <-
as.numeric(summaryMelt_MICROMETER$value)
summaryMelt_MICROMETER$dist_cutoff      <-      factor(x      =
summaryMelt_MICROMETER$dist_cutoff, levels = names(d_cutoff))

## SCALED
summaryMetrics_SCALED <- lapply(summaryMetrics_SCALED, function(x) {
  lnm <- names(x)
  lapply(lnm, function(n, x) {
    data.frame(x[[n]], "dist_cutoff" = n, stringsAsFactors = F)
  }, x = x)
})
summaryMelt_SCALED <- melt(summaryMetrics_SCALED, id.vars = c("samples",
"SummaryVariable", "dist_cutoff"))
summaryMelt_SCALED$variable <- as.character(summaryMelt_SCALED$variable)
summaryMelt_SCALED$value <- as.numeric(summaryMelt_SCALED$value)
summaryMelt_SCALED$dist_cutoff <- factor(x = summaryMelt_SCALED$dist_cutoff,
levels = names(d_cutoff))

```

```
#####

# Plots
#####

## OVERALL
overallPlot_MICROMETER <- ggplot(data = summaryMelt_MICROMETER, mapping =
aes(x = variable, y = value, fill = SummaryVariable)) +
  geom_boxplot() +
  facet_wrap(~SummaryVariable + dist_cutoff, scales = "free_y", ncol = 4) +
  theme_bw() +
  theme(axis.text.x = element_text(angle = 90))
overallPlot_SCALED <- ggplot(data = summaryMelt_SCALED, mapping = aes(x =
variable, y = value, fill = SummaryVariable)) +
  geom_boxplot() +
  facet_wrap(~SummaryVariable + dist_cutoff, scales = "free_y", ncol = 4) +
  theme_bw() +
  theme(axis.text.x = element_text(angle = 90))

## BY PHENO PAIR
survivalPlot_df <- summaryMelt_MICROMETER[1:5]
survival_ipheno <- lapply(survivalPlot_df$variable, strsplit, split = "_to_")
survival_ipheno <- lapply(survival_ipheno, unlist)
survival_ipheno <- sapply(survival_ipheno, "[", 1)
survivalPlot_df <- split(x = survivalPlot_df, f = survival_ipheno)
survivalPlot_list_MICROMETER <- lapply(survivalPlot_df, function(plotdf) {
  ggplot(data = plotdf, mapping = aes(x = variable, y = value, fill =
SummaryVariable)) +
    geom_boxplot() +
    facet_wrap(~SummaryVariable + dist_cutoff, scales = "free_y", ncol = 4) +
    theme_bw() +
    theme(axis.text.x = element_text(angle = 90))
})
```

```

survivalPlot_df <- summaryMelt_SCALED[,1:5]
survival_ipheno <- lapply(survivalPlot_df$variable, strsplit, split = "_to_")
survival_ipheno <- lapply(survival_ipheno, unlist)
survival_ipheno <- sapply(survival_ipheno, "[", 1)
survivalPlot_df <- split(x = survivalPlot_df, f = survival_ipheno)
survivalPlot_list_SCALED <- lapply(survivalPlot_df, function(plotdf) {
  ggplot(data = plotdf, mapping = aes(x = variable, y = value, fill =
SummaryVariable)) +
    geom_boxplot() +
    facet_wrap(~SummaryVariable + dist_cutoff, scales = "free_y", ncol = 4) +
    theme_bw() +
    theme(axis.text.x = element_text(angle = 90))
})

```

```
#####
```

```
# Save
```

```
#####
```

```
## TABLES
```

```
ndir <- paste (getwd(), "ResultTables", sep = "/")
```

```
if (!dir.exists(ndir)) {
```

```
  dir.create(ndir)
```

```
}
```

```
fname <- paste(ndir, "/", "Dataset1_MICROMETER_merge_SummaryTables.txt", sep =
"")
```

```
write.table(x = merge_SummaryTables_MICROMETER, file = fname, sep = "\t",
row.names = F)
```

```
fname <- paste(ndir, "/", "Dataset1_MICROMETER_OverallSummaryTable.txt", sep = "")
```

```
write.table(x = summaryMelt_MICROMETER[,1:5], file = fname, sep = "\t", row.names
= F)
```

```
fname <- paste(ndir, "/", "Dataset1_SCALED_merge_SummaryTables.txt", sep = "")
```



```

write.table(x = merge_SummaryTables_SCALED, file = fname, sep = "\t", row.names =
F)
fname <- paste(ndir, "/", "Dataset1_SCALED_OverallSummaryTable.txt", sep = "")
write.table(x = summaryMelt_SCALED[,1:5], file = fname, sep = "\t", row.names = F)

## PLOTS
ndir <- paste (getwd(), "ResultFigures", sep = "/")
if (!dir.exists(ndir)) {
  dir.create(ndir)
}
fname <- paste(ndir, "/", "Dataset1_MICROMETER_OverallSummaryPlot.pdf", sep = "")
ggsave(filename = fname, plot = overallPlot_MICROMETER, height = 20, width = 40,
scale = 1)
for(p in 1:length(survivalPlot_list_MICROMETER)) {
  fname      <-      paste(ndir,      "/Dataset1_MICROMETER_",      p,      "_",
names(survivalPlot_list_MICROMETER)[p], "_SummaryPlot.pdf", sep = "")
  ggsave(filename = fname, plot = survivalPlot_list_MICROMETER[[p]], height = 20,
width = 20)
}

fname <- paste(ndir, "/", "Dataset1_SCALED_OverallSummaryPlot.pdf", sep = "")
ggsave(filename = fname, plot = overallPlot_SCALED, height = 20, width = 40, scale =
1)
for(p in 1:length(survivalPlot_list_SCALED)) {
  fname      <-      paste(ndir,      "/Dataset1_SCALED_",      p,      "_",
names(survivalPlot_list_SCALED)[p], "_SummaryPlot.pdf", sep = "")
  ggsave(filename = fname, plot = survivalPlot_list_SCALED[[p]], height = 20, width
= 20)
}

```

Renin-angiotensin inhibitors reprogram tumor immune microenvironment: A comprehensive view of the influences on anti-tumor immunity

Dora L. Vallejo-Ardila¹, Theodora Fifis¹, Louise M. Burrell^{2,3}, Katrina Walsh¹ and Christopher Christophi¹

¹Department of Surgery, Austin Health, University of Melbourne, Melbourne, VIC 3084, Australia

²Department of Medicine, Austin Health, University of Melbourne, Melbourne, VIC 3084, Australia

³Department of Cardiology, Austin Health, University of Melbourne, Melbourne, VIC 3084, Australia

Correspondence to: Dora L. Vallejo-Ardila, email: dvallejo@student.unimelb.edu.au

Keywords: renin-angiotensin system; tumor microenvironment; anti-tumor immunity; kallikrein kinin system

Received: June 26, 2018

Accepted: September 08, 2018

Published: October 26, 2018

Copyright: Vallejo-Ardila et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License 3.0 (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

Renin-angiotensin system inhibitors (RASi) have shown potential anti-tumor effects that may have a significant impact in cancer therapy. The components of the renin-angiotensin system (RAS) including both, conventional and alternative axis, appear to have contradictory effects on tumor biology. The mechanisms by which RASi impair tumor growth extend beyond their function of modulating tumor vasculature. The major focus of this review is to analyze other mechanisms by which RASi reprogram the tumor immune microenvironment. These involve impairing hypoxia and acidosis within the tumor stroma, regulating inflammatory signaling pathways and oxidative stress, modulating the function of the non-cellular components and immune cells, and regulating the cross-talk between kallikrein kinin system and RAS.

INTRODUCTION

The main components of the conventional axis of the renin-angiotensin system (RAS) [such as angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE), angiotensin I (Ang I), angiotensin II (Ang II)], function as an intricate peptide signaling network through several receptors [including angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R)] [1]. AGT is produced and released into circulation by the liver, then AGT is hydrolyzed by renin, which is produced by the juxtaglomerular cells of the kidney to form Ang I [1]. Afterwards Ang I is hydrolyzed by ACE in the endothelial cells of the lungs, to produce the biologically active Ang II. Ang II interacts with two different receptors, AT1R and AT2R [1]. The alternative axis comprises ACE2, which is known as ACE-related carboxypeptidase or angiotensin-converting enzyme homolog (ACEH). ACE2 is mostly found in the vascular endothelial cells and renal tubular

epithelium [2] ACE2 cleaves Ang II to Ang-(1-7), whereas ACE produces Ang-(1-7) by cleaving Ang-(1-9). Ang-(1-7) commonly acts via mitochondrial assembly receptor (Mas receptor or MasR) [3]. Another peptide alamandine is generated either by the cleavage of Ang A or Ang-(1-7), by ACE and decarboxylase (DC) respectively. Alamandine binds through MAS-related G protein couple receptor D (MRGD) [3]. Several other truncated bioactive peptides have been previously characterized including Angiotensin III [4], Angiotensin IV [5], and Ang A [6], as part of the RAS network (Figure 1).

In the mid-1970s it became possible for the first time to therapeutically target the components of Ang II/AT1R axis using the angiotensin-converting enzyme inhibitor, captopril [7], and followed by selective blockade of the AT1R with losartan [8]. Renin-angiotensin system inhibitors (RASi) includes two classes of pharmacological agents, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) [8]. A third

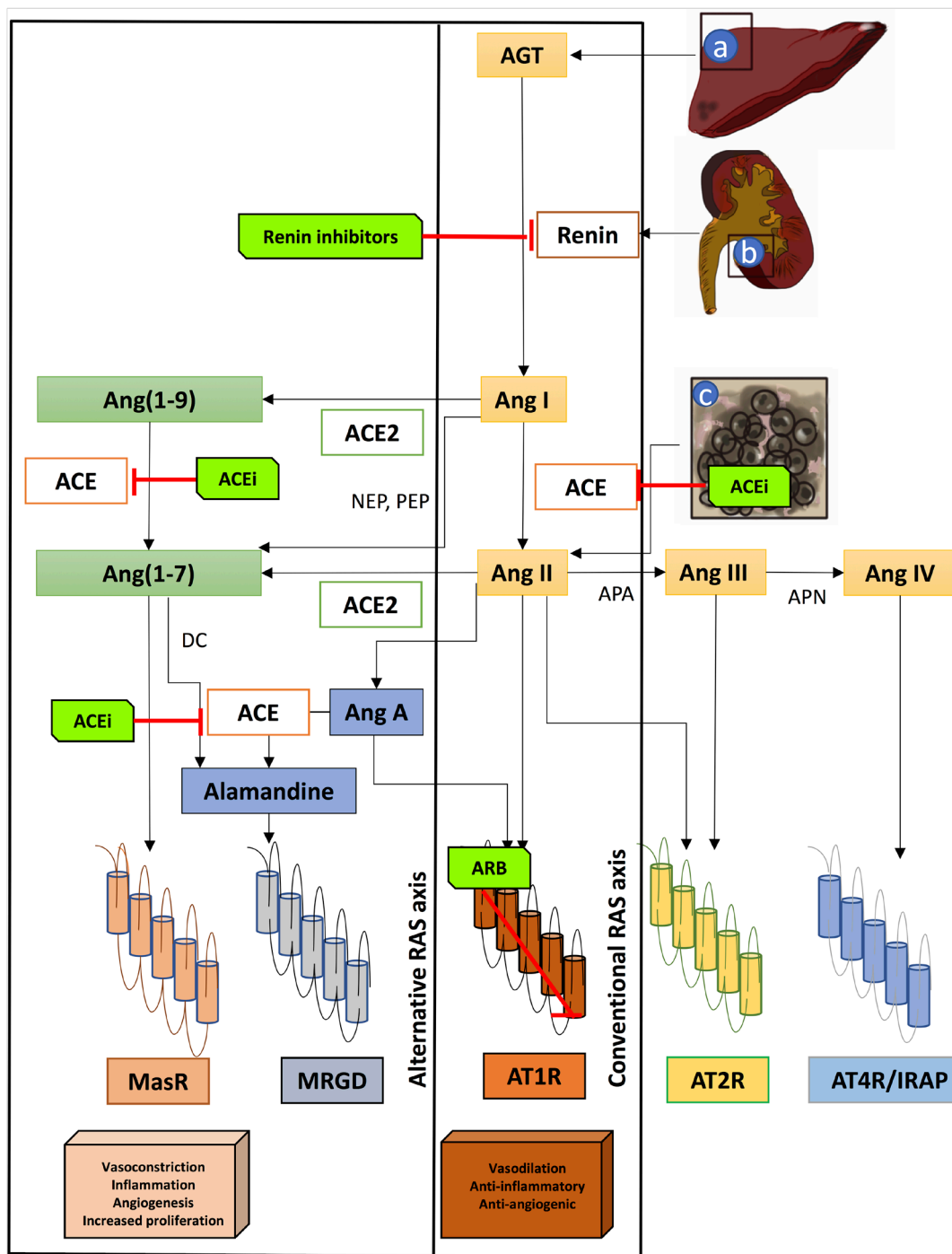


Figure 1: The pro-tumor and anti-tumor mediated effects of the conventional and alternative RAS axis. Angiotensinogen (AGT) is produced and released into circulation by the liver (a), then AGT is hydrolyzed by renin. Renin is produced by the juxtaglomerular cells of the kidney (b) to form Angiotensin I (Ang I). Afterwards Ang I is hydrolyzed by angiotensin-converting enzyme (ACE) in the endothelial cells of the lungs (c), to produce the biologically active angiotensin II (Ang II). Ang II interacts with two different receptors, angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). ACE2 cleaves Ang II to Angiotensin (1-7) (Ang (1-7)), whereas ACE produces Ang (1-7) by cleaving Angiotensin (1-9) (Ang (1-9)). Ang (1-7) commonly acts via mitochondrial assembly receptor (Mas receptor or MasR). Alamandine is generated either by the cleavage of Ang A or Ang (1-7), by ACE and decarboxylase (DC) respectively. Alamandine binds through MAS-related G protein couple receptor D (MRGD). Angiotensin II is metabolized to Angiotensin III (Ang III) by aminopeptidase A (APA), whereas Ang III is metabolized to Angiotensin IV (Ang IV) by aminopeptidase N (APN). AT4R or Insulin-regulated membrane aminopeptidase (IRAP) is activated by Ang IV. NEP, neutral endopeptidase; PEP, prolyndopeptidase. The inhibition of the conventional axis by ACEi or ARB reduces inflammation, vasoconstriction, and angiogenesis means by which also inhibits tumor growth. Opposite effects have been associated with the activation of the alternative axis including vasodilatation, antinflammatory and anti-angiogenic effects.

class of RASi, a direct renin inhibitor such as aliskiren is also included in the list of RASi approved by the U.S. Food and Drug administration (FDA) [9].

RASi are widely used to treat heart failure [10, 11], myocardial infarction [12], and systemic hypertension [13] and have proved to be effective in preventing cardiovascular [14] and renal associated comorbidities [13]. Additionally RASi have shown potential anti-tumor effects that promise a significant impact in cancer therapy [15]. Several studies have yielded diverging findings on the role of RASi on the incidence of recurrence, metastasis and survival in cancer patients [16]. To comprehend the potential benefit of RASi in cancer patients consideration should be given to factors such as tumor heterogeneity, tumor stage, hormone receptor status, human epidermal growth factor receptor 2 (EGFR2) over-expression, and (neo)adjuvant treatment regimen [15]. A meta-analysis including 55 studies suggested that RASi may improve the survival of cancer patients depending on cancer type and class of RASi [16].

Differences between the mediated effects of the RAS conventional and alternative axis has been previously described. In general, the over-expression of RAS components within the Ang II/AT1R axis (such as ACE and AT1R) is associated with tumor growth and with more aggressive tumor features in several types of human cancer, including breast cancer, ovarian cancer and renal cancer [17–19], whereas Ang II/AT2R and Ang(1-7)/MasR showed opposite effects [20]. Nonetheless there are also conflicting evidence, which may suggest tumor-type specific differences [18]. Similarly, ACE2/Ang-(1-7)/MasR axis dysregulation has been shown to be up-regulated or down-regulated depending on the type of cancer [21–23] (Figure 1).

The role of the various RASi in modulating pathological processes involving cell inflammation [24, 25], fibrosis [26–28], and tumor growth [29–32], has been well-documented, but its influence on anti-tumor immunity is uncertain. The aim of this literature review is to analyze current evidence on the effects of RASi on anti-tumor immunity by reprogramming the tumor microenvironment (TME).

The pathological processes and cellular functions inside the TME which appear to be influenced by RASi include tumor angiogenesis, hypoxia and acidosis within the tumor stroma, inflammatory signaling pathways, oxidative stress, immune cell modulation and the role of kallikrein-kinin system (KKS) (Figure 2).

Regulation of tumor angiogenesis using renin-angiotensin inhibitors impacts cancer progression

Conclusive evidence has shown that targeting Ang II/AT1R impairs neovascularization and vascular permeability and decreases microvessel density by

reducing vascular endothelial growth factor (VEGF)-expression [19, 33–35]. Angiogenesis is initiated by the disruption of the endothelial cells (EC) monolayer, and their invasion into the surrounding stroma. The recruitment of pericytes along the basement membrane into the new vessels provides both, mechanical stability and molecular cross talk with EC, which induces VEGF production and survival signaling [36, 37]. The cellular infiltrating populations into the TME can originate either in the blood vessels, the stroma or the bone marrow [37]. For example, cancer-associated fibroblast (CAFs) can be derived from both, tumor stroma and from bone marrow precursors [37]. This cellular plasticity is mediated by the epithelial-mesenchymal transition (EMT) [37]. Many of the different cell types involved in tumor vasculature, and/or are part of the TME such as pericytes [38, 39] and CAFs [40] express RAS components. Regulating angiogenesis using RASi may extend beyond the converging point of VEGF-response. ACE2 acts as potent counter-regulator against ACE and Ang II activity [20, 41, 42]. ACE2 overexpression or ACE2-Ang-(1-7)-MasR activation may suppress angiogenesis either by inhibiting the production of VEGFa in NSCLC [43], or VEGF receptors attenuation in nasopharyngeal carcinoma [44], respectively.

The use of RASi concomitant with anti-VEGF therapy was associated with better survival in metastatic renal cell carcinoma [45–48], metastatic CRC [49], advanced HCC [50] and glioblastoma [51]. Increased resistance has been reported to VEGF inhibition [52, 53]. The tumor vasculature interacts with the TME via several other mechanisms offering potential new therapeutic targets [54]. The regulation of TME by emerging anti-angiogenic therapies are aiming to counteract tumor progression and to achieve greater tumor destruction. Rather than acute vessel disrupting strategies, there is now a focus on anti-inflammatory regimens. For instance, cabozantinib inhibits the activity of VEGFR, c-MET, AXL and other tyrosine kinases, thereby leading not only to impair angiogenesis but also the disruption of other processes inside the TME [55].

Targeting Ang II/AT1R axis to impair hypoxia and acidosis within the tumor stroma

Local RAS works synergistically and independently of systemic RAS in a paracrine fashion [56]. Ang II mediates effects that reduce tumor perfusion and oxygenation, resulting in hypoxia and subsequent acidosis within the tumor stroma [57]. For example, local Ang II, predominantly exists in hypoxic regions of nasopharyngeal carcinoma and breast cancer cells, where it is autocrinely produced by chymase-dependant rather than ACE dependent mechanism [58]. In which case the action of ACEi will not be effective to inhibit tumor growth. Tumor hypoxia and acidosis trigger a cascade of up-regulation of transcription factors, growth factors

and cytokines, including hypoxia inducible factor (HIF), VEGF and Transforming growth factor beta (TGF- β) that together promote immunosuppression within the TME [59, 60]. An immunosuppressive microenvironment includes impaired function of T cells [61], dendritic cells [62] the accumulation of M2-like macrophages [63] and MDSC, but also increased expression of immune check point molecules such as PD-L1 / PD-1 on tumour / stromal cells and the immune cells [64].

In the liver, the local RAS is up-regulated in response to tissue injury and hypoxia [31]. The main effector is Ang II, which normally maintains tissues homeostasis, in this scenario can stimulate the expression pro-angiogenic VEGF via AT1R signaling, leading to wound healing in tissue injury or to tumor neovascularization in hypoxic tumors [65]. ACEi mechanism of action is to reduce the production of Ang II, while ARBs selectively block the action of AT1R receptors inhibiting tumor-associated angiogenesis [16].

Renin-angiotensin inhibitors regulate inflammatory signaling pathways and oxidative stress in tumor biology

The TME is bound to the dynamic between malignant and non-transformed cells [66]. Non-malignant cells are reprogrammed to accomplish tumor-

promoting functions during all stages of carcinogenesis [66]. Intercellular interactions modulate the chemical and physical properties of any tissue through a diverse pool of secreted cytokines, chemokines, growth factors, and inflammatory or matrix remodeling enzymes [67]. TGF- β suppresses the differentiation and function of T helper (T_H), CD8⁺ cells, Natural Killer (NK) cells, and tumor-associated neutrophils (TANs), tumor associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) [67]. Tumor supporting cytokines are released from tumor and stromal cells upon AT1R activation via Ang II including, TGF- β Interleukins (IL-1a, IL-1B, IL-6, IL-8) and MCP-1: (monocyte chemoattractant protein 1), macrophage colony-stimulating factor (M-CSF), cyclooxygenase 2(COX-2), C- reactive protein (CRP) [68]. Immunomodulatory cytokines may up-regulate immunosuppressive pathways, i.e. COX-2 via prostaglandin E2 synthesis, and impair dendritic cell (DC) function by reducing their migration [68, 69]. Ang II/AT1R signaling induces reactive oxygen species (ROS) generation and related proteins such as inducible nitric oxide synthase in the tumor cells and stroma cell [70]. Exposure to ROS in the TME can impair T cell function while enhancing T regs and TAMs, as has been previously reported in prostate cancer [69]. Treatment with the ARB candesartan diminishes ROS generation [71].

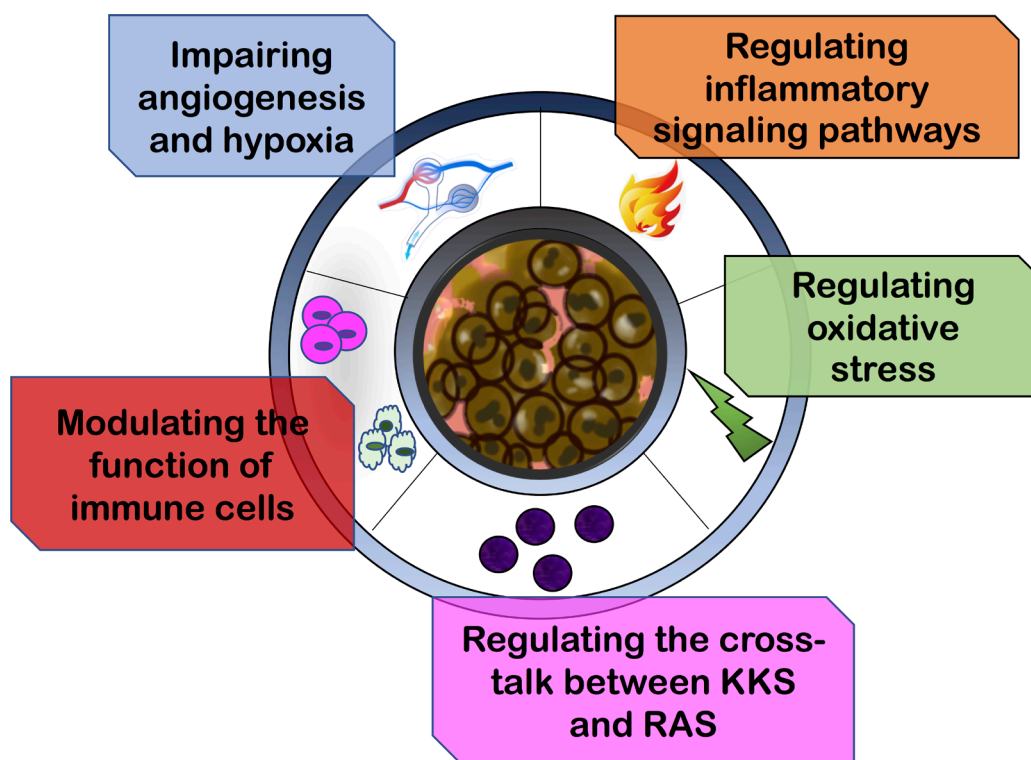


Figure 2: Renin-angiotensin inhibitors reprogram tumor immune microenvironment. The pathological processes and cellular functions inside the tumor microenvironment which appear to be influenced by RASi include tumor angiogenesis, hypoxia and acidosis within the tumor stroma, inflammatory signaling pathways, oxidative stress, immune cell modulation and the role of kalli krein kinin system (KKS).

Renin-angiotensin inhibitors modulate the function of immune cells within the tumor microenvironment

There are three major immunosuppressive cell types within the tumor mass; MDSCs, TAMs and CAFs. MDSC include both myeloid progenitors cells and immature myeloid cells [72]. These cell have been characterized as a population of inhibitory immune cells that lack typical mature myeloid markers, in mouse and human cancers [72]. Murine and human MDSCs exert their suppressive effects through several mechanisms that inhibit CD8⁺T cell activation [73]. Additionally they induce the development of T regs population, the polarization of macrophages to a TAM-like phenotype, the production of ROS, the upregulation of nitric oxide and depletion of nutrients via increased activity of L-arginase [73]. ACE is required for normal myelopoiesis, as an increased level of CD11b(+) bGrl (+) cell-similar to MDSCs phenotype were detected after extramedullary myelopoiesis compensatory to bone marrow incompetence [74]. Likewise, ACE may have a role in the accumulation of MDSCs in the TME. However, the inhibitory function of MDSCs over CD8⁺T cells in the TME requires further investigation [74].

Similarly, TAMs are usually pro-tumorigenic and have shown that a polarized M2-like phenotype contributes to immunosuppression, whereas M1-like phenotype induces anti-tumor immunity [63]. TAMs tend to accumulate in hypoxic and/or necrotic areas of the tumor [75]. Studies with ACE KO and ACE 10/10 mouse model indicated that ACE plays key role in macrophages to regulate the production of pro-inflammatory cytokines such as IL-12, nitride, and TNF- α in response to lipopolysaccharide (LPS) or chitin (a polymer of *N*-acetylglucosamine). These induce M1 macrophages or activate M2, respectively [76]. Additionally, M1 induction through granulocyte-macrophage colony stimulating factor (GM-CSF) upregulate ACE expression in human monocytes [76, 77]. Recent findings remain inconclusive as to how RAS modulation establishes M2 macrophage induction and polarization. Some studies suggest that there is no difference between ACE KO, ACE 10/10 and WT model [74]. Others have found an increased or decreased M2 response by RAS blockade [78, 79]. Our laboratory has shown the immunomodulatory role of RAS in influencing TAMs. Blockade of ACE with captopril increased Kupffer cells infiltration in the tumor-bearing liver during an early stage of tumor progression in CRC liver metastasis [80].

Myofibroblasts also known as CAFs are derived from multiple precursors, such as myoepithelial cells, mesenchymal stem cells, smooth muscle cells and EC [81]. Their capability to secrete EGF family growth factors hepatocyte and fibroblast growth factors and insulin growth factor confer them the potential to induce malignant transformation of cells [82, 83]. CAF express

AT1R [40], through which Ang II [40] stimulates their proliferation and induces the production of various cytokines including TGF- β [40]. TGF- β in addition to its immunosuppressive role as outlined above, can stimulate EMT in malignant cells contributing to an immunosuppressive TME and therapy resistance [84, 85]. Our laboratory found that RAS is implicated in the regulation of EMT in CRC via both AT1 and AT2 receptors by Ang II modulated migration [86]. Additionally, CAF tumor promoting effects have been associated with promoting neovascularization and recruitment of immune cells in the TME via NF- κ B signaling pathway [87]. A recent study concluded that local RAS activity can modulate the function MDSCs, TAMs and CAFs, and indeed is a potent inducer of immunosuppression in the TME [40]. Inhibition of local RAS augments the induction and infiltration of tumor antigen-specific T cells, reduces the T cell suppressive activity of tumor-infiltrating CD11b⁺ cells including MDSCs and enhances the T-cell stimulatory activity of CAFs [47].

In addition to CAFs, other types of immune cells also express RAS components. These include monocytes, neutrophils, dendritic cell and T cells, thus presenting pivotal targets for immune therapy [15]. For example, the stimulatory and coinhibitory interactions between lymphocytes infiltrating tumor stroma and tumor cells expressing receptor-ligands pairs, like immune check point inhibitors [cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), and its ligand (PD-L1)] correlates with T cell dysfunction [37]. The inhibition of AT1R enhances the induction of tumor antigen-specific CD8⁺ cytotoxic T lymphocytes via gp70-Tcells [40]. Additionally, a clinical study of non-metastatic pancreatic ductal adenocarcinoma (PDAC) patients reported significant overall survival in the chronic RASi users, while unbiased gene profiling of resected tumors from this group presented enriched gene signatures associated with antigen processing and presentation, and activity of T cells, and reduced gene signatures associated with tumour aggressiveness. These findings may suggest that RAS signaling could modulate the efficacy of immunotherapy on this type of cancer [88]. Altogether the synergistic use of RASi combined with immune check point inhibitors may improve anti-tumor efficacy by reprogramming the TME towards an immune stimulatory milieu.

Targeting tumor non-cellular components using renin-angiotensin inhibitors may enhance antitumor immunity

RAS has been studied under the scope of chronic wound healing and fibrosis [89]. During stromagenesis activated fibroblasts, have a critical function in orchestrating ECM remodeling. The effects of tissue renin angiotensin (tRAS) on chronic wound healing can be divided into two opposite axes: (a) proinflammatory/

pro-fibrotic and (b) anti-inflammatory/anti-fibrotic [89]. RAS signaling modulates ECM remodeling via AT1 by stimulation in dermal fibroblast, which promotes deposition of fibrotic ECM [90]. The contrary effect was observed via AT2 stimulation, which reduced collagen deposition counteracting AT1 signaling through engagement of tyrosine phosphatase SHP-1 [91]. In the case of tumor-associated stromagenesis, the pathophysiologic response fails to resolve. RAS activated CAFs secrete collagen and other ECM components which leads to a fibrotic reaction so-called tumor desmoplasia [92]. A tumor desmoplastic environment can be either a physical barrier to immune cell infiltration [93], or can provide the substratum to their

interstitial migration [94]. Additionally, RAS activated CAFs and other stromal cells produce immunosuppressive cytokines/ growth factors especially TGF- β in the TME leading to chronic inflammation and effector T- cell disfunction, apoptosis and failure to infiltrate deep into the tumour [61]. RASi targeting of the tumor stroma resulted in collagen I synthesis inhibition by CAFs in a dose-dependent manner in different desmoplastic models including human breast, pancreatic, and skin tumors in mice [95]. A collateral effect of decreasing tumor desmoplasia is the decompression of tumor blood vessels and subsequent reduction of tumor hypoxia by increasing perfusion, which may also enhance antitumor immunity [96].

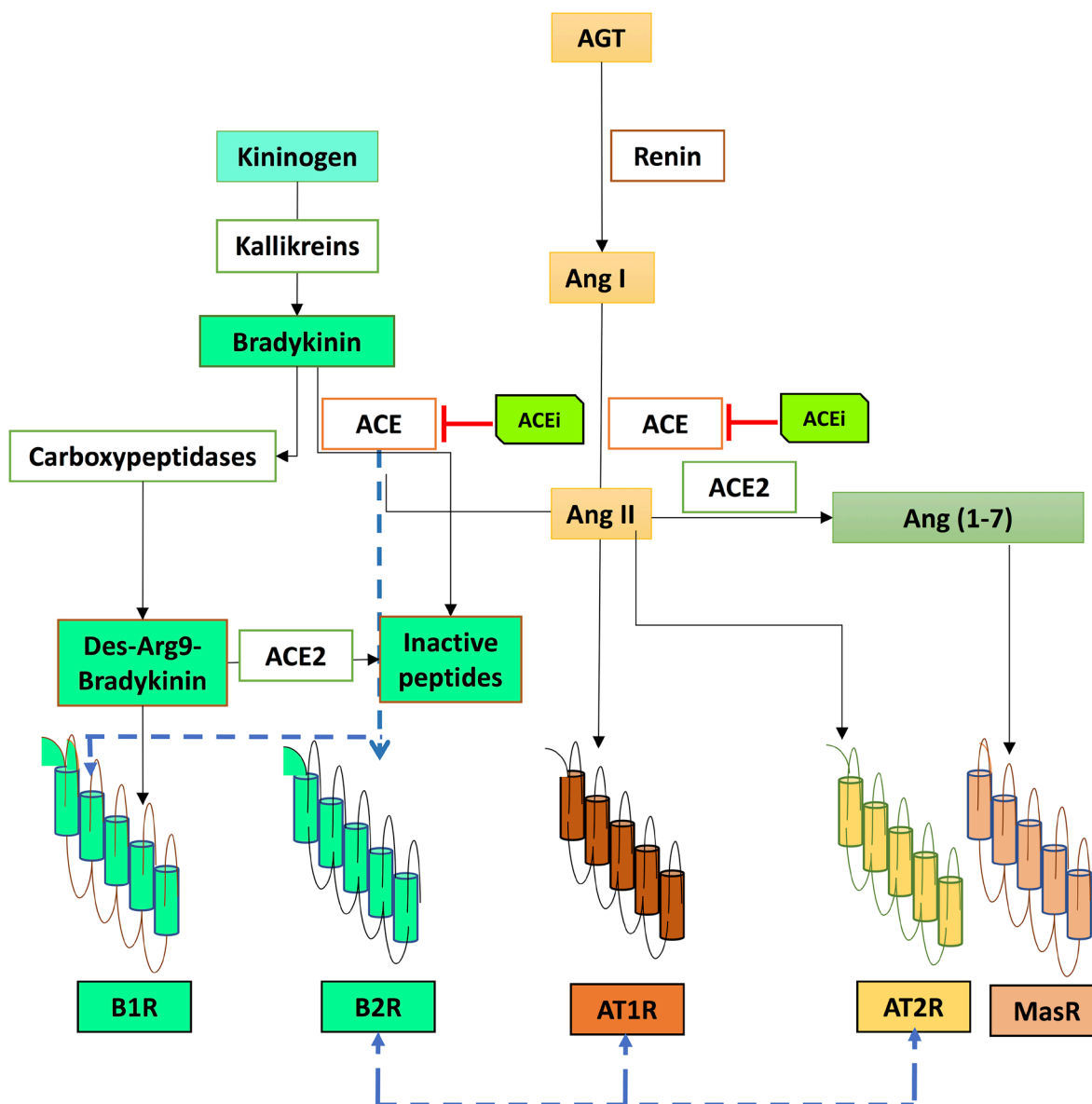


Figure 3: Renin-angiotensin system components intersecting with the kallikrein kinin system. Angiotensin-converting enzyme (ACE) is a major hub intersecting between the crosstalk of both systems, by regulating the levels of Angiotensin II (Ang II) and kinins. ACE metabolizes bradykinin (BK), and converts Angiotensin I (Ang I) into Ang II. Interactions between ACE and kinin receptor 1 (B1R) and/or kinin receptor 2 (B2R) have been reported, same as interactions between angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) with B2R.

The cross-regulation of kalli-krein kinin system and renin-angiotensin system by angiotensin-converting enzyme has pleiotropic effects in the tumor microenvironment

The KKS comprises several peptides that are produced and cleaved in various sites to finally release the vasoactive kinins [97]. Kinins are originated from their parental molecules, the kininogens [97]. Diverse enzymes known also as kininases such as ACE, neutral endopeptidase (NEP), carboxypeptidase N, carboxypeptidase M, cathepsin X and aminopeptidase metabolize the kinins, [98] (Figure 3). Bradykinin (BK) is the final bioactive peptide produced upon the activation of the KKS, which has pleiotropic functions depending on the pattern signaling triggered through its two different receptors [99]. The kinin receptor 1 (B1R) and the kinin receptor 2 (B2R) activate the same signaling pathway, while inducing a different duration and intensity of the signal modulated by calcium influx [100]. B1R and B2R can be expressed in the same cell type, such as EC, fibroblast and several tumor cells [101]. While B1R expression is induced upon inflammatory conditions and tissue injury, B2R is constitutively expressed [102]. The overall effect of BK through either B1R, B2R, or both is to promote vascular cell proliferation [103], influence barrier permeability [104] and possibly stimulate the release of pro-inflammatory cytokines [105]. The role of kinin receptors has been investigated in various types of cancer, including melanoma, renal, prostate, lung and breast and mesothelioma [106–110], among others. Recently our laboratory found that both human and mouse CRC cell lines showed a strong positive expression of B1R and B2R, and while the inhibition of both receptors delayed tumor growth, only B1R blockade reduced tumor load and increased tumor apoptosis [105]. Indistinctively, blockade of B1R or B2R diminished tumor vascularization *in vivo*, and impaired proliferation and migration of CRC cell *in vitro* [111]. The effect of RASi -ACEi- leads to accumulation of kinins and subsequent B1R activation [112], which may influence not only the cellular components part of the TME by activating macrophages and DC, but also can up-regulate cytokine production of IL-6 and IFN- γ [97]. ACEi activate B2R on DC, with increased production IL-12 [113]. Further studies are required to elucidate the therapeutic opportunities of modulating kinin receptors with ACEi and their influence on the TME.

Perspectives and significance

Over the last decade a large number of clinical studies have shown the benefits of using RASi in patients at different stages in several types of cancer. The evidence of RASi impairing tumor growth beyond the function of modulating tumor vasculature is rapidly increasing. A major challenge in the field of cancer therapeutics is

the increasing rate of resistance to chemotherapy [114], and immunotherapy [115]. A better understanding of the complex interaction between non-cellular components, tumor cells, tissue resident immune cells and infiltrating immune cells within TME is required to develop new cancer treatment strategies. An immunosuppressive TME affects the efficacy of immune checkpoint therapy, which is reflected in two different clinical scenarios, overall survival and adverse side effects. RASi are able to reprogram the TME, using mechanisms by which they impair hypoxia and acidosis within the tumor stroma, regulate inflammatory signaling pathways and oxidative stress, modulate the function of the non-cellular components and immune cells, and regulate the cross-talk between KKS and RAS. Targeting RAS conventional axis (Ang II/AT1R), KKS and enhancing RAS alternative axis (ACE2/Ang-(1-7)/MasR) seem to be promising strategies to effectively influence TME toward an immunostimulatory milieu, and subsequently improve immunotherapy outcome for a larger population of cancer patients. Further pre-clinical and clinical studies are necessary to propose that the concomitant use of RASi and immunotherapy could not only improve the overall survival of cancer patients, but also could decrease the immunotherapy related side effects. Lastly, additional studies may be required in order to conclude if the effects of RASi on the tumor stroma, not only would depend upon the degree of tumor desmoplasia, but also may depend on how they can influence tumor immune infiltration by either impairing or promoting their migration throughout the interstitial space.

ACKNOWLEDGMENTS

This work was supported by the Department of Science, Technology, and Innovation (COLCIENCIAS-Colombia) (Ph. D overseas 679 program) and University of Melbourne, Faculty of Medicine, Dentistry and Health Sciences (Scholarship 2018-2021).

CONFLICTS OF INTEREST

The authors of this manuscript have no conflicts of interest to declare.

REFERENCES

1. Carey RM, Siragy HM. Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev.* 2003; 24:261–71.
2. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res.* 2000; 87:E1–9.

3. Lautner RQ, Villela DC, Fraga-Silva RA, Silva N, Verano-Braga T, Costa-Fraga F, Jankowski J, Jankowski V, Sousa F, Alzamora A, Soares E, Barbosa C, Kjeldsen F, et al. Discovery and characterization of alamandine: a novel component of the renin-angiotensin system. *Circ Res*. 2013; 112:1104–11.
4. Moeller I, Allen AM, Chai SY, Zhuo J, Mendelsohn FA. Bioactive angiotensin peptides. *J Hum Hypertens*. 1998; 12:289–93.
5. Mustafa T, Lee JH, Chai SY, Albiston AL, McDowall SG, Mendelsohn FA. Bioactive angiotensin peptides: focus on angiotensin IV. *J Renin Angiotensin Aldosterone Syst*. 2001; 2:205–10.
6. Walmor De M. Beyond the conventional Renin Angiotensin System. *Frontiers*. 2014.
7. Erdös EG. Conversion of angiotensin I to angiotensin II. *Am J Med*. 1976; 60:749–59.
8. Bhardwaj G. How the antihypertensive losartan was discovered. *Expert Opin Drug Discov*. 2006; 1:609–18.
9. Müller DN, Luft FC. Direct renin inhibition with aliskiren in hypertension and target organ damage. *Clin J Am Soc Nephrol*. 2006; 1:221–28.
10. Yusuf S, Pitt B, Davis CE, Hood WB, Cohn JN, and SOLVD Investigators. Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *N Engl J Med*. 1991; 325:293–302.
11. CONSENSUS Trial Study Group. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). *N Engl J Med*. 1987; 316:1429–35.
12. Okura H, Kataoka T, Yoshida K. Renin-angiotensin system inhibitors in patients with myocardial infarction and secondary mitral regurgitation. *Heart*. 2016; 102:694–700.
13. Daien V, Duny Y, Ribstein J, du Cailar G, Mimran A, Villain M, Daures JP, Fesler P. Treatment of hypertension with renin-angiotensin system inhibitors and renal dysfunction: a systematic review and meta-analysis. *Am J Hypertens*. 2012; 25:126–32.
14. Tardif JC, Grégoire J. Renin-angiotensin system inhibition and secondary cardiovascular prevention. *JAMA*. 2013; 310:1130–31.
15. Pinter M, Jain RK. Targeting the renin-angiotensin system to improve cancer treatment: implications for immunotherapy. *Sci Transl Med*. 2017; 9:eaan5616.
16. Sun H, Li T, Zhuang R, Cai W, Zheng Y. Do renin-angiotensin system inhibitors influence the recurrence, metastasis, and survival in cancer patients?: evidence from a meta-analysis including 55 studies. *Medicine (Baltimore)*. 2017; 96:e6394.
17. Arrieta O, Pineda-Olvera B, Guevara-Salazar P, Hernández-Pedro N, Morales-Espinosa D, Cerón-Lizarraga TL, González-De la Rosa CH, Rembao D, Segura-Pacheco B, Sotelo J. Expression of AT1 and AT2 angiotensin receptors in astrocytomas is associated with poor prognosis. *Br J Cancer*. 2008; 99:160–66.
18. Arrieta O, Villarreal-Garza C, Vizcaino G, Pineda B, Hernández-Pedro N, Guevara-Salazar P, Wegman-Ostrosky T, Villanueva-Rodríguez G, Gamboa-Domínguez A. Association between AT1 and AT2 angiotensin II receptor expression with cell proliferation and angiogenesis in operable breast cancer. *Tumour Biol*. 2015; 36:5627–34.
19. Ino K, Shibata K, Kajiyama H, Yamamoto E, Nagasaka T, Nawa A, Nomura S, Kikkawa F. Angiotensin II type 1 receptor expression in ovarian cancer and its correlation with tumour angiogenesis and patient survival. *Br J Cancer*. 2006; 94:552–60.
20. Xu J, Fan J, Wu F, Huang Q, Guo M, Lv Z, Han J, Duan L, Hu G, Chen L, Liao T, Ma W, Tao X, Jin Y. The ACE2/Angiotensin-(1-7)/Mas Receptor Axis: Pleiotropic Roles in Cancer. *Front Physiol*. 2017; 8:276.
21. Yu C, Tang W, Wang Y, Shen Q, Wang B, Cai C, Meng X, Zou F. Downregulation of ACE2/Ang-(1-7)/Mas axis promotes breast cancer metastasis by enhancing store-operated calcium entry. *Cancer Lett*. 2016; 376:268–77.
22. Zheng S, Yang Y, Song R, Yang X, Liu H, Ma Q, Yang L, Meng R, Tao T, Wang S, He J. Ang-(1-7) promotes the migration and invasion of human renal cell carcinoma cells via Mas-mediated AKT signaling pathway. *Biochem Biophys Res Commun*. 2015; 460:333–40.
23. Zhao Y, Chen X, Cai L, Yang Y, Sui G, Fu S. Angiotensin II/angiotensin II type I receptor (AT1R) signaling promotes MCF-7 breast cancer cells survival via PI3-kinase/Akt pathway. *J Cell Physiol*. 2010; 225:168–73.
24. Husain K, Hernandez W, Ansari RA, Ferder L. Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J Biol Chem*. 2015; 6:209–17.
25. Pacurari M, Kafoury R, Tchounwou PB, Ndebele K. The Renin-Angiotensin-aldosterone system in vascular inflammation and remodeling. *Int J Inflamm*. 2014; 2014:689360.
26. Kim G, Kim J, Lim YL, Kim MY, Baik SK. Renin-angiotensin system inhibitors and fibrosis in chronic liver disease: a systematic review. *Hepatol Int*. 2016; 10:819–28.
27. Saber S, Mahmoud AA, Helal NS, El-Ahwany E, Abdelghany RH. Renin-angiotensin system inhibition ameliorates CCl4-induced liver fibrosis in mice through the inactivation of nuclear transcription factor kappa B. *Can J Physiol Pharmacol*. 2018; 96:569–76.
28. Zhu Q, Li N, Li F, Zhou Z, Han Q, Lv Y, Sang J, Liu Z. Therapeutic effect of renin angiotensin system inhibitors on liver fibrosis. *J Renin Angiotensin Aldosterone Syst*. 2016; 17:1470320316628717.
29. Sugimoto M, Yamaoka Y, Shirai N, Furuta T. Role of renin-angiotensin system in gastric oncogenesis. *J Gastroenterol Hepatol*. 2012; 27:442–51.
30. Wegman-Ostrosky T, Soto-Reyes E, Vidal-Millán S, Sánchez-Corona J. The renin-angiotensin system meets the

hallmarks of cancer. *J Renin Angiotensin Aldosterone Syst.* 2015; 16:227–33.

31. Neo JH, Ager EI, Angus PW, Zhu J, Herath CB, Christophi C. Changes in the renin angiotensin system during the development of colorectal cancer liver metastases. *BMC Cancer.* 2010; 10: 134.
32. Ager EI, Neo J, Christophi C. The renin-angiotensin system and malignancy. *Carcinogenesis.* 2008; 29:1675–84.
33. Carbajo-Lozoya J, Lutz S, Feng Y, Kroll J, Hammes HP, Wieland T. Angiotensin II modulates VEGF-driven angiogenesis by opposing effects of type 1 and type 2 receptor stimulation in the microvascular endothelium. *Cell Signal.* 2012; 24:1261–69.
34. Usui T, Sugisaki K, Iriyama A, Yokoo S, Yamagami S, Nagai N, Ishida S, Amano S. Inhibition of corneal neovascularization by blocking the angiotensin II type 1 receptor. *Invest Ophthalmol Vis Sci.* 2008; 49:4370–76.
35. Shirotake S, Miyajima A, Kosaka T, Tanaka N, Maeda T, Kikuchi E, Oya M. Angiotensin II type 1 receptor expression and microvessel density in human bladder cancer. *Urology.* 2011; 77:1009.e19–25.
36. Senger DR, Davis GE. Angiogenesis. *Cold Spring Harb Perspect Biol.* 2011; 3:a005090.
37. Mittal K, Ebos J, Rini B. Angiogenesis and the tumor microenvironment: vascular endothelial growth factor and beyond. *Semin Oncol.* 2014; 41:235–51.
38. Stefanska A, Kenyon C, Christian HC, Buckley C, Shaw I, Mullins JJ, Péault B. Human kidney pericytes produce renin. *Kidney Int.* 2016; 90:1251–61.
39. Berg AC, Chernavsky-Sequeira C, Lindsey J, Gomez RA, Sequeira-Lopez ML. Pericytes synthesize renin. *World J Nephrol.* 2013; 2:11–16.
40. Nakamura K, Yaguchi T, Ohmura G, Kobayashi A, Kawamura N, Iwata T, Kiniwa Y, Okuyama R, Kawakami Y. Involvement of local renin-angiotensin system in immunosuppression of tumor microenvironment. *Cancer Sci.* 2018; 109:54–64.
41. Patel VB, Zhong JC, Grant MB, Oudit GY. Role of the ACE2/Angiotensin 1-7 Axis of the Renin-Angiotensin System in Heart Failure. *Circ Res.* 2016; 118:1313–26.
42. Han CD, Ge WS. Up-Regulation of Angiotensin-Converting Enzyme (ACE) Enhances Cell Proliferation and Predicts Poor Prognosis in Laryngeal Cancer. *Med Sci Monit.* 2016; 22:4132–38.
43. Cheng Q, Zhou L, Zhou J, Wan H, Li Q, Feng Y. ACE2 overexpression inhibits acquired platinum resistance-induced tumor angiogenesis in NSCLC. *Oncol Rep.* 2016; 36:1403–10.
44. Pei N, Wan R, Chen X, Li A, Zhang Y, Li J, Du H, Chen B, Wei W, Qi Y, Zhang Y, Katovich MJ, Summers C, et al. Angiotensin-(1-7) Decreases Cell Growth and Angiogenesis of Human Nasopharyngeal Carcinoma Xenografts. *Mol Cancer Ther.* 2016; 15:37–47.
45. Izzedine H, Derosa L, Le Teuff G, Albiges L, Escudier B. Hypertension and angiotensin system inhibitors: impact on outcome in sunitinib-treated patients for metastatic renal cell carcinoma. *Ann Oncol.* 2015; 26:1128–33.
46. Keizman D, Gottfried M, Ish-Shalom M, Maimon N, Peer A, Neumann A, Hammers H, Eisenberger MA, Sinibaldi V, Pili R, Hayat H, Kovel S, Sella A, et al. Active smoking may negatively affect response rate, progression-free survival, and overall survival of patients with metastatic renal cell carcinoma treated with sunitinib. *Oncologist.* 2014; 19:51–60.
47. Keizman D, Huang P, Eisenberger MA, Pili R, Kim JJ, Antonarakis ES, Hammers H, Carducci MA. Angiotensin system inhibitors and outcome of sunitinib treatment in patients with metastatic renal cell carcinoma: a retrospective examination. *Eur J Cancer.* 2011; 47:1955–61.
48. McKay RR, Rodriguez GE, Lin X, Kaymakcalan MD, Hamnvik OP, Sabbisetti VS, Bhatt RS, Simantov R, Choueiri TK. Angiotensin system inhibitors and survival outcomes in patients with metastatic renal cell carcinoma. *Clin Cancer Res.* 2015; 21:2471–79.
49. Osumi H, Matsusaka S, Wakatsuki T, Suenaga M, Shinozaki E, Mizunuma N. Angiotensin II type-1 receptor blockers enhance the effects of bevacizumab-based chemotherapy in metastatic colorectal cancer patients. *Mol Clin Oncol.* 2015; 3:1295–300.
50. Pinter M, Weinmann A, Wörns MA, Hücke F, Bota S, Marquardt JU, Duda DG, Jain RK, Galle PR, Trauner M, Peck-Radosavljevic M, Sieghart W. Use of inhibitors of the renin-angiotensin system is associated with longer survival in patients with hepatocellular carcinoma. *United European Gastroenterol J.* 2017; 5:987–96.
51. Levin VA, Chan J, Datta M, Yee JL, Jain RK. Effect of angiotensin system inhibitors on survival in newly diagnosed glioma patients and recurrent glioblastoma patients receiving chemotherapy and/or bevacizumab. *J Neurooncol.* 2017; 134:325–30.
52. Vasudev NS, Reynolds AR. Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. *Angiogenesis.* 2014; 17:471–94.
53. Jain RK, Duda DG, Willett CG, Sahani DV, Zhu AX, Loeffler JS, Batchelor TT, Sorensen AG. Biomarkers of response and resistance to antiangiogenic therapy. *Nat Rev Clin Oncol.* 2009; 6:327–38.
54. Schaaf MB, Garg AD, Agostinis P. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis.* 2018; 9:115.
55. Tannir NM, Schwab G, Grünwald V. Cabozantinib: an Active Novel Multikinase Inhibitor in Renal Cell Carcinoma. *Curr Oncol Rep.* 2017; 19:14.
56. Campbell DJ. Clinical relevance of local Renin Angiotensin systems. *Front Endocrinol (Lausanne).* 2014; 5:113.
57. Thews O, Kelleher DK, Vaupel P. Disparate responses of tumour vessels to angiotensin II: tumour volume-dependent

- effects on perfusion and oxygenation. *Br J Cancer*. 2000; 83:225–31.
58. Xie G, Liu Y, Yao Q, Zheng R, Zhang L, Lin J, Guo Z, Du S, Ren C, Yuan Q, Yuan Y. Hypoxia-induced angiotensin II by the lactate-chymase-dependent mechanism mediates radioresistance of hypoxic tumor cells. *Sci Rep*. 2017; 7:42396. <https://www.nature.com/articles/srep42396#supplementary-information>.
 59. Riemann A, Reime S, Thews O. Tumor Acidosis and Hypoxia Differently Modulate the Inflammatory Program: Measurements *In Vitro* and *In Vivo*. *Neoplasia*. 2017; 19:1033–42.
 60. Dayan F, Mazure NM, Brahimi-Horn MC, Pouyssegur J. A dialogue between the hypoxia-inducible factor and the tumor microenvironment. *Cancer Microenviron*. 2008; 1:53–68.
 61. Anderson KG, Stromnes IM, Greenberg PD. Obstacles Posed by the Tumor Microenvironment to T cell Activity: A Case for Synergistic Therapies. *Cancer Cell*. 2017; 31:311–25.
 62. Shurin GV, Ma Y, Shurin MR. Immunosuppressive mechanisms of regulatory dendritic cells in cancer. *Cancer Microenviron*. 2013; 6:159–67.
 63. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel)*. 2014; 6:1670–90.
 64. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, Iyer AK. PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Front Pharmacol*. 2017; 8:561.
 65. Walther T, Menrad A, Orzechowski HD, Siemeister G, Paul M, Schirner M. Differential regulation of *in vivo* angiogenesis by angiotensin II receptors. *FASEB J*. 2003; 17:2061–67.
 66. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci*. 2012; 125:5591–96.
 67. Fearon DT. Immune-Suppressing Cellular Elements of the Tumor Microenvironment. *Annual Review of Cancer Biology*. 2017; 1:241–55.
 68. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, Lichter T, Decker WK, Whelan RL, Kumara HM, Signori E, Honoki K, Georgakilas AG, et al. Immune evasion in cancer: mechanistic basis and therapeutic strategies. *Semin Cancer Biol*. 2015 (Suppl); 35:S185–98.
 69. Zelenay S, van der Veen AG, Böttcher JP, Snelgrove KJ, Rogers N, Acton SE, Chakravarty P, Girotti MR, Marais R, Quezada SA, Sahai E, Reis e Sousa C. Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. *Cell*. 2015; 162:1257–70.
 70. Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol*. 2004; 5:818–27.
 71. Uemura H, Ishiguro H, Ishiguro Y, Hoshino K, Takahashi S, Kubota Y. Angiotensin II induces oxidative stress in prostate cancer. *Mol Cancer Res*. 2008; 6:250–58.
 72. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, Mandruzzato S, Murray PJ, Ochoa A, Ostrand-Rosenberg S, Rodriguez PC, Sica A, Umansky V, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun*. 2016; 7:12150.
 73. Pyzer AR, Cole L, Rosenblatt J, Avigan DE. Myeloid-derived suppressor cells as effectors of immune suppression in cancer. *Int J Cancer*. 2016; 139:1915–26.
 74. Okwan-Duodu D, Landry J, Shen XZ, Diaz R. Angiotensin-converting enzyme and the tumor microenvironment: mechanisms beyond angiogenesis. *Am J Physiol Regul Integr Comp Physiol*. 2013; 305:R205–15.
 75. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood*. 2004; 104:2224–34.
 76. Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, Miyake T, Matsushita K, Okazaki T, Saitoh T, Honma K, Matsuyama T, Yui K, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol*. 2010; 11:936–44.
 77. Fleetwood AJ, Dinh H, Cook AD, Hertzog PJ, Hamilton JA. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J Leukoc Biol*. 2009; 86:411–21.
 78. Aki K, Shimizu A, Masuda Y, Kuwahara N, Arai T, Ishikawa A, Fujita E, Mii A, Natori Y, Fukunaga Y, Fukuda Y. ANG II receptor blockade enhances anti-inflammatory macrophages in anti-glomerular basement membrane glomerulonephritis. *Am J Physiol Renal Physiol*. 2010; 298:F870–82.
 79. Kohlstedt K, Trouvain C, Namgaladze D, Fleming I. Adipocyte-derived lipids increase angiotensin-converting enzyme (ACE) expression and modulate macrophage phenotype. *Basic Res Cardiol*. 2011; 106:205–15.
 80. Wen SW, Ager EI, Neo J, Christophi C. The renin angiotensin system regulates Kupffer cells in colorectal liver metastases. *Cancer Biol Ther*. 2013; 14:720–27.
 81. Xing F, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front Biosci (Landmark Ed)*. 2010; 15: 166–79.
 82. Tao L, Huang G, Song H, Chen Y, Chen L. Cancer associated fibroblasts: an essential role in the tumor microenvironment. *Oncol Lett*. 2017; 14:2611–20.
 83. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers (Basel)*. 2015; 7:2443–58.

84. Terry S, Savagner P, Ortiz-Cuaran S, Mahjoubi L, Saintigny P, Thiery JP, Chouaib S. New insights into the role of EMT in tumor immune escape. *Mol Oncol*. 2017; 11:824–46.
85. Chae YK, Chang S, Ko T, Anker J, Agte S, Iams W, Choi WM, Lee K, Cruz M. Epithelial-mesenchymal transition (EMT) signature is inversely associated with T-cell infiltration in non-small cell lung cancer (NSCLC). *Sci Rep*. 2018; 8:2918.
86. Nguyen L, Ager EI, Neo J, Christophi C. Regulation of colorectal cancer cell epithelial to mesenchymal transition by the renin angiotensin system. *J Gastroenterol Hepatol*. 2016; 31:1773–82.
87. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer Cell*. 2010; 17:135–47.
88. Liu H, Naxerova K, Pinter M, Incio J, Lee H, Shigeta K, Ho WW, Crain JA, Jacobson A, Michelakos T, Dias-Santos D, Zanconato A, Hong TS, et al. Use of Angiotensin System Inhibitors Is Associated with Immune Activation and Longer Survival in Nonmetastatic Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res*. 2017; 23:5959–69.
89. Bernasconi R, Nyström A. Balance and circumstance: the renin angiotensin system in wound healing and fibrosis. *Cell Signal*. 2018; 51:34–46.
90. Min LJ, Cui TX, Yahata Y, Yamasaki K, Shiuchi T, Liu HW, Chen R, Li JM, Okumura M, Jinno T, Wu L, Iwai M, Nahmias C, et al. Regulation of collagen synthesis in mouse skin fibroblasts by distinct angiotensin II receptor subtypes. *Endocrinology*. 2004; 145:253–60.
91. Cui T, Nakagami H, Iwai M, Takeda Y, Shiuchi T, Daviet L, Nahmias C, Horiuchi M. Pivotal role of tyrosine phosphatase SHP-1 in AT2 receptor-mediated apoptosis in rat fetal vascular smooth muscle cell. *Cardiovasc Res*. 2001; 49:863–71.
92. Schnittert J, Bansal R, Storm G, Prakash J. Integrins in wound healing, fibrosis and tumor stroma: high potential targets for therapeutics and drug delivery. *Adv Drug Deliv Rev*. 2018; 129:37–53.
93. Watt J, Kocher HM. The desmoplastic stroma of pancreatic cancer is a barrier to immune cell infiltration. *Oncoimmunology*. 2013; 2:e26788.
94. Mrass P, Kinjyo I, Ng LG, Reiner SL, Puré E, Weninger W. CD44 mediates successful interstitial navigation by killer T cells and enables efficient antitumor immunity. *Immunity*. 2008; 29:971–85.
95. Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci U S A*. 2011; 108:2909–14.
96. Chauhan VP, Martin JD, Liu H, Lacorre DA, Jain SR, Kozin SV, Stylianopoulos T, Mousa AS, Han X, Adstamongkonkul P, Popović Z, Huang P, Bawendi MG, et al. Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels. *Nat Commun*. 2013; 4:2516.
97. da Costa PL, Sirois P, Tannock IF, Chammas R. The role of kinin receptors in cancer and therapeutic opportunities. *Cancer Lett*. 2014; 345:27–38.
98. Blaes N, Girolami JP. Targeting the ‘Janus face’ of the B2-bradykinin receptor. *Expert Opin Ther Targets*. 2013; 17:1145–66.
99. Leeb-Lundberg LM, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev*. 2005; 57:27–77.
100. Moreau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A. The kallikrein-kinin system: current and future pharmacological targets. *J Pharmacol Sci*. 2005; 99:6–38.
101. Wu J, Akaike T, Hayashida K, Miyamoto Y, Nakagawa T, Miyakawa K, Müller-Esterl W, Maeda H. Identification of bradykinin receptors in clinical cancer specimens and murine tumor tissues. *Int J Cancer*. 2002; 98:29–35.
102. Hall JM. Bradykinin receptors. *Gen Pharmacol*. 1997; 28:1–6.
103. Ikeda Y, Hayashi I, Kamoshita E, Yamazaki A, Endo H, Ishihara K, Yamashina S, Tsutsumi Y, Matsubara H, Majima M. Host stromal bradykinin B2 receptor signaling facilitates tumor-associated angiogenesis and tumor growth. *Cancer Res*. 2004; 64:5178–85.
104. Ishihara K, Kamata M, Hayashi I, Yamashina S, Majima M. Roles of bradykinin in vascular permeability and angiogenesis in solid tumor. *Int Immunopharmacol*. 2002; 2:499–509.
105. Bras G, Bochenska O, Rapala-Kozik M, Guevara-Lora I, Faussner A, Kozik A. Extracellular aspartic protease SAP2 of *Candida albicans* yeast cleaves human kininogens and releases proinflammatory peptides, Met-Lys-bradykinin and des-Arg(9)-Met-Lys-bradykinin. *Biol Chem*. 2012; 393:829–39.
106. Maria AG, Dillenburg-Pilla P, Reis RI, Floriano EM, Tefê-Silva C, Ramos SG, Pesquero JB, Nahmias C, Costa-Neto CM. Host kinin B1 receptor plays a protective role against melanoma progression. *Sci Rep*. 2016; 6:22078. <https://www.nature.com/articles/srep22078#supplementary-information>.
107. Chee J, Singh J, Naran A, Misso NL, Thompson PJ, Bhoola KD. Novel expression of kallikreins, kallikrein-related peptidases and kinin receptors in human pleural mesothelioma. *Biol Chem*. 2007; 388:1235–42.
108. Dlamini Z, Bhoola KD. Upregulation of tissue kallikrein, kinin B1 receptor, and kinin B2 receptor in mast and giant cells infiltrating oesophageal squamous cell carcinoma. *J Clin Pathol*. 2005; 58:915–22.
109. Molina L, Matus CE, Astroza A, Pavicic F, Tapia E, Toledo C, Perez JA, Nualart F, Gonzalez CB, Burgos RA, Figueroa

- CD, Ehrenfeld P, Poblete MT. Stimulation of the bradykinin B(1) receptor induces the proliferation of estrogen-sensitive breast cancer cells and activates the ERK1/2 signaling pathway. *Breast Cancer Res Treat.* 2009; 118:499–510.
110. Moodley R, Snyman C, Odhav B, Bhoola KD. Visualisation of transforming growth factor-beta 1, tissue kallikrein, and kinin and transforming growth factor-beta receptors on human clear-cell renal carcinoma cells. *Biol Chem.* 2005; 386:375–82.
 111. da Costa PL, Wynne D, Fifis T, Nguyen L, Perini M, Christophi C. The kallikrein-Kinin system modulates the progression of colorectal liver metastases in a mouse model. *BMC Cancer.* 2018; 18:382.
 112. Ignjatovic T, Tan F, Brovkovich V, Skidgel RA, Erdös EG. Activation of bradykinin B1 receptor by ACE inhibitors. *Int Immunopharmacol.* 2002; 2:1787–93.
 113. Aliberti J, Viola JP, Vieira-de-Abreu A, Bozza PT, Sher A, Scharfstein J. Cutting edge: bradykinin induces IL-12 production by dendritic cells: a danger signal that drives Th1 polarization. *J Immunol.* 2003; 170:5349–53.
 114. Zuccala E. Chemotherapy: clocking up resistance. *Nat Rev Cancer.* 2016; 16:343.
 115. Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. *Br J Cancer.* 2018; 118:9–16.

Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases

Dora Lucia Vallejo Ardila , Katrina A Walsh , Theodora Fifis , Rita Paolini , Georgios Kastrappis , Christopher Christophi , Marcos Vinicius Perini 

To cite: Vallejo Ardila DL, Walsh KA, Fifis T, *et al.* Immunomodulatory effects of renin-angiotensin system inhibitors on T lymphocytes in mice with colorectal liver metastases. *Journal for ImmunoTherapy of Cancer* 2020;**8**:e000487. doi:10.1136/jitc-2019-000487

► Additional material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/jitc-2019-000487>).

DLVA and KAW contributed equally.

Accepted 25 March 2020



© Author(s) (or their employer(s)) 2020. Re-use permitted under CC BY. Published by BMJ.

Surgery, The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne, Victoria, Australia

Correspondence to

Dr Dora Lucia Vallejo Ardila; dlucamed@gmail.com

ABSTRACT

Background It is now recognized that many anticancer treatments positively modulate the antitumor immune response. Clinical and experimental studies have shown that inhibitors of the classical renin-angiotensin system (RAS) reduce tumor progression and are associated with better outcomes in patients with colorectal cancer. RAS components are expressed by most immune cells and adult hematopoietic cells, thus are potential targets for modulating tumor-infiltrating immune cells and can provide a mechanism of tumor control by the renin-angiotensin system inhibitors (RASi).

Aim To investigate the effects of the RASi captopril on tumor T lymphocyte distribution in a mouse model of colorectal liver metastases.

Methods Liver metastases were established in a mouse model using an autologous colorectal cancer cell line. RASi (captopril 750 mg/kg) or carrier (saline) was administered to the mice daily via intraperitoneal injection, from day 1 post-tumor induction to endpoint (day 15 or 21 post-tumor induction). At the endpoint, tumor growth was determined, and lymphocyte infiltration and composition in the tumor and liver tissues were analyzed by flow cytometry and immunohistochemistry (IHC).

Results Captopril significantly decreased tumor viability and impaired metastatic growth. Analysis of infiltrating T cells into liver parenchyma and tumor tissues by IHC and flow cytometry showed that captopril significantly increased the infiltration of CD3⁺ T cells into both tissues at day 15 following tumor induction. Phenotypical analysis of CD45⁺ CD3⁺ T cells indicated that the major contributing phenotype to this influx is a CD4 and CD8 double-negative T cell (DNT) subtype, while CD4⁺ T cells decreased and CD8⁺ T cells remained unchanged. Captopril treatment also increased the expression of checkpoint receptor PD-1 on CD8⁺ and DNT subsets.

Conclusion Captopril treatment modulates the immune response by increasing the infiltration and altering the phenotypical composition of T lymphocytes and may be a contributing mechanism for tumor control.

BACKGROUND

Colorectal cancer (CRC) is the third most common cancer diagnosis, and it is ranked

second as cancer causing death for both sexes combined in high-income countries.¹ Over half of the patients with CRC will develop metastatic liver disease. Subsequently, colorectal liver metastases (CRLM) result in death in at least two-thirds of these patients.² Surgical resection of CRLM is the only potential curative treatment and, when combined with chemotherapy, can achieve 5-year survival rates of 40%–60%.^{2,3} However, the majority of these patients will develop tumor recurrence, most commonly in the liver.⁴ Current research to improve this clinical outcome is focusing on identifying reliable prognostic indicators of treatment response,^{5,6} tumors with a high-risk molecular signature for recurrence,⁷ and in developing novel strategies to immunomodulate the tumor microenvironment (TME).⁸ Established tumors subvert the host antitumor immune response through several mechanisms, including the accumulation of immunosuppressive tumor-associated macrophages (TAMs), myeloid-derived suppressive cells (MDSCs) and cancer-associated fibroblasts (CAFs). Also, the production of immunosuppressive factors influences the infiltration and activation of effector immune cells and the upregulation of T cell inhibitory ligands like programmed death-ligand 1 (PD-L1) on tumor, stromal and immune cells.⁹ During the last few years, many different cancer types have proven responsive to therapeutic targeting of inhibitory T cell receptors by immune check-point inhibitors, such as ipilimumab,¹⁰ nivolumab¹¹ and pembrolizumab.¹² In the case of CRLM, only a small subset of patients, those with deficient mismatch repair pathways, respond to these treatments.¹³ Targeting immunological features that promote metastatic growth, such as molecules that are proinflammatory,

proangiogenic, profibrotic, or specific leukocyte subsets that may change in number, phenotype or function in different types of cancer,^{14–16} could tip the balance towards an antitumor immune response.

Renin-angiotensin inhibitors (RASi) have shown antitumor effects that may have a profound impact in cancer therapy.^{17–19} The role of the various RASi in modulating inflammation²⁰ and fibrosis^{21–22} is well documented. Renin-angiotensin system (RAS) pathway components are expressed by most cells of the innate and adaptive immune system.²³ *In vitro* studies indicate that RAS signaling modulates the activity of various immune cells; on the other hand, *in vivo* studies of cancer immune responses modulated by RASi are still scarce. One study using a mouse renal cancer model reported that captopril increased tumor growth and inhibited the antigen-specific activation of CD8⁺ and CD4⁺ T cells while promoting antigen-specific B cells and their infiltration into tumors.²⁴ In contrast, studies from this laboratory using captopril or angiotensin receptor 1 (AT1R) blockers reported a significant reduction in CRLM and modulation of TAMs.^{18–19–25–26} Supporting these findings, Nakamura *et al* demonstrated that RASi reduced the immunosuppressive activity of TAMs, MDSCs, and CAFs in the TME, leading to the induction and tumor infiltration of tumor antigen-specific T cells.²⁷

In this study, the effects of captopril treatment (RASi) on T lymphocyte subtypes and their expression of some activation or inhibitory factors were investigated in a CRC liver metastasis mouse model.

MATERIALS AND METHODS

Animals and experimental model of CRLM

Male CBA mice (ARC, Perth) were maintained in standard cages with irradiated food and water supplied *ad libitum*. The primary cancer cell line mouse CRC cell (MoCR) was derived from a dimethyl hydrazine-induced primary colon carcinoma in the CBA mouse and maintained *in vivo* by serial passage in the flanks of CBA mice.²⁸ For passage and experimentation, subcutaneous tumors were teased apart, passed through a filter, treated with Ethylenediaminetetra-acetic acid (EDTA) and washed in phosphate-buffered saline (PBS) to make a single cell suspension. CRLMs were induced by intrasplenic injection of 5×10^4 tumor cells followed by splenectomy. Metastases are fully established by 21 days following tumor induction (figure 1A,B).

Experimental design

Tissues were collected at days 15 and 21 post-tumor induction and were used to evaluate tumor growth and T cell immune infiltration by immunohistochemistry (IHC) and fluorescent-activated cell sorting (FACS) (figure 1A).

Treatment protocol

Mice received tumor induction and were separated into two groups: control (saline) and treatment (captopril).

Captopril (pH adjusted to 7.4) was administered daily via intraperitoneal injection adjusted to the body weight of each mouse (750 mg/kg), from the day 1 post-tumor induction to endpoint. Control mice received an equal volume of saline. Tumor growth was assessed by stereology on day 15 or 21 following tumor induction.

At each endpoint, animals were terminally anesthetized; laparotomy was performed; and the abdominal cavity was examined for indications of extrahepatic metastases, paying close attention to the splenic bed. The liver was carefully perfused with 20 mL saline solution, then excised and harvested. Immediately following excision, liver weights were recorded. Livers were then fixed in formalin (10%) (Sigma Aldrich, Castle Hill, New South Wales, Australia) for 24 hours and then transferred to 70% ethanol.

Stereology

Images of the whole liver were taken to examine the tumor distribution and burden load. The liver was then transversely sliced into sections of 1.5 mm thickness using a tissue fractionator. For large livers (saline control), every second slice was sampled for analysis. For smaller livers (captopril-treated tumors), every slice was taken for analysis. Liver slices were placed on a clear plastic plate; a digital camera (Nikon Coolpix 5000, E500) was used to capture the images, and these were analyzed using image analysis software (Image-Pro Plus, Perth, Australia).

Tumors were visualized as distinctive white/cream-colored areas against the red/brown liver tissue. Each tumor outline was traced using image analysis software to determine the area occupied by the tumor. Stereology technique was used to determine tumor burden.

Immunohistochemistry

Formalin-fixed paraffin tissue sections (4 µm) were used with an indirect peroxidase labeling technique (Envision Plus, DAKO, Australia). Following deparaffinization and rehydration, endogenous peroxidase activity was blocked with 3% H₂O₂, and non-specific binding inhibited with 10% normal goat serum (01–6201 Thermo Fisher scientific). Heat-induced epitope retrieval was used. Antigens were visualized by immunohistochemical staining using their respective antibodies diluted as follows: CD3 1:100 (Clone A0452 DAKO), CD4 1:1000 (4SM95 e-Bioscience), PD-L1 1:500 (E1L3N, Cell Signaling Technology) and negative controls were stained without the corresponding primary antibodies. Following primary antibody treatment, sections were incubated with a horseradish peroxidase-labeled polymer secondary antibody. The antigen-antibody complex was visualized by diaminobenzene peroxidase substrate solution (DAKO, Australia). Viable tumor was determined by Haematoxylin and Eosin (H&E) staining.

Image caption and analysis algorithm

Slides were scanned at $\times 20$ magnification (Aperio Scanscope AT Turbo, Leica Biosystems). Image analysis

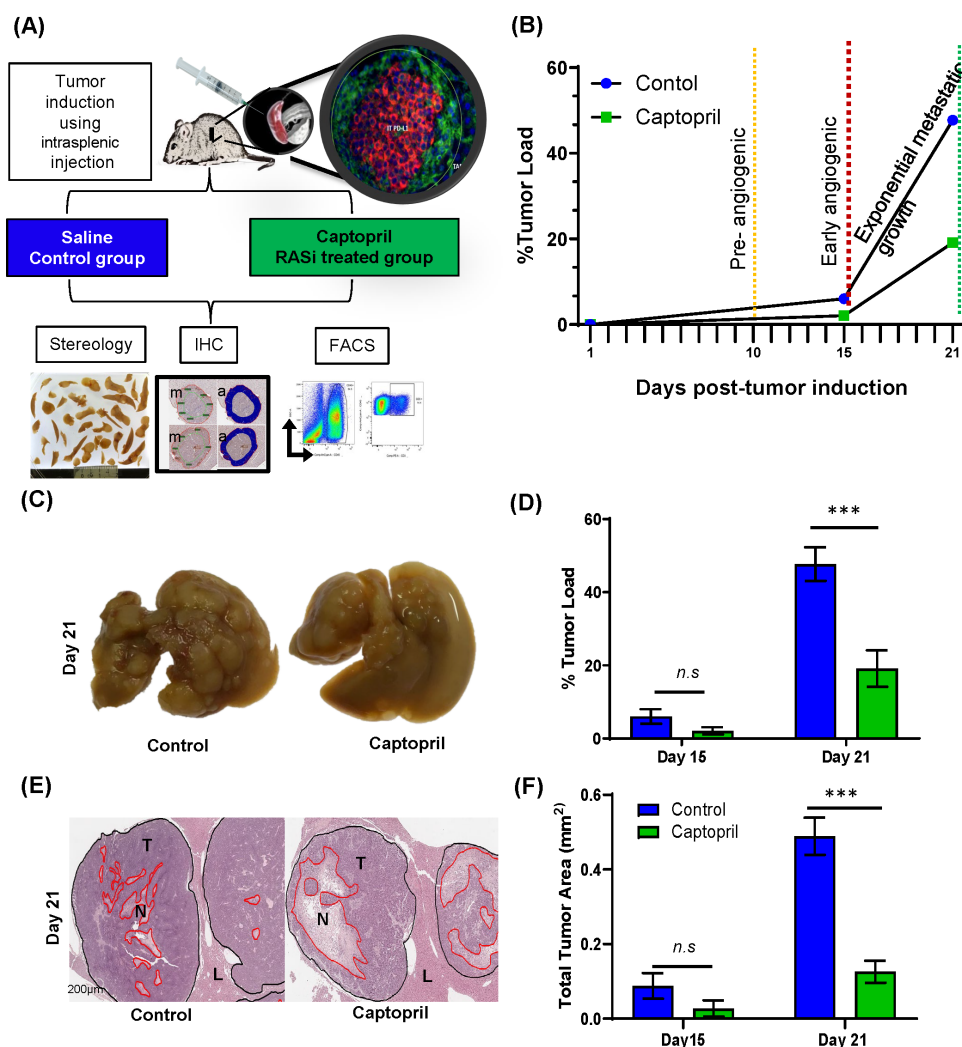


Figure 1 Control of tumor growth and viability by RASi treatment. (A) Liver metastases were induced by intrasplenic tumor induction and allowed to develop for 15 and 21 days. Captopril or saline control treatment was delivered daily by intraperitoneal injection. At each endpoint, mice were terminally anesthetized, and their livers perfused with saline prior to removal. Most of the livers were fixed and prepared for IHC and stereology, while a small proportion was prepared for flow cytometry, including the separation of tumor from the liver tissues. (B) Schematic of tumor growth kinetics following tumor induction and daily treatment. (C) Representative livers of each treatment are pictured. (D) Livers were fixed, sectioned and analyzed by stereology for % tumor burden of the total liver area and (E) stained with H&E for viable tumor analyses (T, tumor; N, necrosis and L, Liver). (F) Proportion of tumor viability. Data represent average $\pm$ SEM of $n=5$ per group; significance was calculated by Student t-test. *** $P<0.001$. FACS, fluorescent-activated cell sorting; IHC, immunohistochemistry; RASi, renin-angiotensin system inhibitor; n.s, not significant.

software (Aperio ImageScope) measured the number of positive cells within designated areas. Given the size of immune cells, the Positive Pixel Count v9 (V.9.1) algorithm was applied as a basis for detecting immune marker positivity, with the intensity thresholds adjusted manually to remove background artifacts and to account for variable differences in cell size (hue value (center): 0.1, hue width: 0.33 and color saturation threshold: 0.15). CD3⁺ cell distribution between tumor periphery and core was examined at day 15 (online supplementary figure S1A) and day 21 post-tumor induction. Same conditions were applied to measure CD4⁺ cells at day 15 (online supplementary figure S1B). The tumor ‘periphery’ was defined to include approximately an equal length of the leading edge of tumor cells and the

adjacent stromal interface defined as the inner invasive margin. The ‘core of the tumor’ was defined as the total tumor area excluding the periphery. For each of these regions (periphery/leading edge or core), a total area up to of 100 μ m per pixel containing the highest density of IHC⁺ cells was drawn to encompass these areas. When the inner invasive tumor margin (TM) or center of the tumor was smaller than 5 μ m per pixel, the positivity of the whole tumor and the invasive margin was considered the same. PD-L1 expression was counted in the membranous and cytoplasmic tumor region using the same staining intensity thresholds (intensity threshold weak: 220–175, medium: 175–100, strong: 100–0, and negative pixels: –1) .²⁹

Flow cytometry

At endpoints of 15 days following tumor induction, livers were excised as described earlier, and caudate lobes were separated for processing for flow cytometry. Tumors were macroscopically isolated from liver parenchyma, and both tumor and macroscopically tumor-free liver were treated with tissue digest medium (0.25 mg/mL Liberase, Sigma and DNase, Sigma) at 37 degrees for 40 min. Tissues were then pushed through a stainless-steel mesh, treated with red blood cell lysis buffer, and resuspended in FACS wash buffer (10% bovine serum albumin/5 mM EDTA/0.01% sodium Azide in PBS PH 7). 1×10^6 cells were stained with antibody cocktails directly conjugated to fluorochromes (CD45-BV510, CD3-PE, CD8-PECy7, PD-1-APC, CD4-APC-Cy7 (BD Bioscience) and CD103-FITC (Miltenyi biotec) and the viability dye, DAPI (additional file 1: online supplementary table 1). Each sample was analyzed on a FACS CANTO II (BD Biosciences) and data were analyzed by FlowJo™ v10. Lymphocytes were identified as live (DAPI negative), single cells that were negative for autofluorescence, CD45⁺ and CD3⁺ (online supplementary figure S2A). Fluorescence minus one (FMO) controls were used to accurately gate CD4⁺ or CD8⁺ T cells and then the expression of PD-1 and CD103 on these T cell subsets (online supplementary figure S2B), including CD3⁺ cells that were expressing neither CD4 nor CD8, termed double-negative T cells (DNTs).

Statistical analysis

All data were presented as the mean value of each group $\pm$ SE of the mean. Correlation matrix was generated using correlation coefficient *r* (or *rs*) for each pair of variables: length (μ m), area (μ m²), TM positivity, tumor core positivity and total positivity. A heat map of *R*² calculating *p* values (two-tail) was generated. Statistical analysis was conducted using GraphPad Prism V.8.3.0 (GraphPad Software, San Diego, California, USA) using both parametric and non-parametric analytical tests as appropriate. All statistical tests were two-sided, and a *p*-value of 0.05 was considered statistically significant.

RESULTS

Captopril inhibits metastatic growth and reduces tumor viability

The stages of metastatic development of the CRLM model have been previously characterized (figure 1B).²⁸ These experiments confirmed previous findings by this laboratory¹⁸ that captopril treatment has profound effects over metastatic growth after the metastases were fully established by 21 days following tumor induction (figure 1C,D). In this study, at day 15 endpoint, there was a trend in the reduction of tumor load by 3.94% difference. In contrast, liver collected at 21 days post-tumor induction from the group treated with captopril had significantly less tumor burden than the control (28.61% difference) (figure 1D). In this study, we also examined tumor viability as this is a better indication of treatment efficacy. Quantitation of

live tumor area demonstrates that the percentage of viable tumor area in the captopril treatment group was significantly less, compared with the control group at 21 days post-tumor induction (figure 1E,F). Similar to the findings for tumor burden, while there is a trend, no significant differences in tumor viability could be determined at day 15 (figure 1F). Captopril treatment reduced the number of metastases, though this reduction did not reach any significance, the size of metastatic foci was in fact significantly reduced (online supplementary figure S3).

Captopril treatment modulates spatial and temporal infiltration of tumor lymphocytes expressing CD3⁺ and CD4⁺

We used IHC to examine changes in CD3⁺ and CD4⁺ lymphocytes within the tumor tissues due to captopril treatment. Significant increases were seen in CD3⁺ lymphocytes in the treated group at day 15 (figure 2A,C). On the other hand, CD4⁺ lymphocytes were significantly decreased at this time point (day 15) as seen in figure 2B,D. Visual examination of the staining shows that lymphocyte infiltration tends to accumulate around the intratumoral vessels (figure 2A,B). Significant increases in the number of CD3⁺ lymphocytes were observed in both TM and intratumoral assessment in the captopril-treated group (figure 2A).

Interestingly, both density and distribution of CD3⁺ tumor-infiltrating lymphocytes inversely correlate with the area of liver metastases (online supplementary figure S4A). While the frequency of CD4⁺ cells in the control group significantly decreased in the same pattern as the CD3⁺ lymphocytes between the two time points; in the treated group, the CD4⁺ T cells did not significantly change compared with day 15, and in fact, at day 21, the frequency of CD4⁺ in the treated group is relatively higher than at day 15, but it is not significantly higher compared with that of the control at day 21 (figure 2C,D). These results suggest that the increased infiltration of CD3⁺ T cell population at day 15 was clearly not CD4⁺ T cells and that at day 21, these are no longer within the tumor in elevated numbers, giving impetus for a thorough investigation of day 15 T cell phenotype changes induced by captopril (figure 2).

Captopril treatment does not alter the tumor PD-L1 expression

Using IHC we also investigated whether the tumor in our model expresses PD-L1 (figure 3A), and whether captopril treatment alters tumor PD-L1 expression. While there is a strong expression of PD-L1 in the tumor, this expression was not significantly altered at any time point due to the treatment (figure 3B) and was not tumor size dependent (figure 3C). No significant difference was found between different populations of tumor cells expressing PD-L1 at different intensities between the control group and captopril at day 15 (figure 3D).

Captopril treatment differentially modulates T cell subpopulations infiltrating into the tumor and liver tissues

Flow cytometry analysis of dissected tumor tissues was used to identify the phenotype and the subpopulations

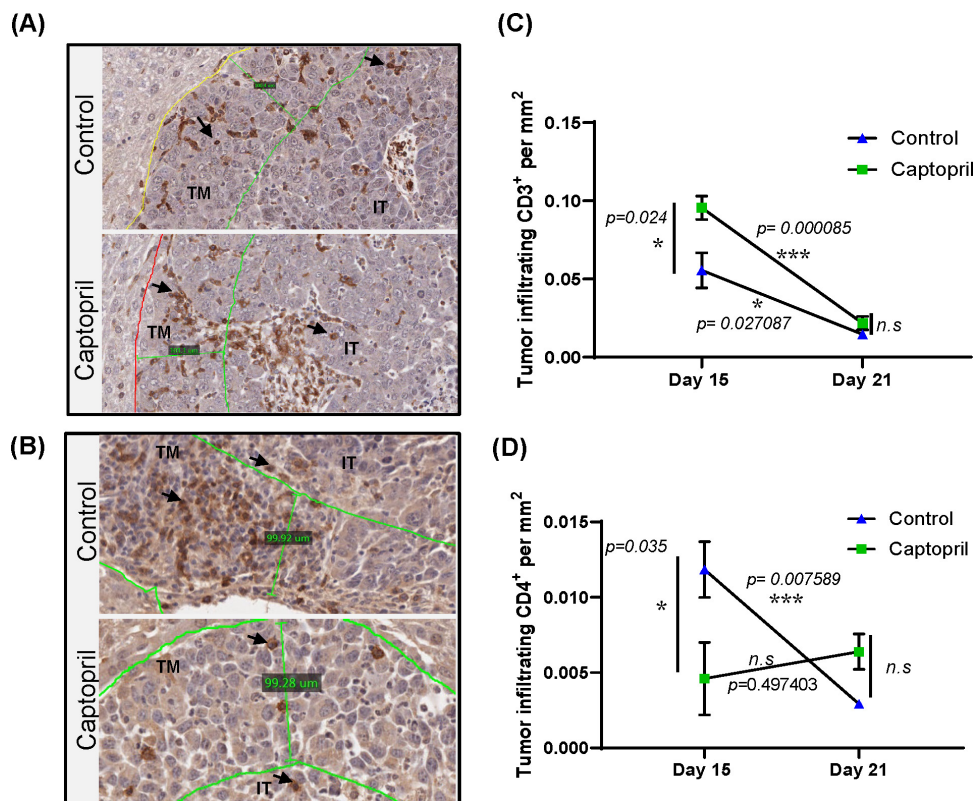


Figure 2 Tumor infiltrating lymphocytes are modulated by RASi. Representative microphotographs of histological quantitation of (A) CD3⁺ lymphocytes and (B) CD4⁺ lymphocytes in tumor tissues at day 15 (scale bar 100µm), including mark up for tumor margin (TM) and intratumoral (IT) areas. Quantitation of the total area staining positive for (C) CD3⁺ or (D) CD4⁺ as an area per mm² of total tumor area, (excluding necrotic areas) for tissues collected at day 15 and day 21. Data n≥5 presented as mean ± SEM. Significantly different data represented by * p<0.05, ** p<0.01, and ***p<0.001. n.s., not significant.

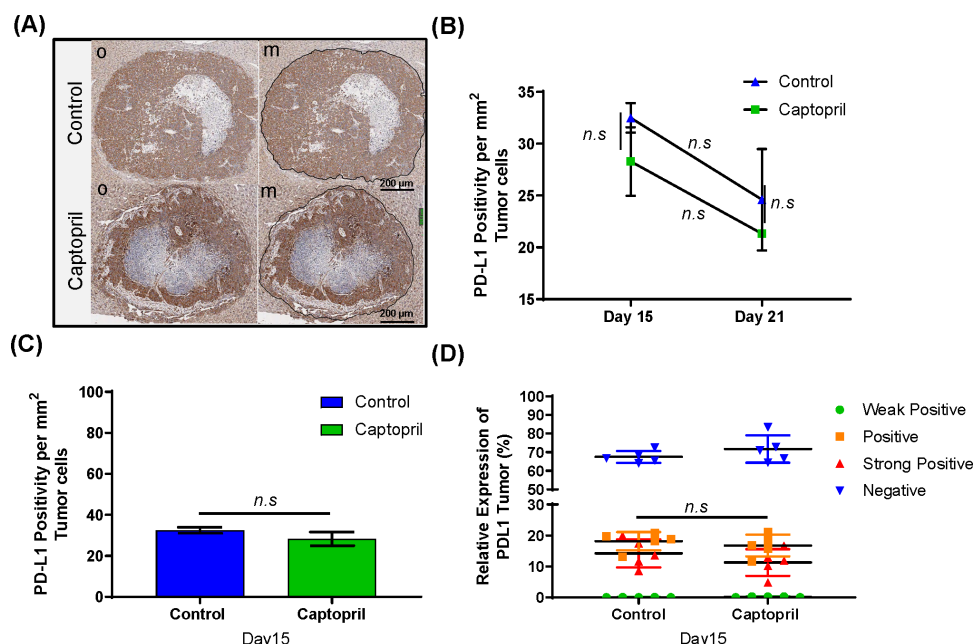


Figure 3 Temporal and spatial PD-L1⁺ expression in tumor cells. (A) Representative microphotographs of IHC PD-L1 staining; original (o) and marked-up (m) at day 15 post-tumor induction (black line) (Scale bar = 200 µm). (B) Histological quantitation of PD-L1 expressions at day 15 and 21 post-tumor induction. (C) Quantitation of the total tumor area staining positive for PD-L1⁺ as an area per mm² of total tumor area, (excluding necrotic areas) for tissues collected at day 15. (D) PD-L1 expression in tumor cells comparison between four levels of staining are shown: weak positive (green), positive (orange), strong positive (red), and negative (blue). Datasets expressed as mean ± SEM with n ≥ 5 mice. n.s., not significant.

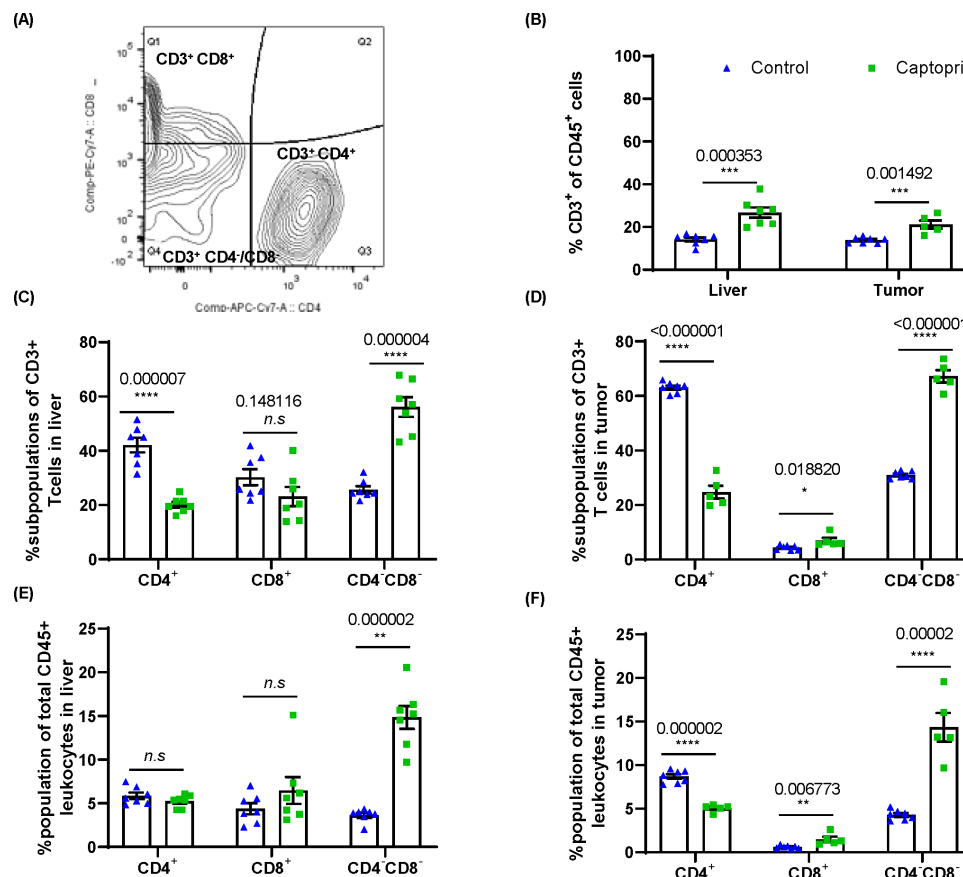


Figure 4 Renin-angiotensin system inhibitor differentially alters the proportions of CD3⁺ subpopulations of lymphocytes in the liver and tumor. (A) Gating strategy for analysis of CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁻CD8⁻. (B) Quantitation of the proportion of CD3⁺ lymphocytes as a proportion of total CD45⁺ leukocytes in the liver and tumor. The proportion (%) of CD3⁺ lymphocytes expressing CD4, CD8 and those negative for both CD4 and CD8 (CD4⁻CD8⁻) in (C) the liver and (D) tumor. The proportion of each subpopulation within the total CD45⁺ leukocyte population in (E) the liver and (F) tumor. Data n≥5 are presented as mean±SEM; significantly different data are represented by *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. n.s., not significant.

of CD3⁺ T cells in tumor that were observed by IHC to be modulated by captopril at day 15 (figure 4A). We also analyzed the T cell subpopulation infiltration into liver tissues that had been macroscopically dissected from tumors. During liver harvesting, livers were perfused via portal vein cannulation with 20 mL of saline to remove peripherally circulating lymphocytes from the analysis; thus, data represent tissue-infiltrating cells.

FACS confirmed that captopril treatment significantly increased the infiltration of CD3⁺ T cells into the tumor. Interestingly, these changes were also seen within the liver parenchyma (figure 4B) indicating that CD3⁺ T cells, trafficking from circulation and/or dividing within the tissue in both liver and tumor, are affected by captopril.

The phenotype of CD3⁺ T cells was examined in terms of the proportion of these cells expressing CD4 and CD8 and those expressing neither CD4 nor CD8, termed DNT (figure 4C,D) and were found to be differentially modulated by captopril. Both in the tumor and liver tissues, there is a decrease in the proportion of CD4⁺ and an increase in the proportion of DNT. On the other hand, there is an increased proportion of CD8⁺ T cells in the tumor, while in the liver, this subpopulation decreases (figure 4C,D).

When considering these populations as a proportion of total leukocytes (CD45⁺) in these tissues, we find that in the liver, captopril treatment does not change either CD4⁺ or CD8⁺ subpopulations, whereas the DNT population is significantly increased (figure 4E). In the tumor, we also see a decrease in the CD4⁺ population while the CD8⁺ and DNT subpopulations significantly increased (figure 4F). This may indicate that in the liver tissue, the increase in CD3⁺ T cells induced by captopril is due to the infiltration of DNTs. Within the tumor, however, the changes induced by captopril are more complex and may reflect changes in the TME that affect the ability of each subpopulation to exist there.

Captopril treatment differentially modulates the activation of T cell subtypes in the tumor and liver tissues

To investigate changes in the activation state of the CD3⁺ T cell subtypes, the cells were also stained for expression of Programmed cell death-1 (PD-1), an inhibitory receptor upregulated following T-cell activation by cognate antigen and is specific for checkpoint ligand PD-L1. Captopril treatment increased the proportion of CD8⁺ and DNT subpopulations expressing PD-1 in both liver and tumor (figure 5A,B).

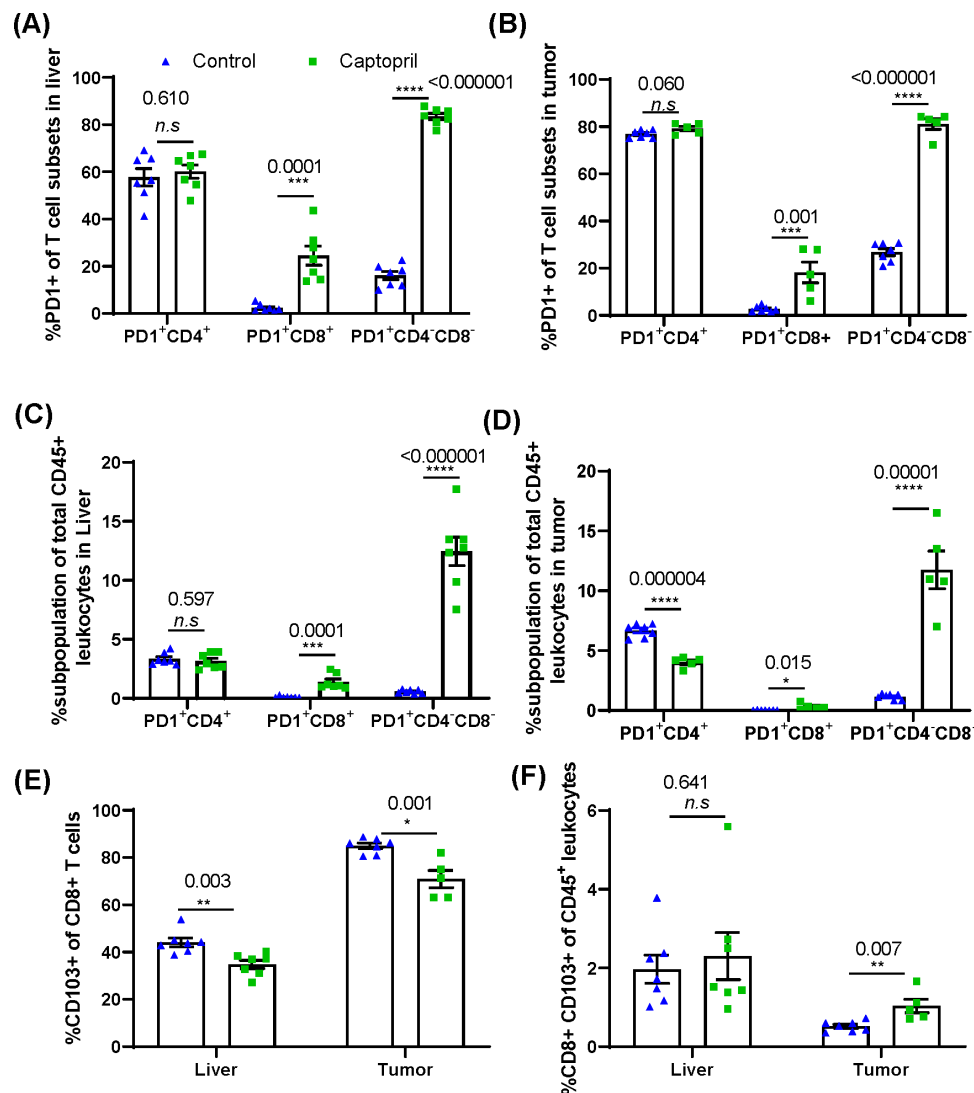


Figure 5 Renin-angiotensin system inhibitor differentially changes the proportions of CD3⁺ subpopulations expressing PD-1⁺ and tissue resident CD8⁺ lymphocytes in the liver and tumor. Quantitation of the proportion of each CD3⁺ subpopulation expressing PD-1⁺ in (A) the liver and (B) tumor. The percentage of PD-1⁺ lymphocyte subpopulations as a proportion of total CD45⁺ leukocytes in (C) the liver and (D) tumor. (E) Proportion of CD8⁺ lymphocytes expressing tissue resident marker CD103 in the liver and tumor. (F) The percentage of CD103⁺ CD8⁺ lymphocytes as a proportion total CD45⁺ leukocytes in the liver and tumor. Data n≥5 are presented as mean±SEM; significantly different data are represented by *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. n.s., not significant.

When considering these activated populations as a proportion of total leukocytes (CD45⁺) in these tissues, we find that in the liver and tumor tissues, captopril treatment again increases activated CD8⁺ and DNTs; however, in the tumor, the proportion of total leukocytes that is activated CD4⁺ significantly decreases (figure 5C,D). This may indicate a role for captopril in the activation of CD8⁺ and DNT but also in suppressing the activation of CD4⁺ T cells within the tumor.

We further investigated the proportion of CD8⁺ T cells expressing CD103 to delineate the effect of captopril on the tissue resident CD8⁺ T cell (T_{RM}) population. The percentage of CD8⁺ T cells expressing CD103⁺ was significantly reduced in both liver and tumor tissues in the treated group (figure 5E). However, within the total leukocyte population, the proportion of CD8⁺ CD103⁺

T cells was significantly increased in tumor and was not affected in the liver (figure 5F).

This suggests a potential mechanism of how captopril may control tumor growth by improving the cytotoxic T cell response.

DISCUSSION

Numerous retrospective clinical studies acknowledge significant improvement in overall survival, progression-free survival, and disease-free survival in patients with cancer using RASi as antihypertensive medication.³⁰ There is a clear upregulation of the RAS component, AT1R within the adenoma-colorectal-liver metastasis axis.^{31 32} Experimental and clinical studies over the last decade indicate that RASi impairs tumor progression

through modulation of the tumor vasculature^{33,34} and tumor desmoplasia³⁵; thus far, its role in tumor immunoregulation is not yet clear. Almost all immune cells express components of the RAS pathway, and several studies implicate a role for RAS in immune dysregulation in several chronic and autoimmune diseases.³⁶ Our previous findings show that the RASi, captopril, reduced tumor inflammation¹⁸ and temporally modified the frequency of TAMs within the TME.²⁵ Additionally, macrophage depletion experiments show that TAMs are important for RASi control of tumor.²⁵

In this study, we investigated the temporal dynamic changes of specific T lymphocyte subpopulations, in response to captopril treatment and their trafficking patterns into the liver and tumor tissues. Captopril treatment significantly and temporally increased CD3⁺ T lymphocyte infiltration both into the tumor and the liver. This finding suggests that increased infiltration of T cells into the tumor may have a role in the observed tumor inhibition as clinical studies report a positive correlation between tumor lymphocyte density and survival outcome.³⁷ The greatest observed changes were seen at day 15 post-tumor induction, possibly reflecting a timely development of antitumor responses and the effect of captopril treatment. By day 21, the infiltration decreased and the significance of this is not clear as the subpopulation composition was not analyzed at this time point. RASi reduces the immunosuppressive TME by altering the activity of TAMs, MDSCs and CAFs,²⁷ and this may account, at least in part, for the increase in CD3⁺ T lymphocyte infiltration observed in this study.

In these experiments, the effect of RASi on the infiltration of T cell subtypes appears specific and possibly local to tumor-affected tissues. It is important to note that the T cells infiltrating the liver may provide a substantial pool, along with those from the peripheral circulation that would be infiltrating the tumor. Remarkably, we found the inhibition of RAS markedly attenuated tumor-infiltrating CD4⁺ T cells. AT1R stimulation has been shown to play a role in the differentiation of CD4⁺ T cells into Th1 cell through the modulation of proteasome function; thus, RASi may inhibit this mechanism of CD4⁺ T cell antigen-mediated activation.³⁸ The proportion of activated circulating CD4⁺ T cells may have been diminished as a result of reduced AngII; therefore, trafficking into the liver and tumor was profoundly affected, as it was reported during deoxycorticosterone acetate-salt hypertension.³⁹

RASi appears to not have a selective effect on the infiltration of CD8⁺ T cells into the liver; however, the significant increase in CD8⁺ lymphocytes into the tumor, particularly those that are identified as tissue resident (CD103⁺) and PD-1⁺, may indicate a recently activated tumor-specific cytotoxic T cell population. Studies reported a positive correlation of CD8⁺-infiltrating lymphocytes and control of tumor growth.⁴⁰ Additionally, the intratumoral presence of T cells expressing the integrin CD103 has been strongly correlated with favorable prognosis for patients with cancer,⁴¹ independently of the infiltration of CD8⁺

T cells.⁴² Moreover, T_{RM} cell express immune checkpoint molecules, which may be a potential surrogate marker of response to immunotherapy as they could expand early during anti-PD-1 treatment.⁴³

Unexpectedly, the most responsive subpopulation of immune cells to captopril treatment was the DNTs. CD3⁺ lymphocytes that do not express CD4 and CD8 may include TcR alpha/beta (TcR $\alpha\beta$ ⁺) and TCR gamma/delta (TcR $\gamma\delta$ ⁺); however, at this stage, identification of the type of DNT has not been examined in this study. DNTs represent only a small fraction of the total peripheral lymphocyte composition (1%–3%) in healthy subjects.⁴⁴ Nevertheless, they are reported to increase in certain diseases with context-specific immune roles such as immune inflammatory and disease-exacerbating activities in autoimmune diseases⁴⁵ to anti-inflammatory⁴⁶ or proinflammatory roles in tissue injury and ischemia.⁴⁷

Interestingly, DNTs have been reported to be present in several solid cancers (melanoma, renal cell carcinoma, and glioblastoma) and to increase on treatment.⁴⁸ The phenotypes of these cells are cancer-specific and distinct from that of resting DNT, but closely resembling DNT in other cancer types and across species.⁴⁸ Furthermore, they positively correlate with treatment outcome, in concordance with the findings in this study.⁴⁸ DNTs have been previously reported to contribute to antimelanoma immunity.⁴⁹ In addition, this immunomodulatory effect is congruent with the reduced frequency of CD4⁺ T cell subpopulation as DNTs have been shown to suppress metabolic activation of CD4⁺ T cells (53) and could account for the CD4⁺ T cell reduction in this study. Nevertheless, the contribution of DNTs to antitumor immunity has only begun to be considered.

In the meantime, cancer immunotherapy strategies have mainly focused on modulating the function of infiltrating CD4⁺ and CD8⁺ lymphocytes. The presence and quality of the immune cell infiltrate of the tumor have been shown to be of prognostic potential for the response of different tumors to immunotherapies, regardless of tumor stage.³⁷ However, when PD-L1 blockers are used, a selective expansion of tumor-infiltrating CD4⁺ and CD8⁺ T cells expressing both activating (inducible T cell costimulator (ICOS, CD278)) and inhibitory (Lymphocyte activation gene 3 (LAG-3) and PD-1) molecules is seen.⁴⁰

This study showed significant upregulation in PD-1⁺ expression on both the CD8⁺ and DNT tumor-infiltrating cells in the captopril-treated mice. A recent study reported that DNT infusion, in combination with anti-PD-1, resulted in increased DNT-mediated antitumor activity during *in vivo* treatment of lung cancer.⁴⁹ Nakamura *et al*, in the only other *in vivo* study on the RASi immunoregulatory effects, report that RASi, in combination with PD-L1 inhibitors, improves the effect on tumor inhibition.²⁷ The increase of lymphocyte populations expressing PD-1 within the RASi-treated tumors, as seen in our study, opens up an opportunity to combine RASi with a combination of checkpoint inhibitor treatments

that may able to increase the efficacy and durability of the antitumor response.⁴⁰

Humans and outbred animal species display wide variability in the antitumor immune responses and drug treatments. In addition, to the genetic contribution, numerous studies report a strong sexual dimorphism in the immune responses.⁵⁰ Furthermore, studies also indicate that some components of the RAS pathway display a sex-biased differential expression in certain tissues that may lead to gender biased response to RASi treatments as well.⁵¹ This study used inbred single-strain male mice to overcome genetic and sexual dimorphism variabilities. However, immune responses to RASi treatments for CRLM need to be investigated in additional animal models of both genders and in clinical studies. Elucidation of genetic and gender differences to RASi response will further define the appropriate patient cohort, thus tailoring treatment to those likely to benefit.

CONCLUSION

This study described the immunomodulatory effect of RASi using captopril, comparing an early and a later stage of tumor development. Treatment significantly increased the infiltration of CD3⁺ T cells into the liver and tumor and altered the phenotypical composition of infiltrating CD4⁺ and CD8⁺ T cells, and DNTs. Additionally, captopril treatment increased the expression of checkpoint receptor PD-1 on the CD8⁺ and DNTs. The significance of these changes needs to be further characterized, especially the identity and function of the DNT population.

Correction notice Since the online publication of this article, the ORCID ID for Theodora Fifiis was updated.

Contributors DLVA, KAW and TF conceived the study, designed the experiments and analyzed the data. DLVA, RP and GK performed and processed animal experiments. DLVA, RP, GK and KAW performed fluorescent-activated cell sorting experiments. DLVA performed immunohistochemistry and wrote the manuscript. DLVA, KAW, TF, MVP and CC discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Funding DLVA is supported by the Department of Science, Technology, and Innovation (COLCIENCIAS-Colombia) (PhD overseas 679 program) and the University of Melbourne, Faculty of Medicine, Dentistry and Health Sciences (Postgraduate Research Scholarship 2018–2021). GK was supported by the Australian Rotary Health and Rotary District 9675 Bowelscan PhD Scholarship award. This work was supported by grants from the Austin Medical Research Foundation: (KAW, 2018; MVP, 2018, TF, 2019).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All animal experiments were conducted in accordance with the guidelines set by the Austin Hospital animal ethics committee (A2018/05549).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data sharing not applicable as no datasets generated and/or analyzed for this study. All data relevant to the study are included in the article or uploaded as supplementary information. All relevant information to the study are included in the article or uploaded as supplementary information.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution 4.0 Unported (CC BY 4.0) license, which permits others to copy, redistribute, remix, transform and build upon this work for any purpose, provided the original work is properly cited, a link to the licence is given,

and indication of whether changes were made. See <https://creativecommons.org/licenses/by/4.0/>.

ORCID iDs

Dora Lucia Vallejo Ardila <http://orcid.org/0000-0001-9863-608X>

Katrina A Walsh <http://orcid.org/0000-0002-0927-2008>

Theodora Fifiis <http://orcid.org/0000-0002-4201-0560>

Rita Paolini <http://orcid.org/0000-0002-2170-4723>

Georgios Kastrappis <http://orcid.org/0000-0002-2219-0007>

Christopher Christophi <http://orcid.org/0000-0002-1349-0884>

Marcos Vinicius Perini <http://orcid.org/0000-0002-0165-1564>

REFERENCES

- 1 Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nat Rev Gastroenterol Hepatol* 2019;16:713–32.
- 2 Tomlinson JS, Jarnagin WR, DeMatteo RP, et al. Actual 10-year survival after resection of colorectal liver metastases defines cure. *Journal of Clinical Oncology* 2007;25:4575–80.
- 3 Van Cutsem E, Cervantes A, Nordlinger B, et al. Metastatic colorectal cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Annals of Oncology* 2014;25:iii1–9.
- 4 Fong Y, Fortner J, Sun RL, et al. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. *Ann Surg* 1999;230:309–18. discussion 18–21.
- 5 Zhang BY, Jones JC, Briggler AM, et al. Lack of Caudal-Type homeobox transcription factor 2 expression as a prognostic biomarker in metastatic colorectal cancer. *Clin Colorectal Cancer* 2017;16:124–8.
- 6 Kemeny NE, Chou JF, Capanu M, et al. KRAS mutation influences recurrence patterns in patients undergoing hepatic resection of colorectal metastases. *Cancer* 2014;120:3965–71.
- 7 Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;21:309–22.
- 8 Mlecnik B, Bindea G, Angell HK, et al. Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. *Immunity* 2016;44:698–711.
- 9 Xia A, Zhang Y, Xu J, et al. T cell dysfunction in cancer immunity and immunotherapy. *Front Immunol* 2019;10:10.
- 10 Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011;364:2517–26.
- 11 Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus docetaxel in advanced Nonsquamous non-small-cell lung cancer. *N Engl J Med* 2015;373:1627–39.
- 12 Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med* 2015;372:2018–28.
- 13 Marshall HT, Djamgoz MBA. Immuno-Oncology: emerging targets and combination therapies. *Front Oncol* 2018;8.
- 14 Affara NI, Ruffell B, Medler TR, et al. B cells regulate macrophage phenotype and response to chemotherapy in squamous carcinomas. *Cancer Cell* 2014;25:809–21.
- 15 Smith HA, Kang Y. The metastasis-promoting roles of tumor-associated immune cells. *J Mol Med* 2013;91:411–29.
- 16 Albini A, Bruno A, Noonan DM, et al. Contribution to tumor angiogenesis from innate immune cells within the tumor microenvironment: implications for immunotherapy. *Front Immunol* 2018;9:527.
- 17 Wegman-Ostrosky T, Soto-Reyes E, Vidal-Millán S, et al. The renin-angiotensin system meets the hallmarks of cancer. *J Renin Angiotensin Aldosterone Syst* 2015;16:227–33.
- 18 Neo JH, Ager EI, Angus PW, et al. Changes in the renin angiotensin system during the development of colorectal cancer liver metastases. *BMC Cancer* 2010;10:134.
- 19 Ager EI, Neo J, Christophi C. The renin-angiotensin system and malignancy. *Carcinogenesis* 2008;29:1675–84.
- 20 Pacurari M, Kafoury R, Tchounwou PB, et al. The renin-angiotensin-aldosterone system in vascular inflammation and remodeling. *Int J Inflamm* 2014;2014:1–13.
- 21 Kim G, Kim J, Lim YL, et al. Renin-angiotensin system inhibitors and fibrosis in chronic liver disease: a systematic review. *Hepatology Int* 2016;10:819–28.
- 22 Saber S, Mahmoud AAA, Helal NS, et al. Renin-angiotensin system inhibition ameliorates CCl₄-induced liver fibrosis in mice through the

- inactivation of nuclear transcription factor kappa B. *Can J Physiol Pharmacol* 2018;96:569–76.
- 23 Haznedaroglu IC, Beyazit Y. Local bone marrow renin-angiotensin system in primitive, definitive and neoplastic haematopoiesis. *Clin Sci* 2013;124:307–23.
 - 24 Wysocki PJ, Kwiatkowska EP, Kazimierczak U, et al. Captopril, an angiotensin-converting enzyme inhibitor, promotes growth of immunogenic tumors in mice. *Clin Cancer Res* 2006;12:4095–102.
 - 25 Wen SW, Ager EI, Neo J, et al. The renin angiotensin system regulates Kupffer cells in colorectal liver metastases. *Cancer Biol Ther* 2013;14:720–7.
 - 26 Neo JH, Malcontenti-Wilson C, Muralidharan V, et al. Effect of ACE inhibitors and angiotensin II receptor antagonists in a mouse model of colorectal cancer liver metastases. *J Gastroenterol Hepatol* 2007;22:577–84.
 - 27 Nakamura K, Yaguchi T, Ohmura G, et al. Involvement of local renin-angiotensin system in immunosuppression of tumor microenvironment. *Cancer Sci* 2018;109:54–64.
 - 28 Kuruppu D, Christophi C, Bertram JF, et al. Characterization of an animal model of hepatic metastasis. *J Gastroenterol Hepatol* 1996;11:26–32.
 - 29 Parra ER, Behrens C, Rodriguez-Canales J, et al. Image analysis-based assessment of PD-L1 and tumor-associated immune cells density supports distinct intratumoral microenvironment groups in non-small cell lung carcinoma patients. *Clin Cancer Res* 2016;22:6278–89.
 - 30 Sun H, Li T, Zhuang R, et al. Do renin-angiotensin system inhibitors influence the recurrence, metastasis, and survival in cancer patients?: evidence from a meta-analysis including 55 studies. *Medicine* 2017;96:e6394.
 - 31 Zhou L, Luo Y, Sato S, et al. Role of two types of angiotensin II receptors in colorectal carcinoma progression. *Pathobiology* 2014;81:169–75.
 - 32 Beitia M, Solano-Iturri JD, Errarte P, et al. Altered expression of renin-angiotensin system receptors throughout colorectal adenoma-adenocarcinoma sequence. *Int J Med Sci* 2019;16:813–21.
 - 33 Carbajo-Lozoya J, Lutz S, Feng Y, et al. Angiotensin II modulates VEGF-driven angiogenesis by opposing effects of type 1 and type 2 receptor stimulation in the microvascular endothelium. *Cell Signal* 2012;24:1261–9.
 - 34 Shirotake S, Miyajima A, Kosaka T, et al. Angiotensin II type 1 receptor expression and microvessel density in human bladder cancer. *Urology* 2011;77:1009.e19–25.
 - 35 Diop-Frimpong B, Chauhan VP, Krane S, et al. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci U S A* 2011;108:2909–14.
 - 36 Capettini LSA, Montecucco F, Mach F, et al. Role of renin-angiotensin system in inflammation, immunity and aging. *Curr Pharm Des* 2012;18:963–70.
 - 37 Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960–4.
 - 38 Qin X-Y, Zhang Y-L, Chi Y-F, et al. Angiotensin II regulates Th1 T cell differentiation through angiotensin II type 1 Receptor-PKA-Mediated activation of proteasome. *Cell Physiol Biochem* 2018;45:1366–76.
 - 39 Vinh A, Chen W, Blinder Y, et al. Inhibition and genetic ablation of the B7/CD28 T-cell costimulation axis prevents experimental hypertension. *Circulation* 2010;122:2529–37.
 - 40 Beyrend G, van der Gracht E, Yilmaz A, et al. Pd-L1 blockade engages tumor-infiltrating lymphocytes to co-express targetable activating and inhibitory receptors. *J Immunother Cancer* 2019;7:217.
 - 41 Dumauthioz N, Labiano S, Romero P. Tumor resident memory T cells: new players in immune surveillance and therapy. *Front Immunol* 2018;9:9.
 - 42 Topham DJ, Reilly EC. Tissue-Resident memory CD8+ T cells: from phenotype to function. *Front Immunol* 2018;9:515.
 - 43 Webb JR, Milne K, Watson P, et al. Tumor-Infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer. *Clinical Cancer Research* 2014;20:434–44.
 - 44 Fischer K, Voelkl S, Heymann J, et al. Isolation and characterization of human antigen-specific TCR $\alpha\beta$ + CD4-CD8- double-negative regulatory T cells. *Blood* 2005;105:2828–35.
 - 45 Hillhouse EE, Lesage S. A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. *J Autoimmun* 2013;40:58–65.
 - 46 Martina MN, Noel S, Saxena A, et al. Double-Negative $\alpha\beta$ T Cells Are Early Responders to AKI and Are Found in Human Kidney. *Journal of the American Society of Nephrology* 2016;27:1113–23.
 - 47 Meng H, Zhao H, Cao X, et al. Double-Negative T cells remarkably promote neuroinflammation after ischemic stroke. *Proc Natl Acad Sci U S A* 2019;116:5558–63.
 - 48 Greenplate AR, McClanahan DD, Oberholtzer BK, et al. Computational immune monitoring reveals abnormal double-negative T cells present across human tumor types. *Cancer Immunology Research* 2019;7:86–99.
 - 49 Fang L, Ly D, Wang S-si, et al. Targeting late-stage non-small cell lung cancer with a combination of DNT cellular therapy and PD-1 checkpoint blockade. *J Exp Clin Cancer Res* 2019;38:123.
 - 50 Capone I, Marchetti P, Ascierto PA, et al. Sexual dimorphism of immune responses: a new perspective in cancer immunotherapy. *Front Immunol* 2018;9:552.
 - 51 Sullivan JC. Sex and the renin-angiotensin system: inequality between the sexes in response to Ras stimulation and inhibition. *Am J Physiol Regul Integr Comp Physiol* 2008;294:R1220–6.

Multiplex Immunohistochemistry protocol in formalin-fixed, paraffin-embedded Colorectal Liver Metastases patient tissues and Machine Learning-based Tissue Segmentation and Cell Phenotyping analysis

Method Article

Abstract

Colorectal cancer liver metastasis (CLM) is the most common cause of death in colorectal cancer (CRC) worldwide. Various immune-suppressive and tumour-promoting mechanisms contribute to the unresponsiveness associated with current checkpoint immunotherapy in CRC. Therefore, techniques such as Multiplex immunohistochemistry (mIHC) and automated image quantitation analysis enable a deeper understanding of the host's anti-tumour immune response diversity and identify potential immunotherapy targets. Herein, we present a 7-plex OPAL™ protocol used to assess the composition and spatial distribution of T cell markers CD3, CD8, Foxp3, and CD103 and the epithelial to mesenchymal transition (EMT) markers alpha-smooth muscle actin (α -SMA) and E-cadherin. The protocol has been manually optimized and validated in two independent cohorts of formalin-fixed, paraffin-embedded CLM patient tissues (n=42) using well-established antibodies, a single spectral library, negative controls, and biological controls for corroborating staining pattern of α -SMA⁺ (sclerosed hemangioma samples) and T-cell infiltrates (benign liver). The accurate profiling of T-cell composition, location, and phenotypic characterization could reveal important insights about the influence of T lymphocytes on prognosis after liver metastasectomy.

Keywords: Colorectal Liver Metastases, Multiplex Immunohistochemistry protocols, Machine Learning-based Tissue Segmentation, and Cell Phenotyping

Introduction

Colorectal cancer liver metastasis (CLM) is the most common cause of death in patients with colorectal cancer (CRC) worldwide¹. Surgical resection of CLM is the only treatment that significantly prolongs their survival². Nevertheless, a multidisciplinary approach has been recommended for CLM treatment as the best strategy to achieve synergy in the efficiency of all treatments³. One of these strategies includes cancer immunotherapy using checkpoint inhibitors⁴, which has proven to achieve long-lasting clinical effects in some patients with advanced disease in some types of cancer⁵. However, in CRC, only patients with deficient mismatch repair respond to these treatments⁴. In addition, there are various immune-suppressive and tumour-promoting mechanisms, which are directly related to the unresponsiveness to cancer immunotherapy⁶. Traditionally, the techniques used for the current stratification of CLM patients are IHC^{7, 8} and flow cytometry⁹, which provide limited information about composition, location and phenotypic characterization, and functional relationships of immune and stromal cells tumour and the adjacent liver parenchyma. Therefore, a better analysis is warranted to deconvolute the complexity within the tumour immune microenvironment (TIME) and reveal informative biomarkers that will aid the current stratification of CLM patients for available therapies, particularly immunotherapies⁷.

Techniques such as Multiplex immunohistochemistry (mIHC) and automated image quantitation analysis enable a deeper understanding of the diversity of the host's anti-tumour immune response by identifying the key players that may inhibit anti-tumour T-cell responses and T-cell infiltration into the tumour tissue⁷⁻¹¹. Here we present mIHC 7-plex OPALTM protocol in formalin-fixed, paraffin-embedded (FFPE) CLM patient tissues and Machine Learning-based Tissue Segmentation and Cell Phenotyping analysis using inForm® software v2.4.2.

Materials and Reagents

1. Microscope slides with CLM, FFPE tissue sections (4 µm).
2. Xylene.
3. Histological grade Ethanol (70% and 100%).
4. 10% Neutral-Buffered Formalin (NBF).
5. Tris Buffered Saline with Tween 20 (0.5%) (TBST) wash buffer, pH 7.5
6. Peroxidase-free water (Milli-Q™ water).
7. RO water.
8. Antibody Diluent/Block (PerkinElmer/Akoya Biosciences cat. N° ARD 1001EA).
9. Antigen retrieval AR6 buffer (10X) (PerkinElmer/Akoya Biosciences cat. No. NEL861001KT)
10. Target Retrieval Solution, pH9 buffer (10x) (Dako code S2367).
11. IHC SELET® Proteinase K (Millipore, concentration 0.2mg/ml)
12. Dako REAL Peroxidase-Blocking Solution (Agilent code S2023)
13. Opal Polymer HRP Ms + Rb (PerkinElmer/Akoya Biosciences)
14. Opal Polaris 7-Color Manual IHC kit (PerkinElmer/Akoya Biosciences cat. No. NEL861001KT)
 - 1X Plus Amplification Diluent
 - Opal 520 Fluorophore
 - Opal 540 Fluorophore
 - Opal 570 Fluorophore
 - Opal 620 Fluorophore
 - Opal 650 Fluorophore
 - Opal 690 Fluorophore

- Opal Polymer HRP Mouse (Ms) + Rabbit (Rb)
- DMSO

15. Primary Antibodies for targets interest:

- CD3 (Agilent Dako, clone A0452; code number M7254)
- CD8 (ThermoFisher, clone SP16; cat. No MA5-14548)
- CD103 (Abcam, [EPR4166(2)]; cat. No ab129202)
- Foxp3 (Abcam, clone 236A/E7; cat. No ab20034)
- E-cadherin (Santa Cruz, clone (H-108) sc-7870)
- Recombinant anti-alpha smooth muscle actin (α - SMA) (Abcam, clone [E184]cat. No ab32575)

16. DAPI solution (1mg/ml) (ThermoFisher scientific; cat. No. 62248)

17. Fluorescent Mounting Medium (Agilent Dako; code number GM304)

18. Coverslips Menzel- Glazer 22x50 mm (Thermo scientific).

Equipment

1. Tissue-tek® slide staining set (12 pp dishes).
2. Tissue-Tek vertical 24 slide rack.
3. Staining and incubation tray covered with aluminum foil.
4. Pipettes and Pipette Tips (1-1000 μ L)
5. Microwave Oven.
6. Vectra® 3.0 Automated Quantitative Pathology Imaging System (Akoya, MA).
7. Phenochart v.1.0.12 (Akoya, MA).
8. inForm® software v2.4.2 (Akoya, MA) installed in a PC with 128GB DDR4 RAM.

Procedure

CLM Specimens were made available from tissues stored in the Victorian Cancer Biobank (VCB) after approval from the Austin Health Human Research Ethics Committee HREC reference HREC/54233/Austin-2019.

Slide preparation:

1. Heat the slides at 60 °C for 60 minutes in an oven

Bath and solvents for deparaffinization and rehydration of FFPE tissue:

2. Xylene for deparaffinization, immerse into three successive baths of xylene for 10 minutes each time. **Note:** Do not let slides dry out between steps.
3. Histological grade ethanol (100%) for rehydration, immerse into two successive baths for 5 minutes each time.
4. Histological grade ethanol (70%) for rinsing, immerse for 5 minutes.
5. After rehydration, rinse in running RO water for ~1 minute.
6. Fix in 10% NBF for 30 minutes.
7. After fixing, wash in running RO water for ~1 minute.

Proceed with antigen retrieval and microwave treatment step as specified for the primary antibody for targets of interest:

8. Place slides in the Tissue-Tek vertical 24 slide rack
9. Fill slide container with antigen retrieval buffer diluted in 1:10 with peroxidase-free water as follows:
 - Day 1- AR6 buffer (PerkinElmer/Akoya Biosciences) (for anti- α -SMA)
 - Day 2- AR6 buffer (PerkinElmer/Akoya Biosciences) (for anti-E-cadherin)
 - Day 3- AR6 buffer (PerkinElmer/Akoya Biosciences) (for anti-Foxp3)
 - Day 4-Target retrieval Solution, pH9 buffer (Agilent Dako) (for anti-CD103)
 - Day 5- AR6 buffer (PerkinElmer/Akoya Biosciences) (for anti-CD8)
 - Day 6- Proteinase K (Millipore) (for anti-CD3).

Note: Proteinase K does not require microwave pre-treatment (steps 10-11); therefore, it should be the last antibody of the panel. Leave at room temperature (RT) for 30 minutes.

10. Heat slides in antigen retrieval buffer in a microwave oven as follows:

- 2 minutes at 100 % power or until boiling point is reached.
- Continue with 10 minutes at 20 % power.

11. Cooldown the slides at RT for 15 minutes.

12. Wash the slides in distilled water (dH₂O) for 1 minute and then rinse in TBST 3 times for 2 minutes each time.

13. Block endogenous peroxidase activity (Dako REAL Peroxidase-Blocking Solution; Agilent) for 30 minutes.

14. Block non-specific binding with Ab Diluent/ Block Buffer (PerkinElmer/Akoya Biosciences) for 30 minutes.

15. Add Primary Antibody diluted at the optimized ratio in Ab Diluent/ Block Buffer (PerkinElmer/Akoya Biosciences) for targets of interest in the following order:

- Day 1- α - SMA at 1:500
- Day 2- E-cadherin at 1:500
- Day 3- Foxp3 at 1:50
- Day 4-CD103 at 1:1000
- Day 5- CD8 at 1:100
- Day 6- CD3 at 1:100

16. Incubate with each antibody overnight at 4°C in a humidified chamber.

17. Wash the slides three times for 2 minutes each time with TBST Buffer at RT with agitation.

18. Add Opal Polymer HRP Ms + Rb (PerkinElmer/Akoya Biosciences) and incubate for 40 minutes at RT.

19. Wash the slides 3 times for 2 minutes each time with TBST Buffer at RT with agitation.
20. Add 50-150µl of TSA-Opal Working Solution per slide (1:50 dilution with 1 x Plus Amplification Diluent) depending on the size of the tissue and the specific conjugated dye to the target of interest:
 - Day 1- To α- SMA, add TSA-Opal690
 - Day 2- To E-cadherin, add TSA-Opal620
 - Day 3- To Foxp3, add TSA-Opal520
 - Day 4- To CD103, add TSA-Opal540
 - Day 5- To CD8, add TSA-Opal570
 - Day 6- To CD3, add TSA-Opal650
21. Incubate the slides for 10 minutes at RT
22. Wash the slides 3 times for 2 min each with TBST Buffer at RT with agitation
23. Repeat the process from point 9, preparing the second day's antigen-retrieval buffer and repeating the process until the final day's procedure is complete.

Counterstaining and Mounting

24. After the last TSA-Opal color treatment, wash the slides in dH₂O for 2 minutes and rinse in TBST for 2 minutes.
25. Add DAPI Solution (1:4000; ThermoFisher scientific) and incubate the slides for 2-5 min at RT.
26. Rinse the slides with TBST 3 times for 2 minutes
27. Rinse in the running RO water for ~ 1 minutes
28. Cover the slide with Fluorescent Mounting Medium (Agilent Dako) and apply coverslip Menzel- Glazer 22x50 mm (Thermo scientific).

29. Leave the slides to set at room temperature overnight and protected from light.
30. Opal stained slides should be protected from light and maintained at 4°C in a humidified chamber for short/long-term image acquisition.

Image acquisition and quantification

31. Use the multispectral fluorescent microscope Vectra® 3.0 Automated Quantitative Pathology Imaging System (Akoya, MA) for whole tissue scan.
32. Use Phenochart® software (low power scanned image) to select high-powered fields (HPF) (equivalent fixed-size stamp 3x3= 2046 x 1530 µm; ×20 object lens, resolution= 0.5 µm equivalent to 1 pixel) across the tumor and adjacent liver area, selecting areas of the tumor with the highest infiltrating lymphocyte density (**Figure 1**).
33. Use inForm® software v2.4.2 Tissue finder algorithm for trainable pattern recognition to reach over 91% accuracy for the following tissue segments: adjacent liver parenchyma depicted in red (LP), invasive tumor margin in blue (IM), and tumor core (TC) in green (**Figure 2**).
34. Use inForm® software v2.4.2 Cell segmentation and phenotyping algorithm to calculate cell count per HPF according to the trainable phenotypes of interest: CD3⁺ T Lymphocytes (CD3⁺ CD8-CD103⁻), CD8⁺ T cells (CD3⁺CD8⁺CD103⁻), TRM (CD3⁺CD8⁺CD103⁺), T regulatory cells (CD3⁺FoxP3⁺), non T cells expressing CD8⁺ or CD103⁺ and cells expressing EMT markers such as α-SMA or E-cadherin. Every sample had background control for the subtraction of autofluorescence (**Figure 3**).
35. Use Pixel-based colocalization analysis using inForm® to quantify fluorescent intensity to discover novel phenotypes not included in the previous algorithm.

Troubleshooting

- a) General immunohistochemistry optimization troubleshooting for single

staining antibodies may be applied.

- b) The single analyte OPAL IHC assay needs to be optimized before combining for use in multiplex detection. Concentrations of each primary antibody/TSA OPAL working solution should be optimized to be detectable within an exposure time of 40-200 milliseconds.
- c) Single spectral libraries need to be developed with control tissues such as HeLa cells FFPE and/or with the tissue of interest.
- d) The order of target and/or OPAL fluorophore detection needs to be optimized and independently validated.

Time Taking

Seven days for staining protocol, allow additional time for image acquisition and inForm analysis depending on the computational capacity of the PC.

Anticipated results

We present mIHC 7-plex OPAL™ protocol in formalin-fixed, paraffin-embedded CLM patient tissues and Machine Learning- based Tissue Segmentation and Cell Phenotyping analysis using inForm® software v2.4.2. The specificity of the antibodies has been independently validated in specimens of sclerosed hemangiomas and benign liver tissues. This novel panel mIHC allows for the characterization of conventional and unconventional T-cells within the CD3⁺ T lymphocytes, offering insights into novel biomarkers for CLM post liver resection and immune surveillance. In addition, we raise the possibility of discovering novel phenotypes and potential immune targets modulated by available cancer immunotherapy.

References

- 1 Keum, N. & Giovannucci, E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nature Reviews Gastroenterology & Hepatology* **16**, 713-732, doi:10.1038/s41575-019-0189-8 (2019).

- 2 Kanas, G. P. *et al.* Survival after liver resection in metastatic colorectal cancer: review and meta-analysis of prognostic factors. *Clin Epidemiol* **4**, 283-301, doi:10.2147/CLEP.S34285 (2012).
- 3 Vera, R. *et al.* Multidisciplinary management of liver metastases in patients with colorectal cancer: a consensus of SEOM, AEC, SEOR, SERVEI, and SEMNIM. *Clinical and Translational Oncology* **22**, 647-662, doi:10.1007/s12094-019-02182-z (2020).
- 4 Overman, M. J., Ernstoff, M. S. & Morse, M. A. Where We Stand With Immunotherapy in Colorectal Cancer: Deficient Mismatch Repair, Proficient Mismatch Repair, and Toxicity Management. *American Society of Clinical Oncology Educational Book*, 239-247, doi:10.1200/edbk_200821 (2018).
- 5 Borcoman, E., Nandikolla, A., Long, G., Goel, S. & Tourneau, C. L. Patterns of Response and Progression to Immunotherapy. *American Society of Clinical Oncology Educational Book*, 169-178, doi:10.1200/edbk_200643 (2018).
- 6 Binnewies, M. *et al.* Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nature medicine* **24**, 541-550, doi:10.1038/s41591-018-0014-x (2018).
- 7 Taube, J. M. *et al.* The Society for Immunotherapy of Cancer statement on best practices for multiplex immunohistochemistry (IHC) and immunofluorescence (IF) staining and validation. *Journal for ImmunoTherapy of Cancer* **8**, e000155, doi:10.1136/jitc-2019-000155 (2020).
- 8 Halse, H. *et al.* Multiplex immunohistochemistry accurately defines the immune context of metastatic melanoma. *Scientific Reports* **8**, 11158, doi:10.1038/s41598-018-28944-3 (2018).
- 9 Blom, S. *et al.* Systems pathology by multiplexed immunohistochemistry and whole-slide digital image analysis. *Scientific Reports* **7**, 15580, doi:10.1038/s41598-017-15798-4 (2017).
- 10 Stack, E. C., Wang, C., Roman, K. A. & Hoyt, C. C. Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods* **70**, 46-58, doi:<https://doi.org/10.1016/j.ymeth.2014.08.016> (2014).
- 11 Lazarus, J. *et al.* Spatial and phenotypic immune profiling of metastatic colon cancer. *JCI insight* **3**, doi:10.1172/jci.insight.121932 (2018).

Figures

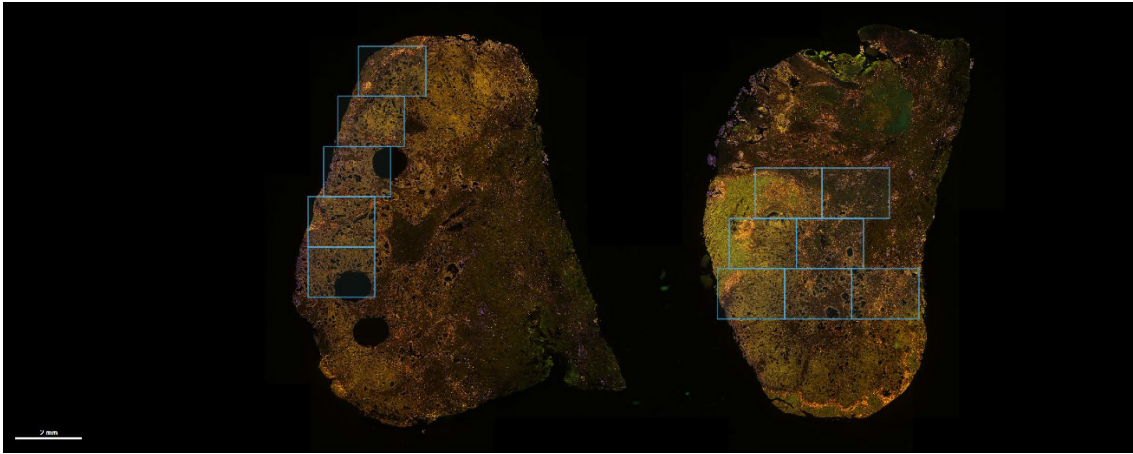


Figure 1. Phenochart. Whole tissue scan (low-powered field) showing 11 High powered Pre-scan field (HPF) (scale bar: 2mm).

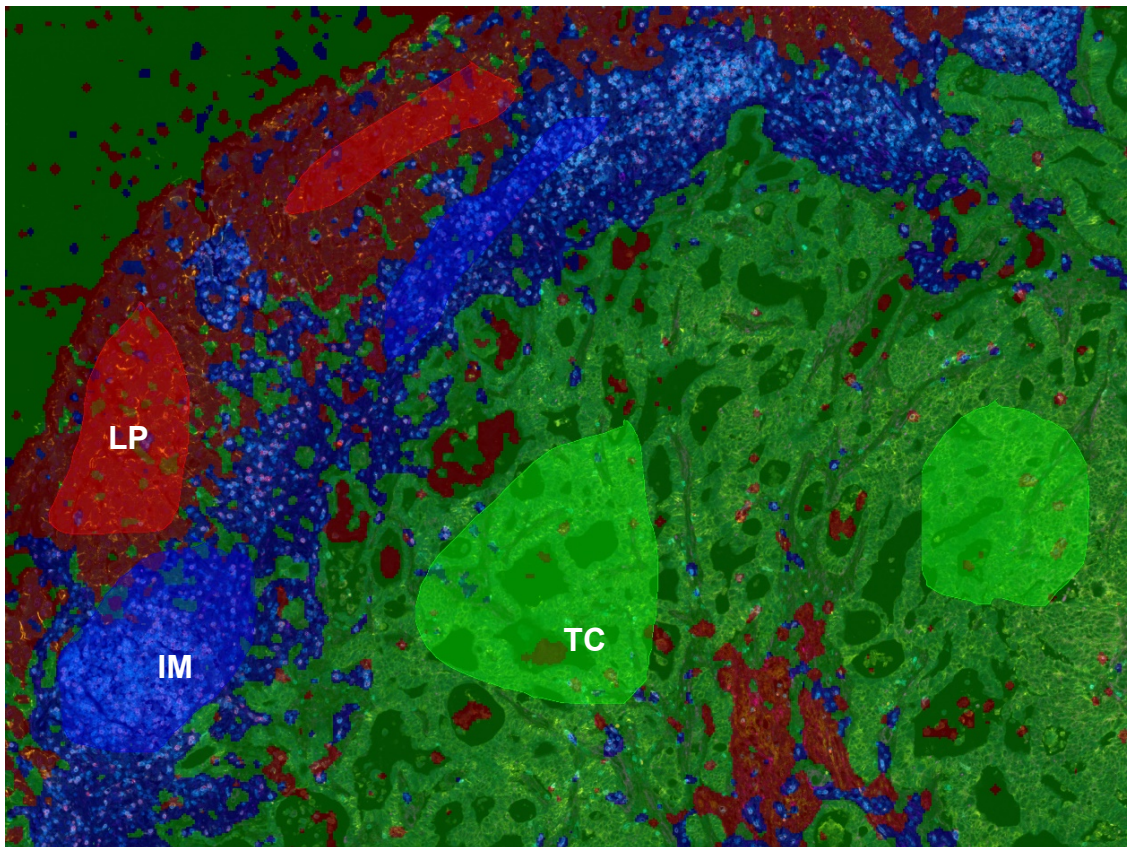


Figure 2. Tissue segmentation. Using machine learning-based algorithm, tissue segmentation was determined as follows: adjacent liver parenchyma in red (LP), invasive tumor margin in blue (IM) and tumor core (TC) in green. (HPF

stamp size 3x3= 2046 x 1530 μm , resolution= 0.5 μm equivalent to 1 pixel, x 20 magnification)

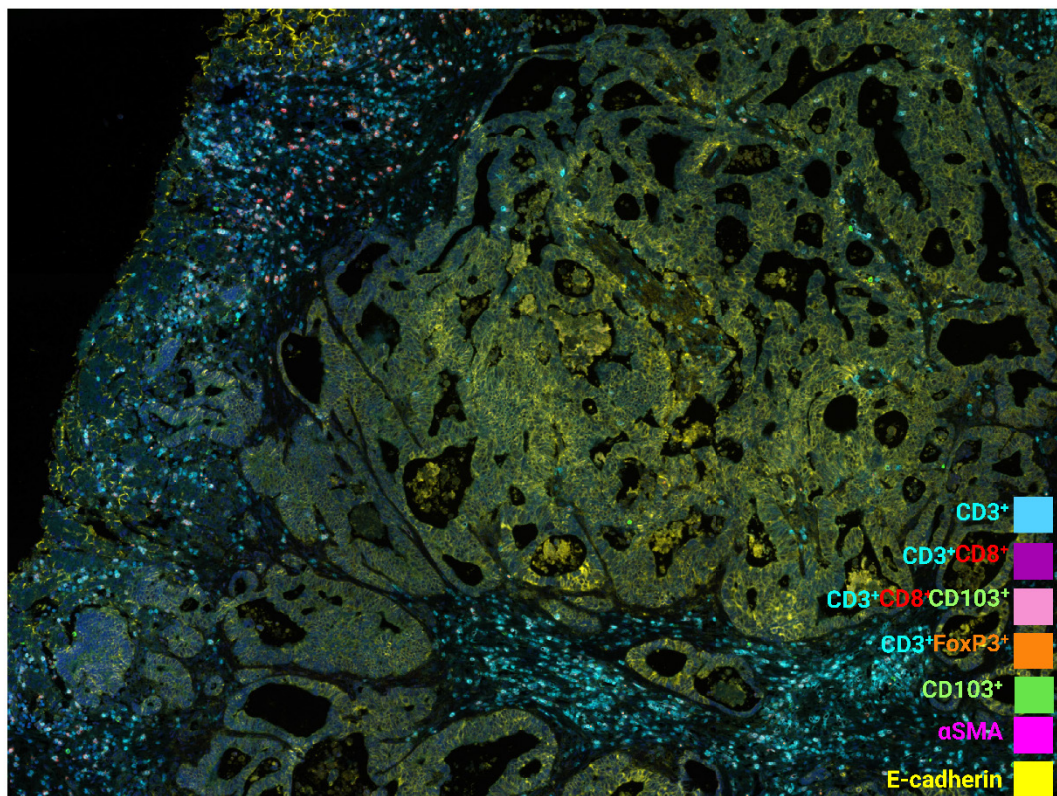


Figure 3. CLM stained by 7-plex OPAL and cell phenotyping.

Representative composite HPF (HPF stamp size 3x3, x 20 magnification) image displayed all phenotypes, including with CD3⁺, CD8⁺ and CD103⁺, Foxp3⁺, α -SMA (tumor stroma and blood vessels) and E-cadherin (epithelial interface of the TC). Key: Cyan: CD3⁺, Red: CD8⁺, Bright Green: CD103⁺, Purple: CD3⁺CD8⁺, Light pink: CD3⁺CD8⁺CD103⁺, Orange: CD3⁺Foxp3⁺ Bright pink: α SMA, and Yellow: E-cadherin.

Acknowledgments

DL Vallejo-Ardila is supported by the Department of Science, Technology, and Innovation (COLCIENCIAS-Colombia) (Ph. D overseas 679 program) and the University of Melbourne, Faculty of Medicine, Dentistry and Health Sciences

(Postgraduate Research Scholarship 2018-2021). This work was supported by grants from the Austin Medical Research Foundation: (Walsh -2018, Perini -2018, Fifis -2019). I would like to acknowledge The Ian Potter Foundation for its generous contribution by purchasing of the VECTRA Quantitative Pathology Imaging System for Imaging Core of the Olivia-Newton John Cancer Research Institute.

Consent for publication

We confirm that the protocol has been read and approved by all named authors and there is no other person who satisfied the criteria for authorship but are not listed. We further confirm that order of authors listed has been approved by all authors.

We have considered intellectual property associated to this work, and there is no impediment to publication. We confirm that we have followed the regulations of our institutions concerning intellectual property.

Competing interests

The authors of this manuscript have no conflicts of interest to declare.