

The Ontogeny of Effector Regulatory T Cells

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

August 2020

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Abstract

Regulatory T (Treg) cells are critical for the maintenance of immune homeostasis and peripheral tolerance. Different subsets of Treg cells have recently been described with many studies showing the importance of context-specific differentiation of Treg cells, in particular within non-lymphoid organs. These non-lymphoid organ Treg cells have a fully suppressive Treg cell phenotype with an effector function and are termed effector (e)Treg cells. However, the ontogeny of eTreg cells have not yet been fully described. Additionally, molecular determinants of the eTreg cell program remain incompletely understood. My thesis examines the transcriptional events that regulate the generation of eTreg cells during their thymic development, their homeostasis and response to infection. Using different gene targeted mouse models at steady state and in viral infection models, I studied the intrinsic molecular mechanisms that contribute to eTreg cell differentiation. In particular, I focused on follicular Treg (T_{FR}) cells, which constitute the eTreg cell subset of the germinal centre.

The molecular control of eTreg cell fate and function converges on the transcription factors IRF4 and Blimp-1. IRF4 is induced by antigen receptor signals and cooperates with AP-1 factors, BATF and JUN, to regulate transcriptional networks involved in lymphocyte differentiation, function and metabolism. For example, these factors regulate genes important for antibody class switch recombination in B cells and functional differentiation of distinct CD4 T helper (Th) subsets, including Th2, Th9, Th17 and T follicular helper cells. IRF4 expression in Treg cells is critical for effector differentiation, yet the precise mechanisms of how IRF4 regulates the transcriptional program of T_{FR} cells remains unknown. Using a novel transgenic IRF4 reporter mouse we found that IRF4 is highly expressed in T_{FR} cells. Using IRF4 knockout mouse models, we demonstrate that IRF4 is necessary for T_{FR} cell generation in a Treg cell-intrinsic manner. IRF4 controls important aspects of the transcriptional program that drives T_{FR} cell differentiation, including genes essential for Treg cell migration. Furthermore, I identified the transcription factor c-Maf to be essential for T_{FR} cell generation and demonstrated its central role in maintaining a follicular program in Treg cells.

Subsequently, using ribonucleic acid (RNA)-sequencing, we generated a “*follicular signature*” of gene expression from the combined analysis of T_{FH} and T_{FR} cells. Integrated transcriptional analyses showed that in the absence of either IRF4 or c-Maf, the majority of

the *follicular signature genes* were downregulated, indicating that these two transcriptional regulators, aside from Bcl6 are indispensable for follicular T_{FR} cell development. Finally, analyses of IRF4 and c-Maf DNA binding sites, identified by chromatin induced precipitation (ChIP)-sequencing, in combination with open chromatin regions in follicular T cell specific loci, we showed that the precise orchestration of distinct sets of genes is required to promote conserved aspects of the follicular T cell fate. In conclusion, my thesis describes how a key transcriptional network orchestrates fundamental steps in T_{FR} cell differentiation and function, which contributes to the understanding of eTreg cell biology.

Declaration

I certify that:

- I. This thesis comprises only my original work toward the degree of Doctor of Philosophy except where indicated in the preface;
- II. Due acknowledgement has been made in the text to all other material used;
and
- III. This thesis is less than 100,000 words in length exclusive of tables, maps, bibliographies and appendices.

Peggy Teh

Preface

The work delineated in this thesis was executed at The Walter and Eliza Hall Institute of Medical Research, The University of Melbourne, under the supervision of Professors Axel Kallies and Daniel Gray.

Based on the regulations of the University of Melbourne, my contributions to the following chapters of the thesis are as follows:

Chapter 3: 95% with contributions from Ajithkumar Vasanthakumar for the generation of the novel reporter mice.

Chapter 4: 85% with contributions from Liana Mackiewicz and Carolina Alvarado for assistance with influenza infections and diphtheria toxin experiments, Ajithkumar Vasanthakumar and Julie Tellier for the ChIP and RNA-sequencing experiments, Yifang Hu and Wei Shi for bioinformatic analysis of RNA and ChIP-sequencing data.

Chapter 5: 75% with contributions from Tom Sidwell and Ajithkumar Vasanthakumar for ChIP and ATAC-sequencing experiments, David Chisanga, and Wei Shi for bioinformatic analysis of ATAC and ChIP-sequencing data.

Chapters 3, 4 and 5 constitute 25%, 50% and 25% of the thesis and thus, my overall contribution to the results presented in this thesis is 85%.

Acknowledgements

I have had the tremendous privilege of pursuing a postgraduate degree with some of the finest immunologists in the world and extremely fortunate to be completing this thesis despite a global pandemic. This journey has highlighted the value of scientific discoveries and I dedicate this thesis to life.

Firstly, I would express my sincere thanks to my supervisors Professors Axel Kallies and Daniel Gray for their patient advice, guidance and insightful feedback through each stage of the process. I am deeply grateful to my committee members Joanna Groom, Ian Wicks and Stephen Nutt for their wise counsel, valuable comments and kind words.

I thank the members of the Kallies Laboratory past and present, and friends from the Molecular Immunology division at WEHI for the stimulating discussions, advice and technical assistance. A big thank you to Julie Tellier, Ajithkumar Vasanthakumar and Jonas Blume for their mentorship. Special thanks to Tom Sidwell, a thoughtful, intelligent and kind soul, for invaluable advice and feedback. I can never repay you for your infinite tolerance and contribution to the success of my postgraduate degree.

A massive thank you to the animal technicians and Bioservices division at WEHI, in particular Teisha Mason, Liana Mackiewicz, Carolina Alvarado, Tania Camillieri, Jamie Leahy and Lauren Wilkins.

To Renee, Mel, Amy, Sarah, Tina, Mun-Ching, Mona, Leanda, Tony, Jim and Jo – know that each one of you have been there for me in more ways than one. For that I am extremely grateful.

Thank you to my medical colleagues at the Alfred Health Renal Unit for their understanding and support. Special mentions to Leny Hidayati and Kristeen Barker for their constant belief in me. Thank you for putting my failures and successes into perspective. To Michael Low, Karen Sanders and Orla O'Brien thank you.

I would also like to acknowledge the generous funding from Kidney Health Australia, and hope that some of the work contained in this thesis will be applicable for translation into clinical practice in the future.

To my wonderful family, thank you for always believing in me. Your unwavering love, support and encouragement carried me through the course of my postgraduate studies.

Last but certainly not the least, thank you Glenn for being by my side through the most challenging part of this PhD. Your patience, love and support made it possible to complete what I had started.

Abbreviations

AIRE	Autoimmune regulator
ATAC-seq	Assay for transposase accessible chromatin sequencing
aTreg	Activated regulatory T cell
Bcl	B cell lymphoma
Blimp-1	B lymphocyte induced maturation protein 1
CD	Cluster of differentiation
ChIP-seq	Chromatin immunoprecipitation sequencing
CSR	Class-switch recombination
cTreg	Central regulatory T cell
CTV	CellTrace Violet
DA	Differentially accessible
DE	Differentially expressed
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eTreg	Effector regulatory T cell
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDR	False discovery rate
Flox	Flanked by <i>loxP</i> sequences
Fox	Forkhead box protein
GFP	Green fluorescent protein
gMFI	Geometric mean fluorescence intensity
IF	Immunofluorescence
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IRF	Interferon regulatory factor
JAK	Janus activating kinase
Kb	Kilobase

Kda	Kilodalton
KLRG1	Killer cell leptin-like receptor subfamily G member 1
KO	Knockout
LP	Lamina propria
LN	Lymph node
MACS	Magnetic activated cell sorting
MARE	Maf recognition element
MHC	Major histocompatibility complex
mLN	Mediastinal lymph nodes
mTEC	Medullary thymic epithelial cell
MTPBS	Mouse tonicity phosphate buffered saline
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
P.f.u.	Plaque forming units
PCR	Polymerase chain reaction
PP	Peyer's patches
pTreg	Peripherally induced regulatory T cell
qPCR	Quantitative polymerase chain reaction
Rad	Radiation absorbed dose
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROR γ t	RAR-related orphan receptor γ t
RPKM	Reads per Kilobases per Million mapped reads
S.D.	Standard deviation
SEM	Standard error of the mean
Seq	Sequencing
SHM	Somatic hypermutation
SI	Small intestine
STAT	Signal transducer and activator of transcription
Tbet	T-box expressed in T cells
TCF-7	T cell factor 7
TCR	T cell receptor
TEC	Thymic epithelial cells

T _{FH}	Follicular helper T cell
T _{FR}	Follicular regulatory T cell
TGF	Transforming growth factor
Th	Helper T cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell
tTreg	Thymic regulatory T cell
WEHI	Walter and Eliza Hall Institute
WT	Wild type

Table of Contents

Chapter One: Introduction.....	1
Published Literature Review	2
Addendum	22
The Adaptive Immune Response	22
T and B lymphocytes.....	23
I) T cell development.....	23
II) CD4+ T lymphocytes.....	25
III) B lymphocytes and T-B cell zone interactions.....	27
Regulatory T cells	29
I) Treg cell origins	30
II) Treg cell function.....	31
III) Treg cell homeostasis and specialization	31
IV) Effector Regulatory T cells	32
The Role of IRF4 in the Transcriptional Regulation of Effector Treg cells	34
Research questions and Thesis Aims	36
Chapter Two: Methods and Experimental Procedures	37
2.1 Animal models	37
2.1.1 Mice	37
2.1.2 Bone marrow reconstitution of chimeric mice	37
2.1.3 Infections	38
2.2 Buffers and Media	38
2.3 Experimental procedures for cellular immunology.....	39
2.3.1 Organ harvests and processing for lymphocyte isolation.....	39
2.3.2 Cell culture	39
2.3.3 Antibodies and Flow Cytometry.....	40
2.3.4 Cell staining for division tracking	41
2.3.5 Treg cell depletion	42
2.3.6 Tamoxifen treatment.....	42
2.4 Experimental procedures for molecular immunology.....	42
2.4.1 Polymerase chain reaction (PCR) for genotyping	42
2.4.2 Immunoblotting	43
2.4.3 Immunofluorescence microscopy	44
2.4.4 Quantitative (q)PCR and gene expression profiling.....	44

2.4.5 Assay for transposase-accessible chromatin (ATAC) sequencing and bioinformatics analysis	45
2.4.6 Chromatin immunoprecipitation (ChIP) sequencing.....	46
2.5 Statistical Analysis	46
Chapter Three: Ontogeny of Effector Regulatory T cells	47
Introduction	47
Results.....	51
3.1 Effector Regulatory T cell development	51
I) Subclassification of Treg cells based on activation status.	51
II) Ontogeny of Treg cells.	53
III) Effector regulatory T cell kinetics in lymphoid organs during neonatal ontogeny.....	53
IV) Neonatal eTreg cells accumulate in non-lymphoid organs.	55
V) Dynamics of ICOS expression during Treg cell differentiation.....	58
3.2 The role of AIRE in early effector Treg cell program	60
I) Ontogeny of neonatal thymic Treg cells and eTreg cells in Aire-deficient mice.	60
II) Kinetics of lymphoid and non-lymphoid organ Treg cells in the absence of Aire.....	60
III) The requirement of Aire in the regulation of splenic eTreg cell compartment.	63
IV) AIRE and eTreg cells in the SI.	63
V) AIRE does not affect the generation of a subset of Treg cells during influenza infection. .	65
3.3 The expression of IRF4 in Treg cells	68
I) Generation and validation of a novel <i>Irf4</i> reporter mouse.	68
II) High <i>Irf4</i> transcription in thymic Treg cells.	69
III) <i>Irf4</i> transcription in splenic Treg cells.	72
Discussion	74
Ontogeny of effector regulatory T cell.....	74
<i>Aire</i> limits tissue-specific inflammation.....	76
<i>Irf4</i> in early Treg cell development.....	77
Chapter Four: T Follicular Regulatory cell differentiation is dependent on the Transcription Factor IRF4	79
Introduction	79
Follicular T helper (T _{FH}) cell generation.....	79
Phenotypic characteristics of follicular T helper cells.....	80
Cytokines essential for the generation of follicular T helper cells.....	80

Transcription factors required for follicular T helper cell generation	81
Discovery of T Follicular Regulatory cells	82
Key T _{FR} cell characteristics	83
T _{FR} cell differentiation and function.....	84
Transcriptional regulation of T _{FR} cell differentiation	85
Results	87
4.1 Kinetics of the germinal centre response to influenza infection.	87
4.2 T _{FR} cells express the transcription factor Blimp-1 and its target IL-10.	90
I) Blimp-1 is essential for the terminal differentiation of effector Treg cells.	90
II) Blimp-1 expression is restricted to eTreg cells where it is required for Il10 expression.	90
III) T _{FR} cells continually experience T cell receptor signals.	92
4.3 IRF4 is highly expressed by T _{FR} cells.	94
4.4 Foxp3-specific deletion of IRF4 results in dysregulated germinal centre responses.	94
4.5 The requirement for IRF4 in T _{FR} cell differentiation is cell intrinsic.....	98
I) Global IRF4-deficient mice demonstrated an absent T _{FR} cell compartment.	98
II) Treg-specific deletion of IRF4 resulted in an absent T _{FR} cell compartment.	100
III) <i>Irf4</i> -deficient Treg cells from hemizygous <i>Irf4^{fl/fl} Foxp3^{Cre}</i> transgenic mice fail to generate T _{FR} cells.	101
4.6 T _{FR} cell function is dependent on the expression of IRF4.....	103
I) Minor plasma cell dysregulation in the absence of T _{FR} cells.....	103
II) An <i>in vitro</i> approach to assess the functional capacity of T _{FR} cell.	104
4.7 IRF4 is essential for T _{FR} cell maintenance.	106
4.8 IRF4 regulates molecules essential for T _{FR} cell migration and localization.	108
4.9 Gene regulation of IRF4 in naïve CD4, regulatory T and follicular T cells.	110
I) Identification of ‘follicular signature genes’ expressed in T cell associated with germinal centre B cells.	110
II) Regulation of the T _{FR} cell transcriptome by IRF4.....	112
4.11 c-Maf in T _{FR} cell differentiation.	114
I) c-Maf is required for T _{FR} cell differentiation.....	114
II) Treg cell intrinsic requirement for c-Maf in T _{FR} cells.....	117
III) T _{FR} cells jointly express IRF4 and c-Maf.....	117
4.12 The transcription factor Foxo1 in T _{FR} cell differentiation.....	121
4.13 TGF- β signalling does not alter the T _{FR} cell generation.	124
Discussion	127

Chapter Five: Molecular regulation of T follicular regulatory cells 131

Introduction	131
Results.....	132
5.1 Chromatin accessibility profiling.	132
I) The chromatin architecture of naïve and follicular CD4 T cells.	132
II) Differences in the chromatin landscape of follicular T cells.	133
5.2 IRF4 and c-Maf regulate common and distinct genes in follicular T cell compartment.	137
5.3 A minority of the follicular gene expression signature is affected by changes in the chromatin landscape.	139
5.4 Genomic binding of c-Maf.	142
5.5 IRF4 and c-Maf establish the gene expression profile of T _{FR} cells.	144
5.6 Epigenetic modification of T _{FR} cells is partially regulated by IRF4 and c-Maf.	145
Discussion	148
Changes in chromatin landscape during CD4 T cell activation	148
Regulation of the T _{FR} cell transcriptional program by IRF4 and c-Maf	150
Regulation of the T _{FR} cell chromatin landscape by c-Maf and IRF4	151
Chapter Six: Final discussions.....	153
I) Blimp-1 expression in neonatal eTreg cell ontogeny.	153
II) IRF4 is central to eTreg cell generation.	154
III) T _{FR} cells are a subset of eTreg cells that have a cell-intrinsic requirement for IRF4. ...	155
IV) Molecular regulation of T _{FR} cells by IRF4 and c-Maf.	156
V) Regulation of the chromatin landscape of T _{FR} cells by c-Maf and IRF4	158
Conclusion.....	159
Bibliography.....	160

List of Figures

Figure 1: Overview of effector CD4 T cell differentiation.	26
Figure 2: Germinal centre structure.....	29
Figure 3: Effector Treg cell differentiation.	34
Figure 4. Subclassification of Treg cell based on activation status.....	52
Figure 5. Treg cell kinetics during neonatal ontogeny.	54
Figure 6. Effector Treg cell kinetics in lymphoid organs during neonatal ontogeny.	55
Figure 7. The ontogeny of effector Treg cells in the liver and small intestine.....	57
Figure 8. ICOS expression during eTreg cell development.	59
Figure 9. Ontogeny of Treg cells in Aire-deficient mice.	61
Figure 10. Kinetics of lymphoid and non-lymphoid organ Treg cells in the absence of Aire.	62
Figure 11. Phenotype of lymphoid organs eTreg cells from adult Aire-deficient mice.	64
Figure 12. Phenotype of eTreg cells within the lamina propria of the SI.....	65
Figure 13. Aire-deficiency does not affect T _{FR} cell differentiation.....	67
Figure 14. Generation and validation of novel <i>Irf4</i> reporter mice.....	70
Figure 15. Thymic Treg cells express high levels of <i>Irf4</i>	71
Figure 16. <i>Irf4</i> transcription in splenic Treg cells.	73
Figure 17. Kinetics of the germinal centre response after influenza infection.	89
Figure 18. T _{FR} cell express the transcription factor Blimp-1 and its target IL-10.....	91
Figure 19. Blimp-1 expression is restricted to eTreg cells where it is required for <i>Il10</i> expression.	92
Figure 20. T _{FR} cells perceive TCR signals.	93
Figure 21. IRF4 is highly expressed by T _{FR} cells.	95
Figure 22. IRF4 regulates T _{FR} cell differentiation.....	97
Figure 23. IRF4 is intrinsically required for T _{FR} cell differentiation.	99
Figure 24. Treg cell specific deletion of <i>Irf4</i> results in a near complete absence of T _{FR} cells.	100
Figure 25. T _{FR} cell differentiation is dependent on IRF4 expression.	102
Figure 26. IRF4 is required for the expression of T _{FR} cell migration and functional molecules.	105
Figure 27. IRF4 is required for T _{FR} cell maintenance.	107
Figure 28. T _{FR} cell localization is dependent on chemokine receptor expression.....	109
Figure 29. T _{FR} cell gene signature.	111

Figure 30. Identification of direct IRF4 target gene candidates in Treg cells.	113
Figure 31. c-Maf is required for T _{FR} cell commitment.	116
Figure 32. c-Maf is intrinsically required for T _{FR} cell generation.	118
Figure 33. Effector Treg and T _{FR} cells jointly express IRF4 and c-Maf.	120
Figure 34. T _{FR} cell generation in the absence of Foxo1.	123
Figure 35. TGF- β deletion does not restrict follicular T cell differentiation.	126
Figure 36. The chromatin architecture of naïve and follicular CD4 T cells.	134
Figure 37. Identification of the chromatin landscape of follicular CD4 T cells.	136
Figure 38. IRF4 and c-Maf regulate common and distinct genes in the follicular T cell compartment.	140
Figure 39. Some of the gene expression profile of follicular T cells is regulated by epigenetic modification.	141
Figure 40. Genomic binding of c-Maf and IRF4.	143
Figure 41. Key T _{FR} related genes are targets of c-Maf and IRF4.	145
Figure 42. Epigenetic modification of T _{FR} cells is partially regulated by IRF4 and c-Maf.	147

List of Tables

Table 1. List of buffers and media ingredients.	38
Table 2. List of primers for genotyping.	43
Table 3. Number of open chromatin regions identified in ATAC-seq samples.	133

Chapter One: Introduction

Part of this chapter has been published as a chapter in the following book series. All authors have given permission for this publication to be used as a part of Chapter 1 of this thesis.

Teh, P. P., A. Vasanthakumar and A. Kallies (2015). "Development and Function of Effector Regulatory T Cells." Prog Mol Biol Transl Sci 136: 155-174.

<https://doi.org/10.1016/bs.pmbts.2015.08.005>

This research project evolved out of my interest in the molecular regulation of tissue-regulatory T (Treg) cell development and maintenance. To further understand the current relevant concepts, I performed a comprehensive literature review, which resulted in the following publication. It provides a broad overview on the development and function of Treg cells, and a more detailed review on a Treg cell subset termed effector (e)Treg cells, which are predominantly found within non-lymphoid organs. In addition to this review, my thesis also focuses on aspects of thymic T cell development, Treg cell differentiation and transcription factors essential for eTreg cell differentiation and function. There have been numerous publications that have contributed to the field since the review's initial publication and I have co-authored three peer reviewed publications, containing some of the experimental results in this thesis.



Development and Function of Effector Regulatory T Cells

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Contents

1. Introduction	156
2. Features of Effector T Regulatory Cells	157
3. Role of TCR Signaling in Effector T Regulatory Cell Differentiation	158
4. Cytokine Requirements of Effector T Regulatory Cells	159
5. Distinct Migratory Properties of Effector T Regulatory Cells	159
6. Functional Specialization of Effector T Regulatory Cells	161
7. Effector T Regulatory Cells in Nonlymphoid Organs	162
7.1 Visceral Adipose Tissue	162
7.2 Gastrointestinal Tract	163
7.3 Skin	164
7.4 Muscle	164
7.5 Central Nervous System	165
7.6 Kidney	165
8. A Unified Model of Effector T Regulatory Cell Differentiation	166
9. Future Perspectives	168
Acknowledgments	169
References	169

Abstract

Distinguishing self from nonself is a unique feature of the immune system. Although most self-reactive T cells are eliminated in the thymus, a few rogue cells escape the negative selection process and have the potential to mediate autoimmune disease. Over the last decade, there has been a vast improvement in our understanding of the cellular mechanisms that evolved to dampen the deleterious effects of these self-reactive T cells. In particular, T cells expressing the transcription factor FoxP3, known as regulatory T (Treg) cells, play a central role in maintaining immune homeostasis and suppressing autoimmune responses. In addition, Treg cells are endowed with the ability to suppress diverse inflammatory responses both in lymphoid and in nonlymphoid tissues. This requires Treg cells to undergo a peripheral differentiation and specialization program that results in the emergence of effector Treg (eTreg) cells that are characterized by their

ability to produce high amounts of immunosuppressive molecules, including IL-10. This chapter discusses the recent advances in our understanding of the mechanisms governing the differentiation, migration, and maintenance of eTreg cells, in particular in nonlymphoid tissues, in health and disease.



1. INTRODUCTION

Immune homeostasis critically depends on the balance between anti- and proinflammatory processes. Excessive and prolonged inflammation, whether in response to infection, tissue injury, or perturbed metabolic homeostasis, is deleterious to the organism. Treg cells, a specialized subset of CD4⁺ T cells, maintain immunological homeostasis in a nonredundant manner by suppressing the activation of self-reactive and inflammatory T cells. The development, function, and maintenance of Treg cells are dependent on the expression of the transcription factor FoxP3.¹ The essential nature of Treg cells is exemplified in mice or humans with loss-of-function mutations in FoxP3 that result in a fatal, early-onset, T-cell-dependent multiorgan inflammatory disease.^{2–4}

Approximately 10–15% of the murine and 5–10% of the human CD4⁺ T-cell population are comprised of Treg cells. This population can be further subdivided based on their developmental origin into cells that acquire FoxP3 expression in the thymus and those that are induced to differentiate into Tregs in the periphery. Treg cell development in the thymus is a two-stage process with the first stage governed by T-cell receptor (TCR) signaling and the second dependent on the cytokine interleukin (IL)-2.^{5–7} In the first stage, transcription factors and epigenetic regulators downstream of the TCR facilitate the development of partially committed Treg precursors, in which the FoxP3 locus is poised for activation. Transcriptional induction of FoxP3 takes place in the second stage, which is driven by the IL-2-dependent transcription factor STAT5 (signal transducer and activator of transcription 5).⁸ FoxP3 subsequently establishes the Treg cell transcriptional program, which reinforces stable maintenance of FoxP3 itself and confers functional fitness to the cells. IL-2 and TCR signaling are not only required for the development of Treg cells but are also essential for their continuous survival and homeostasis.^{6,9,29}

Treg cells execute their suppressor function by diverse mechanisms (reviewed in Refs. 10–12). These include limiting IL-2 availability to conventional T cells, CTLA-4 (cytotoxic T-lymphocyte-associated protein

4)-mediated inhibition of costimulation, secretion of IL-10 and IL-35, or expression of suppressor molecules such as CD39 and TIGIT (T-cell immunoreceptor with Ig and ITIM domains).^{13–18} Although it is clear that Treg cells can utilize multiple pathways that lead to the suppression of immune responses, the relative contribution of each of these mechanisms is unknown.



2. FEATURES OF EFFECTOR T REGULATORY CELLS

Most Treg cells localize to secondary lymphoid organs, and the majority of them have a naïve phenotype. These Treg cells have been termed central Treg (cTreg) cells. They express high amounts of CD25, the alpha chain of the high-affinity IL-2 receptor, and, therefore, can deprive surrounding conventional T cells of IL-2 and limit their proliferation. However, the expression of functionally important molecules such as IL-10, CTLA-4, ICOS (inducible costimulator), and TIGIT is restricted to or greatly increased in a subpopulation of Tregs, which have an activated phenotype. These cells have been termed effector Treg (eTreg) cells.²⁰ Depending on their localization and surrounding cytokine milieu, eTreg cells are phenotypically diverse. eTreg cell development is regulated by TCR signals that act together with cytokine and tissue-specific factors. T helper (T_H) lineage-specific and other transcription factors cooperate with FoxP3 during this differentiation process to generate functional and spatial diversity, which is critical to contain immune-mediated damage. Mature eTreg cells express the transcription factor B-lymphocyte-induced maturation protein 1 (Blimp-1) and are potent producers of the anti-inflammatory cytokine IL-10.^{19,20} Several proinflammatory cytokines are known to upregulate the expression of Blimp-1 in Treg cells. Transcriptional profiling of Blimp-1⁺ eTreg cells demonstrated their unique transcriptional signature and has provided further insights into key molecular events that underpin the eTreg cell differentiation program.²¹ Importantly, the FoxP3-dependent transcriptional program is conserved in eTreg cells. Although Blimp-1 expression was used to initially define eTregs, it is not required for their differentiation. It is, however, essential for IL-10 production and plays a significant role in the migration of eTreg cells to nonlymphoid tissues.²⁰ While eTreg cells represent a minor population within lymphoid organs, they predominate in nonlymphoid tissues, reflecting their essential role in the resolution of local inflammation and tissue homeostasis.¹⁹

Analogous to the murine Treg cell differentiation scheme, human cTreg cells are CD45RA⁺FoxP3^{low}, while eTreg cells are CD45RO⁺CD45RA⁻FoxP3^{high}.^{22,23} eTreg cells in humans are also known to express TIGIT, CD39, ICOS, and IL-10.^{24–26} Thus, mounting evidence suggests that similar to murine Treg cells, human Treg cells can undergo a peripheral differentiation program critical for the maintenance of immune tolerance.



3. ROLE OF TCR SIGNALING IN EFFECTOR T REGULATORY CELL DIFFERENTIATION

TCR signaling is critical for the first stage of Treg cell development in the thymus. Several TCR-induced transcription factors such as c-Rel and nuclear factor of activated T cells (NFAT) are directly involved in priming the FoxP3 locus and in inducing its transcription. Deletion of these transcription factors results in severe Treg cell deficiency in the thymus and periphery.^{27,28} Recently, two studies have shed light on the role of TCR signaling in Treg cell homeostasis demonstrating the continuous requirement of TCR signaling.^{29,30} Strikingly, loss of TCR β specifically affected the eTreg cell population, indicating that TCR signals are required for the development of functionally mature Treg cells. This is consistent with our observations that expression of Nur77, a surrogate marker for TCR signaling, was elevated in eTreg cells in nonlymphoid tissues compared to their cTreg counterparts.^{21,31}

We have previously described that the TCR-induced transcription factor interferon regulatory factor (IRF)4 is required for eTreg cell differentiation.²⁰ IRF4 deficiency severely reduced the number and function of eTreg cells in both lymphoid and nonlymphoid tissues.²⁰ Although the exact role of IRF4 in eTreg cell differentiation is unknown, evidence from other lymphocyte populations suggests that it affects cell differentiation and function at multiple levels. In CD4⁺ T cells, IRF4 is required for the differentiation of multiple T_H cell subsets, including T_H2, T_H17, and T follicular helper (T_{FH}) cells,^{32–34} while in CD8⁺ T cells, IRF4 regulates metabolic functions and clonal population expansion in a TCR-affinity-dependent manner.³⁵ IRF4 is also essential for germinal center B-cell development and plasma cell differentiation, where it is critical in inducing Blimp-1.^{36,37} IRF4 also activates expression of Blimp-1 in Treg cells and cooperates with Blimp-1 to activate IL-10.²⁰

eTreg cells, in particular those residing in nonlymphoid tissues, have distinct TCR repertoires, suggesting that their development can be influenced directly by local cues such as tissue antigens.³⁸ Differences in TCR-ligand

affinity are known to have direct effects on the characteristics and intensity of downstream signaling pathways, which might also influence the differentiation of eTreg cells.³⁹ Taken together, these observations demonstrate an essential role for TCR signaling in eTreg cell differentiation.



4. CYTOKINE REQUIREMENTS OF EFFECTOR T REGULATORY CELLS

In addition to TCR signaling, cytokines are essential mediators required to shape immune responses. Many cytokines execute their function by inducing the phosphorylation of downstream STAT transcription factors, which independently or in cooperation with other TCR-induced transcription factors reprogram naïve T cells to effector T cells. eTreg cells are dependent upon the local cytokine milieu, suggesting that their differentiation mirrors this paradigm. In line with this idea, STAT3 deficiency in Treg cells disarms their ability to suppress T_H17 responses.⁴⁰ Treg cells depend on IL-2 for their development and ongoing survival, which is mediated by the prosurvival molecule Mcl-1.⁴² Interestingly, cTregs are particularly responsive to IL-2 and preferentially localize in the T-cell zones of lymphoid organs in a chemokine receptor (CCR)7-dependent manner.⁴³ Consistent with this, we demonstrated that the Blimp-1⁺ eTreg cells that do not express CCR7 were excluded from the T-cell zones.²¹ Together, these observations suggest that eTreg cells may use alternate cytokines for their survival. Transcriptional profiling revealed that several cytokine receptors including the IL-33 receptor (ST2) were specifically upregulated in eTregs.²¹ In line with this finding, we and others have recently demonstrated the specific requirement for IL-33 in the homeostasis of visceral adipose tissue (VAT) and colonic Treg cells.^{21,44,45} Similarly, Treg cells present in the muscle during inflammation or injury also express high levels of ST2, although its Treg-specific role in this tissue is unknown.⁹¹ Collectively, these studies support the notion that Treg cells require site-specific cytokines to differentiate to eTreg cells.



5. DISTINCT MIGRATORY PROPERTIES OF EFFECTOR T REGULATORY CELLS

The localization of different Treg populations depends on the expression of distinct chemokine receptors. CCR7 and CD62L (L-selectin) are highly expressed by cTreg cells and allow their efficient trafficking into lymph nodes and the white pulp of the spleen (reviewed in Ref. 46).

Downregulation of CD62L and CCR7 is one of the early events of eTreg cell differentiation that facilitates their egress from lymphoid organs and subsequent migration to sites of inflammation^{46–48} (see Table 1). This event is important, as tissue-specific inflammatory disease occurs when eTreg cell migration is impaired.⁴⁹ While downregulation of CCR7 is essential in this process, concerted upregulation of other chemokine receptors facilitates the migration and retention of eTreg cells in various peripheral tissue microenvironments. CCR4 expression in Treg cells is required for migration to the skin and lung to suppress aberrant inflammation⁴⁹ and promotes Treg cell migration to the gut to limit colitis.⁵⁰ In addition to CCR4, Tregs in the small intestinal lamina propria express high amounts of CCR9.⁵¹ Similarly, CCR6 directs trafficking of Treg cells to the intestine and the central nervous system (CNS) during inflammation and allows for the accumulation of Treg cells in mucosal and inflamed sites.^{52–54} Furthermore, CXCR3 is specifically expressed on a subset of Treg cells that develop under T_H1 inflammatory conditions,⁵⁵ while CXCR5 is expressed on Treg cells in the germinal center.⁵⁶

Integrins also play critical roles by promoting retention of Tregs within inflamed tissues. For example, expression of α E integrin (CD103) on Treg cells poises them for recruitment and retention within nonlymphoid organs during infection or inflammation.⁵⁷ Consequently, CD103⁺ Treg cells can be found in nonlymphoid organs such as the skin, lungs, and the epithelial lining of the gut, and also at sites of inflammation.^{49,58} Overall, these observations demonstrate that the migrational properties of Treg cells are intimately linked to their functional specialization and critical for efficient immune suppression.

Table 1 Characteristics of Central (c)Treg Cells Compared to Effector (e)Treg Cells

Central Treg Cells	Effector Treg Cells
Dominant in secondary lymphoid organs, including lymph nodes and the white pulp of the spleen	Dominant in nonlymphoid tissues and also present in secondary lymphoid organs, such as the red pulp of the spleen
FoxP3 ⁺	FoxP3 ⁺
Blimp-1 [−]	Blimp-1 ⁺
CD62L ⁺	CD62L [−]
CCR7 ⁺	CCR7 [−]
IL10 [−]	IL10 ⁺



6. FUNCTIONAL SPECIALIZATION OF EFFECTOR T REGULATORY CELLS

Analogous to other T cells, Treg cells exhibit functional plasticity and have the capacity to adapt to diverse inflammatory conditions.^{20,59–63} In line with this notion, Treg cells are able to functionally diversify in response to the same cytokine signals and co-opt the similar transcriptional modules that polarize conventional CD4⁺ T cells to T_H1, T_H2, T_H17, and T_{FH} cells. This allows Tregs cells to specifically antagonize their conventional counterparts that differentiated in the same inflammatory microenvironment (reviewed in Refs. 19,62,64).

eTreg cells that develop under T_H1 inflammation have been particularly well studied. In addition to FoxP3, these Treg cells rely on the transcription factor T-bet for their differentiation and depend on cytokines such as interferon gamma (IFN γ) and IL-27.^{55,65} T-bet expression in Treg cells induces upregulation of CXCR3, thereby promoting colocalization of Tregs and conventional CD4⁺ T cells during Th1 inflammation.⁵⁵ On the other hand, expression of the canonical T_H2 transcription factor GATA3, although not essential for Treg cell homeostasis, is required for T_H2-type Treg cell development during inflammation, particularly at mucosal sites.⁶⁶ In the absence of GATA3, Treg cells are unable to control T_H2 responses and instead produce IL-17.⁶⁷ Interestingly, a recent study revisiting the role of the transcription factors T-bet and GATA3 has demonstrated that the absence of these transcription factors individually in Treg cells does not result in pathology under homeostatic conditions. However, the combined deletion of both transcription factors in Treg cells resulted in severe autoimmunity, highlighting the importance of these regulators in coordinating the transcriptional program that maintains Treg cell function.⁶⁸

Another well-studied example of codifferentiation of conventional CD4 T cells and Treg cells is the germinal center response, which is required for affinity maturation and immune globulin class switching of B cells. Germinal center development and organization are facilitated by a specialized T_H cell subset called T_{FH} cells.⁴¹ Treg cells can differentiate into a specialized population termed T follicular regulatory (T_{FR}) that modulate the T_{FH} response.⁵⁶ Similar to T_{FH} cells, T_{FR} cells depend on the transcription factor Bcl6, and akin to other eTreg populations, T_{FR} cells express Blimp-1.⁵⁶ This is in contrast to T_{FH} cells, whose development is inhibited by Blimp-1.⁵⁶ Further studies are required to understand commonalities and differences in the differentiation program of T_H cell populations and their corresponding Treg cell subsets.



7. EFFECTOR T REGULATORY CELLS IN NONLYMPHOID ORGANS

Recent murine studies have established the existence of multiple distinct Treg cell subsets residing in nonlymphoid tissues. Depending on their anatomical localization, these Treg cells express distinct molecular signatures and display unique phenotypic features.^{61,69,91}

In particular, barrier tissues, such as the gastrointestinal tract and the skin that are constantly exposed to commensal microbiota, have a constitutive eTreg cell presence. Similarly, tissues rich in proinflammatory cytokines, such as the VAT, harbor large numbers of Treg cells. This is in contrast to other nonlymphoid tissues such as the muscle and the CNS, which recruit Treg cells only after injury or inflammation. In the following section, we will discuss some of the well-characterized tissue-resident Treg cells.

7.1 Visceral Adipose Tissue

VAT Treg cells constitute one of the better-known specialized populations of tissue-resident Treg cells. Lean adipose tissue is rich in Treg cells that produce large amounts of IL-10 and are critical for the suppression of local inflammation, which contributes to glucose intolerance and insulin resistance.^{70,72} Obesity is a major driver of VAT inflammation and is characterized by infiltration and activation of inflammatory leukocytes resulting in insulin resistance (reviewed in Ref. 73). Genetic or diet-induced obesity leads to a pronounced loss of Treg cells from the VAT,⁷⁰ which is also seen in humans, where obesity is directly linked to development of the metabolic syndrome and type 2 diabetes.⁷⁴

VAT Treg cells express high amounts of peroxisome proliferator-activated receptor gamma (PPAR- γ), a transcription factor that regulates adipocyte differentiation and is critical for the development of VAT Treg cells.⁷⁵ *In vitro* studies demonstrated that PPAR- γ cooperated with FoxP3 resulting in upregulation of the VAT Treg cell signature.⁷⁵ VAT Treg cells have a distinct TCR repertoire and cytokine and chemokine receptor expression pattern compared to cTreg cells.⁷⁰ For example, they express high amounts of CCR2 and the killer cell lectin-like receptor G1 (KLRG1), which are not expressed on cTreg and only on some eTregs in other anatomical locations.⁷⁰

We recently discovered that IL-33 is essential for VAT Treg cell differentiation and homeostasis.²¹ IL-33, known for its role in enhancing

T_H2-type immunity, is produced by a range of different cell types.⁷⁶ It serves as an “alarmin” that signals inflammation and tissue damage to responder cells.⁷⁷ Administration of IL-33 to mice resulted in massive VAT Treg population expansion and was able to rescue Treg cell numbers within the VAT of obese mice, leading to attenuated VAT inflammation and improved insulin sensitivity.²¹ Induction of both the IL-33 receptor (ST2) and PPAR- γ is controlled by the TCR-induced transcription factor IRF4, and mice lacking either of these factors lacked Treg cells in the VAT.^{21,75} This confirms the critical roles of TCR signaling and IRF4 in eTreg cell differentiation.

7.2 Gastrointestinal Tract

The gastrointestinal tract constitutes an important barrier that is constantly exposed to benign commensals, pathological microbes, and food-derived antigens. There is immense interest in understanding the interplay of the various immune cell types and the local microbiota that maintain the balance between pro- and anti-inflammatory stimuli. Gut bacteria modulate the mammalian immune system in a variety of ways, with some bacteria producing metabolites and proteins that enhance the development of induced Treg cells.^{78,79} Emerging evidence in the field suggests that disruption to the normal composition of the gut microbiota (dysbiosis) can alter the local immune cells from a tolerogenic state to an inflammatory, pathogenic one, resulting in inflammatory and metabolic diseases (reviewed in Refs. 80,81).

Intestinal Treg cells reside in the lamina propria and gut-associated lymphoid tissues and are essential for regulating local mucosal homeostasis, preventing the development of inflammatory diseases within the gut.^{82,113} Intestinal Treg cells secrete high amounts of IL-10, which is critical for the prevention of colitis.¹⁶ In particular, polysaccharide A of the commensal bacterium *Bacteroides fragilis* facilitates the development of IL-10-producing FoxP3⁺ Treg cells, which can provide protection from experimental colitis.⁷⁹ Intestinal Treg cells can originate from either thymic-derived or peripherally induced Treg cells, though there is some uncertainty as to the relative contribution of each group to intestinal Treg pool.^{69,83} Interestingly, a subset of Treg cells within the lamina propria coexpress FoxP3 and the transcription factor ROR γ t, suggesting a unique developmental origin.⁸⁴ Similar to other tissue-resident Treg populations, intestinal Treg cells are dependent on the local cytokine milieu for their expansion and maintenance, and analogous to the VAT, IL-33 plays an important role.⁸⁵ Disruption of homeostasis in the gastrointestinal system results in the release of

IL-33 from epithelial cells, and IL-33 enhances TGF β (transforming growth factor beta)-mediated differentiation of Treg cells through serine phosphorylation of GATA3, which is required for maintaining high expression of FoxP3.⁸⁵

7.3 Skin

The skin is a barrier tissue, which offers protection from a variety of environmental insults. It is host to a multitude of commensals but is also frequently exposed to pathogens (reviewed in Ref. 86). A range of immune cells, including Treg cells, reside in the skin. They are required for maintaining immune tolerance but also contribute to wound healing.⁷¹ Similar to results from murine studies, the homeostasis of Treg cells in the human skin is also determined by skin commensals. Human skin Treg cells display unique TCRs, express distinct cell surface markers, produce different cytokines compared to circulating Treg cells, and are preferentially located within hair follicles.⁸⁷ Both human and murine skin Treg cells accumulate upon antigenic stimulation and require IL-7 (derived from keratinocytes) for their maintenance.^{87,88} They express higher levels of the activation markers such as CTLA-4 and ICOS.^{87,89} The recruitment of skin Treg cells is dependent upon the expression of the chemokine receptor CCR4,^{49,90} and deficiency in CCR4 expression on Treg cells after an inflammatory insult resulted in the development of severe cutaneous inflammation.⁴⁹

7.4 Muscle

Healthy muscle tissue is almost completely devoid of hematopoietic cells and does not contain Treg cells. However, two recent studies discovered that a specialized population of Treg cells can be found within the muscle after injury and is required for tissue repair processes.^{91,92} Muscle Treg cells regulated the differentiation of satellite cells necessary for the regeneration of skeletal muscle.⁹¹ Upon injury, Treg cells accumulated and proliferated within the muscle and were found in close proximity to regenerating muscle fibers.⁹² Muscle Treg cells showed enhanced expression of activation and differentiation markers such as glucocorticoid-induced tumor necrosis factor receptor family-related gene, programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin protein 3, and KLRG1 and displayed molecules associated with Treg suppressive function such as CTLA-4 and IL-10.^{91,92} Interestingly, Treg cells persisted for weeks in the muscle despite the removal of the inciting insult. Analogous to the other tissue-resident

Treg cells, muscle Treg cells show largely distinct TCR repertoires when compared to conventional Treg cells.⁹¹ Muscle Treg cells are characterized by their high production of amphiregulin, an epidermal growth factor-like molecule that is essential for tissue repair (reviewed in Ref. 93). Furthermore, muscle Treg cells upregulate the chemokine receptor CCR2, which is necessary for the recruitment of leucocytes to injured skeletal muscles.⁹⁴

7.5 Central Nervous System

The role of immune cells within the CNS has been intensely studied. While lymphocytes are excluded from the healthy CNS, in inflammatory conditions, Treg cells can enter and accumulate within the CNS and suppress inflammation.^{95,97} In line with this, patients diagnosed with multiple sclerosis (MS), a chronic demyelinating inflammatory disorder of the CNS, have increased numbers of Treg cells detected within their cerebrospinal fluid.⁹⁶

In studies utilizing the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, Treg cells are critical to attenuate inflammation.⁹⁷ Interestingly, the presence of IL-6 in the local cytokine milieu can abolish Treg cell-mediated suppression of activated T cells in the CNS.⁹⁵ Treg cells require CCR6 to infiltrate the CNS compartment and to attenuate the inflammation associated with EAE.⁵³ In addition, CD103⁺ Treg cells are found within the CNS during the recovery phase,⁹⁸ suggesting that Treg cells are retained in the CNS in a tissue-specific manner.

Intriguingly, although the mechanism of action is not clear, melatonin, a hormone that controls circadian rhythm, was shown to improve EAE and attenuate T-cell responses. Mice that received melatonin showed decreased T_H1- and T_H17-type responses and interestingly displayed increased Treg cell frequencies and IL-10 production in the CNS.⁹⁹ Consistent with the eTreg cell model, CNS Treg cells have an activated phenotype and express high levels of IL-10 to facilitate disease remission.¹⁰⁰

7.6 Kidney

The implications for Treg cells residing in the kidney are important as they can have a significant impact on the outcomes of acute kidney injury (AKI) occurring as a consequence of acute renal allograft rejection, autoimmune diseases, or ischemic reperfusion injuries. Although only low numbers of Treg cells reside in the healthy kidney,¹⁰¹ their numbers increase after an insult, both in mice and in humans.¹¹⁴ Kidney Treg cells have been shown to reduce the severity of inflammation and injury related to ischemia

reperfusion, a common cause for AKI and a critical cause for early allograft dysfunction in kidney transplantation.¹⁰² These cells express high levels of TGF β , IL-10, and CXCR3¹⁰⁶ and act to dampen interstitial inflammation by reducing the production of proinflammatory cytokines such as IFN γ and TNF α (tumor necrosis factor alpha), thereby facilitating tissue repair.¹⁰³ In line with these findings, Treg cell-deficient mice subjected to ischemia reperfusion injury (IRI), experimental murine glomerulonephritis or nephrotoxic agents show increased renal inflammation, acute glomerular or tubular necrosis, and deterioration in kidney function.^{102,104,105} In addition, depletion of Treg cells in mismatched mouse models of kidney transplants resulted in renal allograft rejection.¹⁰⁶

Although it has been reported that Treg cells upregulate CCR6 in order to home to the kidneys,¹⁰⁷ little is known about the molecular determinants that may facilitate their differentiation and function. Furthermore, while kidney Treg cells are able to secrete IL-10 to ameliorate AKI after IRI¹⁰² and crescentic glomerulonephritis,¹⁰⁸ it is unclear whether they perform any kidney-specific functions. Thus, more detailed studies are required to define the precise role and nature of kidney-resident Tregs in health and disease.



8. A UNIFIED MODEL OF EFFECTOR T REGULATORY CELL DIFFERENTIATION

At present, the developmental pathways of eTreg cells are under intense study. Although it is clear that they can differentiate from cTreg cells in the periphery, a recent report suggests that tissue-seeding Treg cells develop early during ontogeny in the thymus.⁷² Phenotypically, eTreg cell differentiation is characterized by the downregulation of CCR7 and CD62L and the upregulation of suppressor molecules such as IL-10, TIGIT and CTLA-4. As outlined above, eTreg cells also upregulate the transcription factor Blimp-1, which is required for IL-10 production (Fig. 1).²⁰ Transcriptome analyses of Treg cells isolated from various organs, including lymphoid tissues, VAT, colon, and muscle, have vastly improved our understanding about the specific requirements for eTreg cell differentiation.⁵⁹ Furthermore, our direct comparison of Treg cell subsets based on Blimp-1 expression has revealed dramatically different transcriptional profiles of cTregs and eTregs.²¹ Importantly, TCR signaling and transcription factors downstream of TCR have emerged as key players in the eTreg cell differentiation process.²⁹ In particular, IRF4 plays a key role during eTreg differentiation. Loss of IRF4 leads to eTreg cell deficiency in lymphoid tissues and Treg cell deficiencies in nonlymphoid

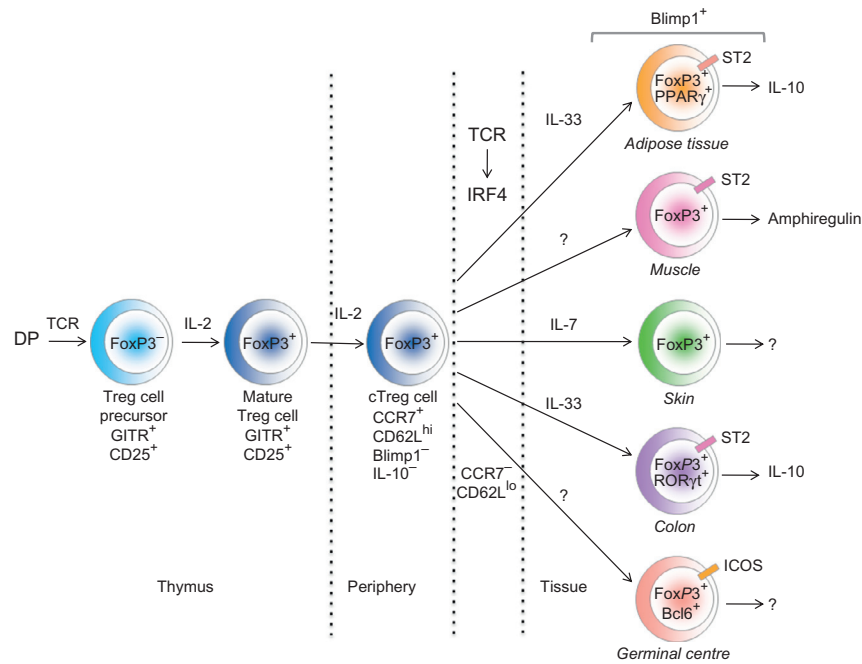


Figure 1 Schematic representation of effector regulatory (eTreg) cell development. Early Treg cell development in the thymus occurs in two stages where T-cell receptor (TCR) and IL-2 signaling cooperate to upregulate the lineage-specific transcription factor FoxP3. Mature Treg cells migrate to the secondary lymphoid tissues where they continue to require IL-2 for their survival. These Treg cells express molecules that promote lymph node homing such as the chemokine receptor CCR7 and are called central (c)Treg cells. cTreg cells do not express IL-10 and display a naïve phenotype (CD62L^{hi}). cTreg cells undergo further differentiation upon receiving TCR stimulation. TCR signaling results in the expression of the transcription factor IRF4, which controls the key events during eTreg cell differentiation such as upregulation of the transcription factor Blimp-1. At this stage, Treg cells downregulate CCR7 and tissue-specific chemokine receptors that guide the migration of eTreg cells to nonlymphoid tissues. IRF4 together with Blimp-1 induce IL-10 production in eTreg cells. IRF4 also regulates the adipose tissue eTreg cell-specific transcription factor PPAR- γ and the receptor for IL-33, ST2.

tissues such as the lung and VAT.^{20,21} Specifically, IRF4 is required for the expression of Blimp-1 and IL-10, the correct expression of CCR6, and the upregulation of the costimulatory molecule ICOS.^{20,32} Treg cells also depend on IRF4 for the upregulation of the VAT Treg-specific transcription factor PPAR- γ and the IL-33 receptor ST2, a process which requires cooperation with the transcriptional regulator BATF (basic leucine zipper transcription factor, ATF-like).^{21,75} This transcriptional module may also be active in other

tissue-resident Treg cells as BATF was also found to be required for CCR9 expression and efficient localization of Treg cells to the gut.¹⁰⁹ Together, these findings indicate that IRF4, in cooperation with BATF is central for the differentiation of multiple types of eTreg cells. These data also provide strong support for a model for eTreg differentiation in which IRF4 establishes a common transcriptional program characterized by induction of multiple suppressive molecules, including IL-10, and results in changes to the migratory capacities of eTreg cells. This program is further shaped by a second layer of transcriptional regulators that are directed by distinct inflammatory signals or tissue-specific factors and cytokines. However, precisely how IRF4 establishes this transcriptional framework remains to be determined.



9. FUTURE PERSPECTIVES

Numerous studies over the past few years have addressed the role of immune cells located within nonlymphoid tissues. Accumulating evidence shows that Treg cells are critical to limit or prevent inflammation within these nonlymphoid tissues and have an essential role in maintaining homeostasis. However, the developmental pathways of Treg cells located in nonlymphoid tissues are only now being elucidated, and many intriguing questions remain unanswered. One attractive model would suggest that eTreg cells arise from cTreg cells that are recruited to nonlymphoid organs in a TCR-specific manner, and tissue antigens may play an important role in this process. Once these cells have reached the target organ, they may proliferate and differentiate in response to local tissue-specific or inflammatory signals. Such a model is supported by a number of studies, all of which strongly implicate IL-33 into this process.^{21,72,85,110,111} However, it remains to be seen whether other regulatory modules exist that may be responsible for the establishment of organ-specific Treg cell populations. Another, not necessarily exclusive model would predict that eTreg cell differentiation is programmed in the thymus. This is supported by a recent study, which demonstrated that a distinct subset of Treg cells develops in the thymus in an autoimmune regulator (AIRE)-dependent manner and contributes significantly to the maintenance of immune tolerance.¹¹² It is intriguing to speculate that it is these Treg cells, generated perinatally, that are responsible for “seeding” the tissue-resident Treg cell population. Thus, more studies are required to understand the factors that guide the differentiation program of eTreg cells within nonlymphoid organs.

Much of the success in Treg cell research comes from the study of murine models. While informative, the results of these studies cannot be directly extrapolated to human diseases. They do, however, lead to important considerations, such as the question of how much can be deduced from numbers of Treg cells circulating in the peripheral blood, without assessing the phenotype or differentiation status of these cells. It is conceivable that rather than the absolute number of Treg cells, it is the phenotype or subset of Treg cells that is important for protection from specific insults such as inflammation or autoimmune disease. Therefore, much work is required to understand the role of eTreg cells in general, and tissue-resident Treg cells in particular in humans, and novel approaches to identify and manipulate specialized eTreg populations in patients are needed. Defining and distinguishing tissue-resident eTreg cell populations in healthy and diseased organs will enable a better understanding of the cellular defects that result in the development of autoimmune diseases, malignancy, or transplant rejection.

ACKNOWLEDGMENTS

The authors would like to thank the members of the Kallies laboratory, in particular Tom Sidwell, for helpful discussion. We also thank A. Banerjee and G. Grigoriadis for their critical comments on the review. This work was supported by grants and fellowships from the National Health and Medical Research Council of Australia (A.K.), a fellowship from the Sylvia and Charles Viertel Foundation (A.K.), the Victorian State Government Operational Infrastructure Support and Australian Government NHMRC Independent Research Institute Infrastructure Support scheme and Kidney Health Australia (P.T.).

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Addendum

The immune system protects the host from an immense variety of microbial challenges but is tolerant to self. It consists of two lines of defence – the innate and the adaptive arms, which are both essential to control and shape the immune response (Marshall, Warrington et al. 2018). The innate response, which includes the physical barrier protection provided by the skin and mucous membranes, provides the early and immediate reaction against invading pathogens but it is not antigen-specific (Turvey and Broide 2010). In contrast, the adaptive immunity found in vertebrates provides a more specific and longer-lasting response with the capacity for immunological memory. This unique feature of the adaptive immunity is important for a rapid and efficient response upon subsequent re-exposure to the same antigen (Bonilla and Oettgen 2010). Although the innate and adaptive immune responses are distinct, the cooperative interaction of both arms of the immune system is a major determinant of an adequate immune response. The cells of the adaptive immune response form the basis of my study and are therefore the focus of this review.

The Adaptive Immune Response

B and T lymphocytes are key players responsible for the adaptive arm of the immune response. Both B and T cells express distinct but specific receptors that are able to recognize a specific antigen and respond to it in different ways to establish immune control.

T cells constitute the cell-mediated arm of the adaptive immune response. The two major subclasses are CD4⁺ or CD8⁺, depending on the surface expression of these coreceptors and their T cell receptor (TCR) restriction to peptides presented by major histocompatibility complex (MHC)I or MHCII respectively (Bernard and Bousnell 1984). CD8⁺ T cells are involved in the defence against intracellular pathogens through a direct killing mechanism, and are termed cytotoxic T cells (Zinkernagel and Doherty 1974). CD4⁺ T cells orchestrate an immune response through the provision of contact-dependent stimulation to other cell types such as B cells and antigen presenting cells (APCs) (Mosmann, Cherwinski et al. 1986). Interaction between an antigen and its cognate TCR results in proliferation of a broad array of antigen-specific clones.

B cells constitute the antibody-mediated arm of the adaptive immune system, responsible for the production of antibodies that target the clearance of pathogens through neutralization, opsonization and by activation of the complement cascade (Bonilla and Oettgen 2010). B cells can also function as APCs in inflammatory conditions (Crotty 2011). Though there is a

clear distinction between B and T cell function, the optimal antibody response is dependent on multifaceted interactions between B cells and ‘helper’ T cells.

T and B lymphocytes

I) T cell development

T cell development occurs in the thymus via a multistep process which is coordinated by a set of transcription factors that are activated by signalling pathways (reviewed in Miller 2020). Thymic T cell progenitors are derived from haematopoietic stem cells that are found in the yolk sac, fetal liver or bone marrow that migrate through the blood to the thymus. T cell precursors then undergo stringent selection events within the thymus to produce an immunocompetent T cell population.

The thymus is divided into two major anatomical areas – an outer cortex and inner medulla. T cell precursors enter through the thymic cortex where they dynamically interact with cortical thymic epithelial cells (cTECs), which induces the selection of newly generated T cells (Anderson and Takahama 2012). The interaction of thymocytes with the TECs within the medulla (mTECs) is essential for thymic tolerance. The medulla is also where a large number of tissue-restricted self-antigens are exclusively expressed (Derbinski, Schulte et al. 2001) and is regulated by the activity of the transcription factor, Autoimmune Regulator (AIRE) (Anderson, Venanzi et al. 2002). The expression of various chemokines and chemokine receptors coordinates the stepwise migration of thymocytes from the cortex to the medulla where the final step of T cell differentiation occurs (Ueno, Saito et al. 2004).

The development of a functional repertoire of T cells with useful TCR specificities is dependent on a number of selection events, which begins with their education in the thymus (Klein, Kyewski et al. 2014). CD4-CD8- double negative (DN) thymocytes comprise the early T cell precursors and are found in the cortex. DN thymocytes undergo random gene arrangement of the TCR β -chain locus in a process termed V(D)J recombination, that generates one of a diverse range of possible rearranged TCR. This serves to increase the repertoire of TCR specificities (Schlissel 2003). DN cells that have functionally recombined β chains will upregulate CD4 and CD8 molecules, becoming double positive (DP) thymocytes. DP thymocytes then recombine their TCR α loci, and successful expression of both α and β chains allows the formation of the dimeric T cell antigen receptor (Petrie, Livak et al. 1993). In this way, somatic recombination of TCR genes generates a pool of T cells

with a broad repertoire of TCRs with random specificities, but can give rise to potentially self-reactive T cells.

During their intrathymic journey, immature thymocytes come into contact with various MHC molecules provided by cells in the thymic microenvironment (Ladi, Yin et al. 2006, Benz, Martins et al. 2008, Love and Bhandoola 2011). The signal strength perceived by the TCR and self-peptide MHC (pMHC) complex interactions determines the fate of the thymocytes (Hogquist and Jameson 2014). Thymocytes that express TCRs that do not facilitate pMHC binding at all or bind with low avidity are destined to death by neglect (Egerton, Scollay et al. 1990, Hernandez, Newton et al. 2010). The remainder of the thymocytes undergo one of two distinct developmental fates (reviewed in (Starr, Jameson et al. 2003). A T cell is positively selected if it binds pMHC complexes with intermediate affinity, promoting survival signals and further differentiation (Hogquist, Jameson et al. 1994, von Boehmer 1994). On the other hand, thymocytes that express a TCR that binds self-pMHC complexes with high affinity are removed through apoptotic cell death (clonal deletion), a primary mechanism of negative selection (Sprent and Kishimoto 2002, Klein, Kyewski et al. 2014). This process imparts an important characteristic of the immune system to prevent autoimmune pathology by ensuring that T cells that react to self are eliminated, establishing the state of immune tolerance (Mathis and Benoist 2004, Kyewski and Klein 2006). Thus, the ability of TCR to recognize self can elicit completely opposing outcomes. Only thymocytes with a functional TCR, which is competent to trigger low-level signalling but does not cross the activation threshold to self-peptides, is positively selected and permitted to undergo further differentiation and eventually exit the thymus.

However, not all self-reactive T cells are eliminated during the process of central tolerance. A number of CD4 single positive thymocytes that receive strong TCR signals are able to escape deletion. Some of these self-reactive thymocytes have the potential to mediate autoimmune disease, whilst a proportion are enriched for cells that are instructed to differentiate into Foxp3⁺ Treg cell lineage in a process termed clonal diversion (Jordan, Boesteanu et al. 2001, Klein and Jovanovic 2011). Treg cells are highly effective at suppressing self-reactive T cells that have escaped into the periphery. Other peripheral tolerance mechanisms, such as the induction of anergy, receptor editing, clonal deletion and suppression also exist to ensure tolerance to self (Xing and Hogquist 2012).

Foxp3⁺ Treg cells differentiation occurs in the medulla, with Treg cells possessing TCRs with higher affinity to self-antigens (Moran, Holzapfel et al. 2011). Antigens for TCR stimulation and costimulatory signals are provided by thymic stromal cells (mTECs and dendritic cells). mTECs highly express the transcriptional regulator Autoimmune regulator (Aire), which is responsible for the promiscuous gene expression of various tissue-restricted self-antigens (Derbinski, Schulte et al. 2001, Mathis and Benoist 2009, Kawano, Nishijima et al. 2015). As such, Aire promotes thymic Treg cell development and controls the peripheral repertoire of Treg cells (Aschenbrenner, D'Cruz et al. 2007, Mathis and Benoist 2009). Genetic abnormalities of Aire expression result in autoimmune diseases collectively termed autoimmune polyglandular syndrome 1 or autoimmune polyendocrinopathy candidiasis ectodermal dysplasia (APECED), which is characterized by the loss of self-tolerance affecting multiple organs (reviewed in (Peterson, Org et al. 2008). This will be further discussed in Chapter 3.

II) CD4⁺ T lymphocytes

CD4 T lymphocytes exit the thymus as mature resting T cells that can differentiate into distinct effector CD4 T subsets (Zhu, Yamane et al. 2010). In response to inflammation or intracellular and extracellular pathogens, effector CD4 T cells, also known as helper T (Th) cells, upregulate the expression of lineage-specific transcription factors in the presence of specific polarizing cytokines that promote their differentiation. This process involves the concurrent inhibition of alternate pathways (Zhu, Yamane et al. 2010), though there is growing evidence that there is some plasticity in the Th lineage commitment (Oestreich, Huang et al. 2011). CD4 T cells that differentiate into Th1 cells upregulate the transcription factor, T-bet (encoded by *Tbx21*) and express the Th1 lineage cytokine IFN- γ (Zhu, Yamane et al. 2010, Lazarevic, Glimcher et al. 2013). IL-4 expression promotes Th2 differentiation, in which the master transcriptional regulator GATA binding protein (GATA)-3 drives the production of Th2-related cytokines IL-4, IL-5 and IL-13 (Zhu, Yamane et al. 2010). Extracellular bacteria and fungi induce the differentiation of Th17 cells, which express retinoic acid receptor-related orphan receptor (ROR)- γ t, essential for the expression of *Il17a*, *Il17f* and *Ccr6* (Korn, Bettelli et al. 2009, Mitsdoerffer, Lee et al. 2010). Follicular T helper (T_{FH}) cells represent another effector CD4 T cell lineage that differentiate in response to the master transcriptional factor B cell lymphoma (BCL)6 (Chtanova, Tangye et al. 2004,

Nurieva, Chung et al. 2009) (**Fig. 1**). T_{FH} cells provide costimulatory and survival signals to germinal centre B cells to promote B cell responses (Crotty 2019). Treg cells expressing the lineage-specific transcription factor, Foxp3, are another subset of effector CD4 T cells that are characterized by their enhanced suppressor function (Cretney, Kallies et al. 2013).

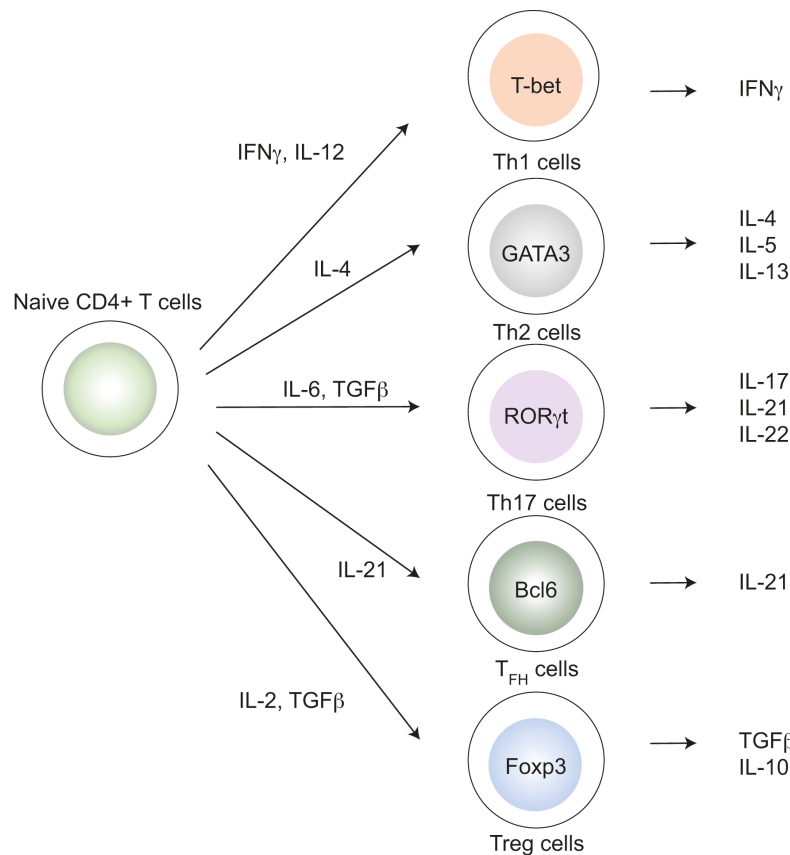


Figure 1: Overview of effector CD4 T cell differentiation.

Naïve CD4 T cells can undergo stimulus-specific differentiation in the presence of polarizing cytokines and lineage-defining transcription factors. These effector CD4 T or helper T (Th) cells produce lineage-specific cytokines. For example, in the presence of IFN γ and IL-12, Th1 T cells express the transcription factor T-bet to promote the secretion of IFN γ , Th2 T cells are induced by IL-4 to express GATA3 for the production of IL-4, IL-5 and IL-13, whilst IL-6 and TGF- β presence induced the expression of ROR- γ t by Th17 T cells to produce IL-17, IL-21 and IL-22. Follicular T helper (T_{FH}) cells are an effector CD4 T cell subset found within the germinal centre that express the master transcription factor, Bcl6 and secretes IL-21. The suppressor CD4 T cell subset, Treg cells, upregulate the expression of Foxp3 in the presence of IL-2 and TGF β , to secrete the immunosuppressive cytokines IL-10 and TGF- β .

III) B lymphocytes and T-B cell zone interactions

Each B cell expresses a different cell surface receptor generated through random gene rearrangement to produce a diverse repertoire of antibodies. This results in the generation of a population of lymphocytes that are able not only to recognize and respond to a wide variety of pathogens, but also bind with high affinity (reviewed in (Cyster and Allen 2019)). Naïve B cells generated in the bone marrow migrate to secondary lymphoid organs and remain in a quiescent state until antigenic encounter. Following activation by cognate antigen, mature B lymphocytes undergo rapid clonal expansion resulting in the proliferation of cells bearing the same antigen-specific receptor. Activated B cells can either differentiate into antibody secreting cells (ASC) or undergo class-switch recombination to one of several immunoglobulin (Ig) isotypes (Nutt, Hodgkin et al. 2015). T cell-independent B cell activation occurs outside the B-cell follicles and results in short-lived extra-follicular plasmablasts that primarily secrete low affinity IgM (Ho, Lortan et al. 1986, MacLennan, Toellner et al. 2003).

ASCs generated during a T cell-dependent response involves the recruitment of B cells into a specialized but transient microstructure called the germinal centre (Victora and Nussenzweig 2012). Found within B cell follicles of secondary lymphoid organs, such as the spleen or lymph nodes, the germinal centre can be divided into a dark and light (**Fig. 2**). Germinal centre B cell precursors undergo multiple rounds of cell division and selective clonal expansion in the process of migration between the dark zone and the light zone (Hauser, Junt et al. 2007, Schwickert, Lindquist et al. 2007). In the dark zone, antigen-specific B cells undergo further rounds of proliferation and somatic hypermutation of Ig genes mediated by the enzyme, activation-induced cytidine deaminase (AID; which is encoded by *Aicda*) to produce high affinity antibodies (Victora, Schwickert et al. 2010, Nutt, Hodgkin et al. 2015). This antibody affinity maturation process allows for the selection of B cell receptors with enhanced antigen specificity (Chan and Brink 2012, Bannard and Cyster 2017). B cells that bind antigen with low affinity or that become autoreactive undergo apoptosis (Nemazee and Buerki 1989, Hartley, Crosbie et al. 1991, Shlomchik and Weisel 2012). At the end of this process, the majority of B cells that exit the germinal centre will become long-lived ASCs while the remainder will become memory B cells (Dorner and Radbruch 2005). Antigen-experienced memory B cells have the capacity to respond rapidly

upon future encounter with the same pathogen (Ahmed and Gray 1996, Yoshida, Mei et al. 2010).

B cells receive help that is provided by a subset of effector CD4 T cells called follicular helper T (T_{FH}) cells, which is key to the germinal centre response (Fazilleau, Mark et al. 2009). T_{FH} cells express CXC chemokine receptor 5 (CXCR5) to allow colocalization of T_{FH} cells and B cells within the germinal centre (Vinuesa, Linterman et al. 2016). T_{FH} cells specialize in providing costimulatory signals through the expression of CD40L, and cytokines, such as interleukin (IL)-4 and IL-21 to germinal centre B cells, which promote B cell responses (reviewed in (Crotty 2014). Additionally, the secretion of IL-4 by T_{FH} cells provides pro-survival signals to germinal centre B cells, which are prone to apoptosis (Deenick, Ma et al. 2011).

Interactions between T and B cells result in the production of high affinity antibodies by plasma cells and subsequent generation of immunological memory and is a crucial element of adaptive immunity (Nutt and Tarlinton 2011). Importantly, precise regulation of the germinal centre reaction is fundamentally required to limit autoimmunity.

The next section explores the key aspects of the immunosuppressive regulatory T (Treg) cell generation, differentiation and function, with particular emphasis on effector (e)Treg cells that are the focus of this thesis.

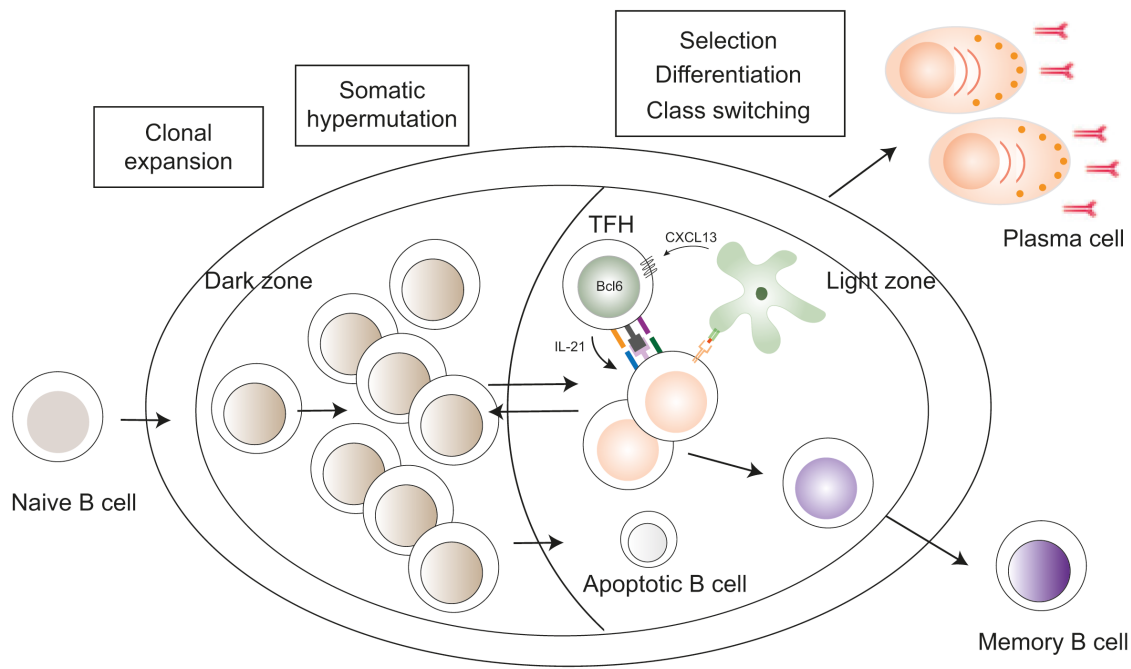


Figure 2: Germinal centre structure

Germinal centres are specialised sites located within secondary lymphoid organs, which consist of a dark and light zone. Mature B cells expand, differentiate and undergo somatic hypermutation in the dark zone. Interactions with antigen presenting and follicular T helper cells in the light zone provides survival and differentiation signals. Germinal centre B cells with the highest affinity for antigen are selected in a process called clonal selection. Germinal centre B cells migrate between the two zones with the process culminating in the formation of long-lived antibody secreting plasma cells and memory B cells. FDC, follicular dendritic cells; T_{FH}, follicular T helper cells.

Regulatory T cells

Regulatory T (Treg) cells are integral players of the adaptive immune system. Treg cells constitute the suppressor compartment of CD4 T cells and express the lineage-specific transcription factor, Foxp3 (Vignali, Collison et al. 2008, Rudensky, Sakaguchi et al. 2011). Treg cells comprise approximately 10-15% of the murine CD4⁺ T cell compartment and play an indispensable role in the maintenance of immunological homeostasis and prevention of lethal immune pathology (Sakaguchi, Yamaguchi et al. 2008). An exaggerated immune response followed by the development of severe autoimmune disease is observed in mice and men that have genetic anomalies of Treg cell development (Bennett, Christie et al. 2001,

Brunkow, Jeffery et al. 2001, Wildin, Ramsdell et al. 2001, Fontenot, Gavin et al. 2003, Josefowicz, Lu et al. 2012, Ohkura, Kitagawa et al. 2013). In recent years, Treg cells have also been shown to play key roles in resolving tissue inflammation and participate in tissue metabolism (Feuerer, Herrero et al. 2009, Vasanthakumar, Moro et al. 2015) and repair (Burzyn, Kuswanto et al. 2013, Arpaia, Green et al. 2015). The expression of Foxp3 is required for all aspects of Treg cell biology, including development, function and identity (Lin, Haribhai et al. 2007, Williams and Rudensky 2007, Zheng and Rudensky 2007). In addition to Foxp3, Treg cells are dependent on signalling from various transcription factors, epigenetic regulators and cytokines for their development, function and survival.

I) Treg cell origins

Treg cells can be categorized into two distinct subsets based on their developmental origin (Lee, Bautista et al. 2011). In the thymus, thymocytes undergo a series of regulated differentiation events including positive and negative selection (Starr, Jameson et al. 2003) leading to selection into the Treg cell lineage and induction of Foxp3 expression (Lio and Hsieh 2008). The majority of Treg cells derive from this pathway and are termed thymic or tTreg cells. The second group of Treg cells are peripherally derived and are enriched at the interface between the host and the environment where they suppress inflammatory responses (Rudensky 2011, Ohkura, Kitagawa et al. 2013). Here, in the presence of cytokines and transcriptional activators, naïve peripheral conventional CD4⁺ T cells encounter their cognate antigen and ‘turn on’ the expression of Foxp3 to acquire the Treg cell phenotype (Yadav, Stephan et al. 2013). These peripherally-derived (p)Treg cells are found at mucosal sites, such as the lung or gut, which are constantly exposed to air and food-borne allergens (Belkaid and Tarbell 2009). pTreg cells can also be generated *in vitro* through the activation of naïve CD4 T cells with IL-2 and transforming growth factor (TGF)- β (Chen, Jin et al. 2003, Abbas, Benoist et al. 2013). Both tTreg and pTreg cells are critical for the maintenance of peripheral tolerance and immune homeostasis (Curotto de Lafaille and Lafaille 2009). Distinguishing these two subsets of Treg cells with reliable markers remains controversial. Both neuropilin-1 (Nrp1) and the transcription factor, Helios (encoded by *Ikzf2*) have been reported to be highly expressed by tTreg cells (Thornton, Korty et al. 2010, Weiss, Bilate et al. 2012, Yadav, Louvet et al. 2012). However, there are reports that Helios expression can also be detected on activated T cells and in specific conditions, on pTreg cells (Akimova,

Beier et al. 2011, Serre, Benezech et al. 2011, Gottschalk, Corse et al. 2012). In certain situations, using stimulus-specific transcription factors, such as GATA-3 expression for tTreg cells, and ROR- γ t for pTreg cells have been useful to discriminate Treg cells in the gastrointestinal tract (Wohlfert, Grainger et al. 2011, Burzyn, Kuswanto et al. 2013, Sefik, Geva-Zatorsky et al. 2015).

II) Treg cell function

Treg cells have the capacity to restrain immune responses through a variety of mechanisms that are directed towards pathogens and commensal microbiota (reviewed by (Pandiyani, Zheng et al. 2011). This can be indirect through the sequestration of cytokines. For instance, Treg cells mediate their immunosuppressive function through the local consumption of IL-2 thereby limiting the proliferation of CD4 T cells (Fontenot, Rasmussen et al. 2005). Additionally, Treg cells can upregulate a variety of activation-associated molecules and release several anti-inflammatory cytokines including IL-10, IL-35 and TGF- β (Vignali, Collison et al. 2008, Cretney, Xin et al. 2011). Treg cells can be directly suppressive via cell-to-cell interactions through the inhibition of APCs by inhibitory molecules such as CTLA-4 binding to B7 on APCs (Oderup, Cederbom et al. 2006). Other means of suppression include direct cytotoxicity via the release of cytolytic molecules such as granzyme B and cAMP, the release of exosomes and the disruption of metabolic pathways (Sakaguchi, Yamaguchi et al. 2008, Tang and Bluestone 2008, Wing and Sakaguchi 2012). Stringent control of effector CD4 T cell responses is crucial for immune homeostasis. As such, the appropriate balance of CD4 T cell and Treg cells at the various stages of an immune response is essential for effective protection against infection whilst preventing autoimmunity.

III) Treg cell homeostasis and specialization

tTreg cell lineage commitment is a two-step process which is dependent on TCR signalling and IL-2 (Lio and Hsieh 2008). Strong TCR activation is critical for the generation of partially committed Treg cell precursors, in which the Foxp3 locus is poised for activation (Moran, Holzapfel et al. 2011). Additionally, these thymic Treg cell precursors also upregulate the high affinity interleukin (IL)-2 receptor alpha chain, CD25 and tumour necrosis factor receptor superfamily member 18 (TNFRSF18 or glucocorticoid-induced TNF related protein (GITR)) (Lio and Hsieh 2008). These CD25⁺GITR⁺ Treg cell precursors

receive IL-2 signalling, which subsequently activates STAT5 that induces Foxp3, resulting in the development of mature Foxp3⁺ Treg cells (Mahmud, Manlove et al. 2013).

IL-2 is a well-studied cytokine in a myriad of immune cell types and is important in many aspects of T cell biology (Malek and Castro 2010, Boyman and Sprent 2012). Numerous studies have described the role of IL-2 in T cell activation and proliferation (Pipkin, Sacks et al. 2010, Liao, Lin et al. 2011, Ballesteros-Tato, Leon et al. 2012). IL-2 is produced primarily by activated CD4 T cells and elicits its effect via the IL-2 receptor (IL-2R), with Treg cells constitutively expressing elevated levels of CD25 (Fontenot, Rasmussen et al. 2005). During Treg cell development, IL-2 binding and signal transduction induces a number of downstream pathways including JAK/ STAT and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) (Burchill, Yang et al. 2007), which is critical for the continuous survival and homeostasis of Treg cells in the periphery (Fontenot, Rasmussen et al. 2005, Josefowicz and Rudensky 2009). IL-2 also promotes the generation of pTreg cells (Davidson, DiPaolo et al. 2007). Though Treg cells are highly dependent on this potent T-cell growth factor, they are unable to secrete IL-2 as Foxp3 has been shown to inhibit nuclear factor of activated T cells (NFAT), required for IL-2 transcription (Bettelli, Dastrange et al. 2005, Wu, Borde et al. 2006). Additionally, IL-2 mediated signals are required to induce Blimp-1 expression in favour of terminal effector differentiation of Treg cells (Cretney, Xin et al. 2011). Whilst IL-2 is essential for Treg cell homeostasis, other cytokines such as IL-33 are important for the maintenance of Treg cell in other non-lymphoid organs such as the visceral adipose tissue (Schiering, Krausgruber et al. 2014, Kolodin, van Panhuys et al. 2015, Vasanthakumar, Moro et al. 2015).

Studies have shown that Treg cells are a phenotypically diverse and functionally heterogeneous population (Tang and Bluestone 2008, Sakaguchi, Vignali et al. 2013, Liston and Gray 2014). Treg cells represent a subset of Th cells that are endowed with the ability to suppress polarized immune responses and inflammation in various lymphoid and non-lymphoid tissues (Feuerer, Hill et al. 2009, Campbell and Koch 2011, Burzyn, Benoist et al. 2013, Chaudhry and Rudensky 2013, Cretney, Kallies et al. 2013).

IV) Effector Regulatory T cells

The most suppressive Treg cells are the terminally differentiated effector (e)Treg cells predominantly found in non-lymphoid tissues (**Fig. 3**). In response to TCR and various

cytokine signals, antigen-inexperienced central (c)Treg cells adopt specific transcriptional programs to differentiate into eTreg cells (Cretney, Xin et al. 2011). These differentiation programs are regulated by distinct transcription factors typical of the Th cell population that they suppress (Feuerer, Herrero et al. 2009, Koch, Tucker-Heard et al. 2009, Zheng, Chaudhry et al. 2009, Linterman, Pierson et al. 2011, Cretney, Kallies et al. 2013). This allows for the appropriate colocalization of eTreg and effector CD4 T cells in the relevant inflammatory environments and mucosal sites; and is required for Treg cells to acquire functional specialization allowing for context dependent immune regulation (Burzyn, Benoist et al. 2013). For example, the prototypical T_{FH} cell transcription factor, Bcl6 is essential for the development of a specialised subset of Treg cells found within the germinal centre, the T follicular regulatory (T_{FR}) cells (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011, Wollenberg, Agua-Doce et al. 2011). T_{FR} cells differentiate from tTreg cells and are essential to regulate the proliferation of T_{FH} cells and limit germinal centre reactions, important to prevent the development of autoimmunity (reviewed in (Sage and Sharpe 2016). Key T_{FR} cell characteristics and function will be discussed further in Chapter 4.

Importantly, all eTreg cells can be identified by their expression of the transcription factor B lymphocyte-induced maturation protein (Blimp)-1 (encoded by gene *Prdm1*), which is required for the production of the immunoregulatory cytokine IL-10 (Cretney, Kallies et al. 2013). Blimp-1 also regulates Treg cell survival through the control of the anti-apoptotic factor, Bcl2 expression (Cretney, Xin et al. 2011). As such, Blimp-1-expressing Treg cells constitute the active effector stage of the Treg cell lineage. eTreg cells represent a minor population in the lymphoid organs, but dominate in non-lymphoid tissues such as the lung, kidney, visceral adipose tissue and gastrointestinal tract (reviewed in (Teh, Vasanthakumar et al. 2015). Treg cell effector molecules such as ICOS, CTLA-4 and other activation-associated molecules such as killer cell lectin-like receptor subfamily G member 1 (KLRG1) and T cell immunoreceptor with Ig and ITIM domain (TIGIT) are highly expressed by eTreg cells compared to cTreg cells, contributing to enhanced suppressive activity of eTreg cells (Wing, Onishi et al. 2008, Cretney, Xin et al. 2011, Cheng, Yuan et al. 2012).

Transcription factors, including IRF4 are known to orchestrate the transcriptional network of eTreg cell development, maintenance and suppressive function (Cretney, Xin et al. 2011, Vasanthakumar, Liao et al. 2017). However, the molecular mechanisms that are regulated by

IRF4 remain ill-defined. Using T_{FR} cells as an eTreg cell subset, we were interested to elucidate the role of IRF4 in T_{FR} cell biology.

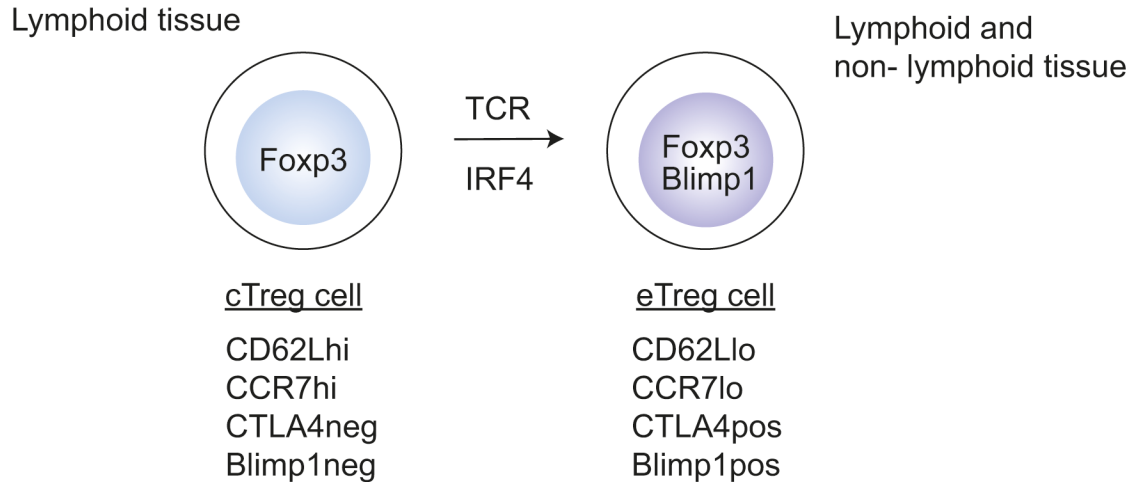


Figure 3: Effector Treg cell differentiation.

Mature Treg cells exit the thymus as central (c)Treg cells that have a naive phenotype. Upon antigenic encounter and T cell receptor engagement, Treg cells upregulate IRF4 and can further differentiate into a population of Treg cells that have an effector phenotype, termed effector (e)Treg cells. eTreg cells are characterised by the expression of Blimp-1 (encoded by *Prdm1*) and are predominantly found within non-lymphoid organs. cTreg, central Treg; eTreg, effector Treg; IRF4, interferon regulatory factor 4; TCR, T cell receptor activation; Treg, T regulatory.

The Role of IRF4 in the Transcriptional Regulation of Effector Treg cells

The transcription factor, IRF4 (also known as pip, MUM1, LSIRF, NFEM5 and ICSAT) has been shown to be a key transcriptional regulator in lymphocyte biology, required for both B and T cell differentiation and function (Mittrucker, Matsuyama et al. 1997, Lohoff, Mittrucker et al. 2002, Klein, Casola et al. 2006, Sciammas, Shaffer et al. 2006, Zheng, Chaudhry et al. 2009, Cretney, Xin et al. 2011, Man, Miasari et al. 2013, Willis, Good-Jacobson et al. 2014, Mahnke, Schumacher et al. 2016). IRF4 belongs to a the IRF family of transcription factors, that consists of nine family members and contains a highly conserved N-terminal DNA binding domain (DBD) (Brass, Kehrli et al. 1996). But unlike other IRF family members, IRF4 is not induced by interferon. Instead, T and B cell receptor stimulation, lipopolysaccharides and CD40 signalling can induce the expression of IRF4

(Mittrucker, Matsuyama et al. 1997). IRF4 binds the DNA sequence GAAA but requires heterodimerization with other factors for high affinity binding (Escalante, Yie et al. 1998). In B cells, IRF4 cooperatively binds to factors such as PU.1 or SPIB, which increases its DNA binding affinity (Brass, Kehrli et al. 1996). IRF4 regulates genes via complexes that contain the ETS/ IRF consensus elements (EICE) (Brass, Kehrli et al. 1996, Escalante, Brass et al. 2002). In T and B cells, IRF4 forms a complex with the activator protein (AP)-1 transcription regulators, basic leucine zipper activating transcription factor-like (BATF) and Jun, forming the AP-1/ IRF4 composite element (AICE) that promotes the transcription of various genes in T cells (Ciofani, Madar et al. 2012, Glasmacher, Agrawal et al. 2012, Li, Spolski et al. 2012, Murphy, Tussiwand et al. 2013). IRF4 expression is induced downstream of TCR signalling and is critically required to promote effector differentiation of helper T cell subsets (Lohoff, Mittrucker et al. 2002, Rengarajan, Mowen et al. 2002, Zheng, Chaudhry et al. 2009, Bollig, Brustle et al. 2012, Mahnke, Schumacher et al. 2016). It was first shown that mice that had IRF4-deficient Treg cells displayed an exaggerated Th2 response (Zheng, Chaudhry et al. 2009). Subsequently, Cretney, Xin and colleagues described the essential nature of IRF4 for the establishment of effector Treg cell function demonstrating a severe activation defect and impaired suppressive function in the absence of IRF4 expression in Treg cells (Cretney, Xin et al. 2011). IRF4 expression is restricted to antigen-experienced Treg cells and is essential for the expression of eTreg cell related genes (Cretney, Xin et al. 2011, Levine, Arvey et al. 2014, Vahl, Drees et al. 2014). The expression level of IRF4 increases proportionally to TCR signal strength, and correlates with clonal expansion and expression of target genes required for effector differentiation and the metabolic program in T cells (Man, Miasari et al. 2013, Yao, Buzo et al. 2013). Additionally, in Treg cells, IRF4 directly induces the transcription factor, *Prdm1* (encoding Blimp-1), which promotes the production of the immunosuppressive cytokine, IL-10 (Cretney, Xin et al. 2011). In the absence of IRF4, Treg cells also failed to upregulate migratory molecules such as CD103 and CCR6 (Cretney, Xin et al. 2011). Furthermore, IRF4 is an essential regulator of T and B cell interactions, regulating many genes critical for germinal centre responses (Kwon, Thierry-Mieg et al. 2009, Ise, Kohyama et al. 2011). Thus, IRF4 and downstream targets of IRF4 regulate multiple aspects of eTreg cell differentiation and suppressor function. In this way, IRF4 is indispensable for the control of autoimmune responses.

Research questions and Thesis Aims

This thesis focused on identifying the role of IRF4 in the differentiation and function of T_{FR} cells, which has not been previously characterized in detail. We hypothesize that the development and function of Treg cells follows a common transcriptional pathway that is dependent on IRF4, but the adaption of Treg cells to a specific microenvironment requires Treg cells to adopt a specific transcriptional program. To this end, the research aims of this thesis were:

- I. To map the ontogeny of eTreg cell beginning with thymic development.
- II. To investigate the requirement for IRF4 in differentiation and function of T_{FR} cells.
- III. To define the molecular program of T_{FR} cell development.

Chapter Two: Methods and Experimental Procedures

2.1 Animal models

2.1.1 Mice

Irf4^{-/-} mice (Mittrucker, Matsuyama et al. 1997), *Il10*^{GFP} mice (Kamanaka, Kim et al. 2006), *Il21*^{GFP/+} mice (Luthje, Kallies et al. 2012) and *Prdm1*^{GFP/+} mice (Kallies, Hasbold et al. 2004) were described previously and crossed with *Foxp3*^{RFP} (Wan and Flavell 2005). *c-Maf*^{fl/fl} mice (Wende, Lechner et al. 2012) and *Irf4*^{fl/fl} mice (Klein, Casola et al. 2006) were crossed to *Foxp3*^{Cre} mice (Rubtsov, Rasmussen et al. 2008). *Rag1*^{-/-} mice, *Foxp3*^{DTR} mice (Kim, Rasmussen et al. 2007), *Foxo1*^{fl/fl}*pLck*^{Cre} mice (Ouyang, Liao et al. 2012), *Nur77*^{GFP}*Foxp3*^{RFP} mice (Moran, Holzapfel et al. 2011) and *Tgfb2*^{fl/fl}*pLck*^{Cre} (Zhang and Bevan 2012) mice were described previously. *Aire*^{-/-} (Anderson, Venanzi et al. 2002) mice were crossed with the *Foxp3*^{RFP}*Blimp1*^{GFP/+} mice. *Irf4*^{tdTom/GFP} dual reporter mice were generated by our laboratory and had either single reporters for IRF4 protein (*Irf4*^{tdTom}) or *Irf4* mRNA (*Irf4*^{GFP}). *Irf4*^{GFP} reporter mice were crossed to *Foxp3*^{RFP} reporter mice to generate *Irf4*^{GFP/+}*Foxp3*^{RFP} mice. All mice were maintained on a C57BL/6 background.

Mice were bred and maintained in a specific pathogen free facility at The Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Australia. Mice aged between 3 days and 20 weeks old were used in accordance with the National Health and Medical Research Council of Australia guidelines. Animal experiments were approved by the Animal Ethics Committee guidelines of The Walter and Eliza Hall Institute of Medical Research.

2.1.2 Bone marrow reconstitution of chimeric mice

Mixed bone marrow chimeras were generated by reconstitution of sub-lethally irradiated (2 x 350 Rads) *Rag1*^{-/-} mice with a mixture of mutant or control (CD45.2) and Ly5.1 donor bone marrow cells as indicated. *Irf4*^{fl/fl}*Foxp3*^{Cre}: *Foxp3*^{DTR} and *Irf4*^{+/+}*Foxp3*^{Cre}: *Foxp3*^{DTR} mixed bone marrow chimeras were generated in a similar fashion in lethally irradiated (2 x 550 Rads) *Foxp3*^{DTR} host. Irradiated mice were provided with neomycin supplemented drinking water for three weeks following irradiation. Chimeric mice were allowed to reconstitute for 6-8 weeks prior to experiments.

2.1.3 Infections

Mice were anaesthetized with inhaled penthrane and inoculated with 1×10^4 p.f.u. of HKx31 (H3N2) influenza virus intranasally (Flynn, Belz et al. 1998). Mice were sacrificed and the lungs, spleen, mediastinal lymph nodes and Peyer's patches were removed for analysis at day 9 after infection at the peak of the anti-viral response.

2.2 Buffers and Media

Name	Ingredients and pH
Assay for transposase accessible chromatin (ATAC) lysis buffer	10mM Tris-Hcl pH7.4, 10mM NaCl, 3mM MgCl ₂ .1% Igepal CA-630 in nuclease free water
Cell lysis buffer	135 mM NaCl, 20 mM Tris-HCL (pH 7.5), 1.5 mM Mg ₂ Cl,1 mM EGTA, 10% glycerol, 1% Triton X-100, protease and phosphatase inhibitors (Roche).
Hanks 2% FCS	Hanks balanced salt solution (HBSS) with glucose, phenol red, no calcium and no magnesium (Gibco) was supplemented with 2% FCS.
PLP buffer	(1% paraformaldehyde, 0.075M L-lysine, and 2.1 mg/ml sodium periodate in 0.0375 M phosphate buffer (pH 7.4))
Red cell lysis buffer	8.34g crystalline NH ₄ Cl (0.156M), 1g crystalline NaHCO ₃ (12mM), 0.037g crystalline EDTA tetra sodium salt (0.097mM) (pH 7.25-7.35)
T cell medium	Mouse tonicity Iscove's modified Dulbecco's medium (Sigma) was supplemented with 1x non-essential amino acids (Sigma), 1mM sodium pyruvate, 10mM HEPES (Sigma), 2mM L-glutamine, 50µg/ml gentamicin (Sigma), 50mM β-mercaptoethanol (Sigma) and 10% fetal calf serum (FCS) and sterile filtered.

Table 1. List of buffers and media ingredients.

2.3 Experimental procedures for cellular immunology

2.3.1 Organ harvests and processing for lymphocyte isolation

Spleens, peripheral lymph nodes, mediastinal or mesenteric lymph nodes, Peyer's patches and thymi were collected in media and passed through a 70µm nylon filter (Falcon). Single cell suspensions of splenocytes were then incubated for 2 minutes at room temperature with red cell lysis buffer for removal of erythrocytes, and washed with FACS buffer.

Following perfusion of PBS through the right ventricle, livers were passed through a 70µm nylon filter. Single cell suspensions were then overlaid on a 37.5% Percoll (GE Healthcare) gradient and centrifuged at 693g for 15 minutes, with minimum break and accelerator. Pelleted lymphocyte-enriched fractions were collected after centrifugation. Erythrocytes were lysed with red cell lysis buffer and washed in FACS before staining.

Intestinal LPLs were extracted from the small intestine of mice cut into sections, with the Peyer's patches removed. The sections were dissected longitudinally, cleaned and cut into 0.5 cm² pieces. Samples were washed in MTPBS twice by vortex. The epithelial layer was removed by incubation in 5mM EDTA Ca²⁺ Mg²⁺ free Hanks balanced solution with 2% FCS for 25 minutes at 37°C on a rotor (230rpm). The intestinal pieces were washed with chilled MTPBS, followed by 30 minute digestion with Collagenase III (1 mg/mL; Worthington)/DNase I (200 µg/mL; both from Roche) and Dispase (4U/mL;Sigma) at 37°C. Supernatant and intestinal pieces were passed through a 70µm nylon strainer and washed with 30ml Hanks 2% FCS. LPL fractions were purified by 40/80% Percoll (GE Healthcare) gradient and washed twice before staining with various antibodies against surface and intranuclear molecules.

2.3.2 Cell culture

2.3.2.1 T cell enrichment and cell culture

After erythrocyte lysis, CD4 T cells were enriched from pooled splenocytes and peripheral lymph nodes prior to culture using antibodies against MHCII (M5/114), CD11b (M1/70), F4/80 (F4/80), Gr-1 (RB6-8C5), Ly76 (Ter-119) and CD8 (53.6.7), followed by depletion with Goat anti-Rat magnetic beads (Polysciences), or using commercial naïve CD4 T cell isolation kit (Miltenyi) according to manufacturer's instructions, or flow cytometrically using the BD FACSAria II, BD FACSAria Fusion or BD Influx (BD Biosciences). Purified CD4

T cells were cultured in the presence of plate bound α CD3 (clone 145-2C11 (WEHI)) at 10 μ g/ml, 100 IU/mL of soluble IL-2 (Peprotech) and 2 μ g/ml of soluble α CD28 (clone 37.51) in complete T cell media. All cultures were incubated at 37°C with 5% CO₂ and humidity control between 3 to 7 days.

For Th subset polarizing conditions, we included the following cytokines and antibodies.

Th1: 10mg/ml; IL-12 (Peprotech), 10mg/ml α IL-4 antibody (BVD4).

Th2: 20ng/ml IL-4 (R%*D* Systems), 10mg/ml α IFN- γ antibody.

pTreg: 0.5ng/ml TGF- β , 10mg/ml α IL-4 antibody and 10mg/ml α IFN- γ antibody.

Some T cell cultures were further supplemented with 10ng/ml IL-6 (eBioscience).

2.3.2.2 B cell culture

Mouse B cells were isolated from pooled spleen and peripheral lymph nodes using the anti-CD19 positive selection kit (Miltenyi Biotec) according to manufacturer's instructions. Purified B cells were enumerated and then stained with CTV as detailed below. Cells ($4-8 \times 10^4$) were placed into a 48-well tissue culture plate and stimulated in complete RPMI 1640 media with α IgM and CD40L. In most cases, purity of the CD19⁺ B cells were >90% when assessed by flow cytometry.

2.3.2.3 B, T and Treg cell assay

B and T cells from pooled peripheral lymph nodes and spleens of wild type and *Irf4*^{-/-} *Foxp3*^{RFP} were isolated as described above.

Where indicated, in some experiments, lymphocytes from pooled spleen and mediastinal LNs were sorted via flow cytometry for CXCR5^{hi} PD1^{hi} CD4⁺ CD19⁻ *Foxp3*^{RFP} reporter negative T_{FH} cells, and *Foxp3*^{RFP} reporter positive cells from influenza infected *Foxp3*^{RFP} and *Irf4*^{-/-} *Foxp3*^{RFP} mice at day 9 after infection.

B cells (5×10^4), T_{FH} (5×10^4), and either WT or *Irf4*-deficient Treg cells were placed into 48 well tissue culture plates in 750 μ L of media containing soluble α CD3 (2 μ g/ml) and α IgM (5 μ g/ml). All cultures were incubated at 37°C with 5% CO₂ and humidity control.

2.3.3 Antibodies and Flow Cytometry

Single-cell suspension of spleens and lymph nodes were surfaced stained in FACS buffer (PBS + 0.1% FCS) with fluorochrome-conjugated antibodies directed against the following mouse antigens: B220 (RA3-6B2), Bcl6 (K112-91), CCR7 (4B12), CD103 (2E7), CD127

(A7R34), CD19 (1D3, 6D5), CD25 (PC61.5), CD28 (37.51), CD38 (90), CD4 (GK1.5), CD4 (RM4-5), CD44 (IM7.8.1), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD8 (53-6.7), c-Maf (sym0F1), CTLA4 (14D3), CXCR3 (173), CXCR4 (2B11), CXCR5 (2G8), Fas/CD95 (Jo2), Foxp3 (FJK-16s), GATA3 (TWAJ), GITR (DTA-1), Gl7 (Gl7), Helios (22F6), ICOS (15F9), IFN- γ (XMG1.2), IgD (42334), IgM (R6-60.2), IL-10 (JES5-16E3), IL-17 (TC11-18H10), IL-4 (11B11), IRF4 (3E4), KLRG1 (2F1), Lag3 (C9B7W), Neuropilin-1 (FAB566A), Nur77 (12.14), PD1 (J43, 29F.1A12, RMP1-30), ROR γ t (Q31-378), Tbet (4B10), TCF1 (C63D9), TCR β (H57- 597), TIGIT (4D4).

Streptavidin BUV395, PeCy7 was used as a secondary reagent for biotinylated anti-bodies. Fc-receptors were blocked with 2.4G2 (anti-CD16/32). Fixable viability dye eFluor®506 (eBioscience) was used to exclude dead cells.

Antibodies were purchased from e-Biosciences, Biolegend, and BD or were purified from hybridoma supernatants and coupled to fluorophores by standard procedures. Intracellular staining was performed using the Foxp3 staining kit (eBioscience) according to the manufacturer's protocols.

Cells were acquired on the BD LSRFortessa, BD LSRFortessa X-20 or BD FACSCanto II (BD biosciences). Data was analysed with FlowJo (Tree Star) software version 9.9.6 and 10.

2.3.4 Cell staining for division tracking

Undivided cells were stained with division-tracking dye Cell Trace Violet (CellTrace™ Violet Cell Proliferation Kit, Life Technologies, cat#C34557) prior to culture. 5mM CTV in DMSO was diluted 1:10 in PBS+0.1%BSA. Lymphocytes were resuspended in 500 μ L PBS+0.1%BSA. 5 μ L of 0.5mM CTV was added to the lymphocyte suspension, mixed thoroughly and incubation at 37°C for 20 minutes. The staining was quenched with 2.5mL (5x staining volume) of cold 5% FCS in RPMI. The supernatant was removed and lymphocytes were washed again with 5mL 5% FCS-RPMI. Supernatant was removed and cells were resuspended in tissue culture medium.

2.3.5 Treg cell depletion

Foxp3^{DTR} (Kim, Rasmussen et al. 2007) mice and chimeras were transiently depleted of Treg cells by intraperitoneal injection of 1µg diphtheria toxin (DT) (kindly provided by Professor Andrew Lew) at days 0, 1 and 6 of the experiment.

2.3.6 Tamoxifen treatment

For *in vivo* experiments involving tamoxifen-inducible Cre strains, mice were treated with tamoxifen (Sigma-Aldrich, 0.2 mg/g) emulsified in peanut oil via oral gavage for 3 consecutive days.

2.4 Experimental procedures for molecular immunology

2.4.1 Polymerase chain reaction (PCR) for genotyping

PCR was performed with GoTaq Green Master Mix (Promega). A master mix for each reaction was made up of 1mL of nuclease free water, 1mL of GoTaq. green master mix and 5µL of each primer at 200µM stock (see **Table 2**). Mastermix was aliquoted into PCR tubes at 19µL per reaction and 1µL of tail DNA added for each PCR reaction. PCR reaction was undertaken in a T100 Thermal cycler (Biorad). PCR products were run on 2% w/v agarose gel in TAE buffer with ethidium bromide to allow visualization of DNA using ultraviolet light using a Gel Dock XR+ (Biorad). Sequences (5'-3') of primers (Geneworks) are listed in **Table 2**.

Allele	Forward	Reverse
Aire-null	ACCTCAGCCAAAACCCATACTA	ACTGGTTTAGGTCCACATCTGC
Maf flox	ATGATCAGGCTCAGGCTTAAA	CGCACCTGACAACGTG
Foxp3 RFP	CAAAACCAAGAAAAGGT GGGC	Wild type: CAGTGCTGTTGCTGTGT AAGGGTG Reporter: GGAATGCTCGTCAAGAA GACAGG
Foxp3-Cre Wild type	CCAGATGTTGTGGGTGAGTG	TGGACCGTAGATGAATTTGAGTT
Foxp3-Cre Knock in	AGGATGTGAGGGACTACCTCCTGTA	TCCTTCACTCTGATTCTGGCAATTT
Foxo1 flox	GCTTAGAGCAGAGATGTTCTCACATT	Wild type:

		CCAGAGTCTTTGTATCAGGCAAATAA Flox: CAAGTCCATTAATTCAGCACATTGA
Irf4 flox	AATACTGAGCTGCAGTCTAGC	Wild type: TGTTGCTGGTGGAGAGGAAG Flox: GACCACTACCAGCAGAACAC
Irf4 GFP	ACCTGAACACGTTACCACTC	Wild type: TGTTGTTGTCTTCAAGTGA Reporter ATGGTTGTGGCCATATTATC
Irf4-null	Wild type: GGTGTGTGGGTTATGCACTTGAAGG Knock out: TGGCGCCTACCGGTGGATGTGG	AGTGAGCTGCTTTGCTCCTG
Irf4tdTom	ACCTGAACACGTTACCACTC	Wild type: TGTTGTTGTCTTCAAGTGA Reporter CCGTCCTCGAAGTTCATCAC
Lck-Cre	ACTGACCGTACACCAAATTTGCCTG	GATAATCGCGAACATCTTCAGGTTC
Prdm1 GFP	TGAGTAGTCACAGAGTACCCA	Wild type: GGCAAGATCAAGTATGAGTGC Reporter: GCGGAATTCATTTAATCACCCA
Tgfb2 flox	TAAACAAGGTCCGGAGCCCA	ACTTCTGCAAGAGGTCCCCT

Table 2. List of primers for genotyping

2.4.2 Immunoblotting

Activated CD4⁺ T lymphocytes were harvested after *in vitro* cell culture as described in Section 2.3.3.1. Cells were washed in cold PBS to remove excess protein from the medium and then lysed using Triton Lysis Buffer with protease and phosphatase inhibitor and stored at -20°C overnight. The following day, samples were thawed on ice and boiled with SDS sample buffer at 95°C for 5 mins. Samples were then loaded on to a 1.5mm 4-20% gradient gel (ThermoFisher Scientific). Gels were transferred to a nitrocellulose membrane using the BioRad Trans-Blot® Turbo™ Transfer System. The membrane is then blocked using 5% milk PBS 0.1% Tween 20 at room temperature for 1 hour and incubated with primary antibody overnight at 4°C. The membrane was washed 3 times for 5 minutes each in PBS

0.1% Tween 20, then incubated with 5% milk PBS 0.1% Tween 20 and secondary antibody HRP at 1:3000 (Santa Cruz Biotechnology) at room temperature for 1 hour. Then, the membrane was washed with PBS 0.1% Tween 20 3 times for 5 minutes each and finally in PBS. The membrane was transferred onto a transparency sheet and incubated with 1.5mL of developing reagent for a minute prior to digitally developed using Biorad ChemiDoc™ MP System. Actin (Santa Cruz Biotechnology, cat#sc-1616) was used as a control. The following primary antibodies were used: α Bcl6 (7D1-10), α IRF4 (3E4), α Foxo1 (C29H4).

2.4.3 Immunofluorescence microscopy

Spleens, mediastinal lymph nodes and Peyer's patches were collected in PBS and fixed in PLP buffer at 4 degC between 4-6 hours and then dehydrated overnight with sucrose (30%) before embedding in Tissue-Tek® O.C.T. Compound, Sakura at harvest and stored at -80degC. Frozen sections 8 μ m in thickness were made permeable and non-specific binding was blocked in PBS containing 0.1% BSA (?) and 10% donkey serum (Jackson ImmunoResearch), followed by staining in PBS. Sections were stained with antibodies for the detection of CD3, B220, α RFP that were detected with the appropriate secondary antibodies, and primary antibodies to GL7 and Foxp3. Slides were mounted using ProLong Gold antifade reagent (Invitrogen). Images were acquired on a LSM780 confocal microscope (Carl Zeiss MicroImaging) and analysed with ImageJ (Fiji) software.

To quantify the distribution of RFP+ Treg cells in the spleen (draining lymph node or Peyer's patch), two non-consecutive sections were examined for each mouse. T cell zone was defined as the α CD3+ area, GC were identified as GL7+ area and B cell follicles negative for α CD3 but α B220+. The area of the T cell zone, and GC were measured and RFP+ cells within each defined area were counted using Image J.

2.4.4 Quantitative (q)PCR and gene expression profiling

RNA extraction and library preparation on CD4 T cells purified from the spleen and mediastinal lymph nodes of influenza infected *Foxp3^{RFP}Il21^{GFP/+}* dual reporter mice 9 days after infection using flow cytometry were performed by Dr Julie Tellier. RNA-seq reads were aligned to the mm10 build of the *Mus musculus* genome using the Subread aligner software (Rsubread 1.26.0) (Liao, Smyth et al. 2013). Gene level read counts were obtained using featureCounts (Liao, Smyth et al. 2014). Genes were excluded from downstream analysis if

they failed to achieve a CPM (counts per million mapped reads) value of 1 in at least 1 library. An FDR cut-off of 0.1 was applied for calling differentially expressed genes. A linear model was fitted to each gene, and empirical Bayes moderated t-statistics was used to assess differences in expression.

We also performed RNA extraction and library preparation on Treg cells purified from the spleen and mediastinal lymph nodes of *Irf4*^{-/-} *Foxp3*^{RFP} and *Irf4*^{+/+} *Foxp3*^{RFP} mice.

We reanalysed the published RNA-seq dataset comparing wild-type and *c-Maf*-deficient Treg cells isolated from the Peyer's patches (Wheaton, Yeh et al. 2017). Description of the analyses is found within the appropriate chapters.

2.4.5 Assay for transposase-accessible chromatin (ATAC) sequencing and bioinformatics analysis

ATAC-sequencing was performed as published (Buenrostro, Giresi et al. 2013, Buenrostro, Wu et al. 2015). 50,000 flow cytometry sorted CD4 T cell subsets from spleens and mediastinal lymph nodes of influenza infected *Foxp3*^{RFP} mice at day 9 after infection were resuspended in 50µl of chilled ATAC lysis buffer and centrifuged for 10 minutes at 500xg at 4°C. Nuclei of an equivalent of 25,000 cells were resuspended in 25µl of Tagmentation buffer (2x Tagment DNA buffer, 20x Tagment DNA enzyme (Nextera library preparation kit) and incubated 37°C for 30 minutes. Next, Tagmented DNA was isolated using the MinElute PCR purification kit (Qiagen). Samples were PCR-amplified with a common forward primer and unique barcoding reverse primers before tagmentation efficiency and concentration was assessed with a bioanalyzer high sensitivity DNA analysis kit (Agilent). Samples were depleted for small (<150bp) fragments using solid phase reversible immobilization beads and then libraries were pooled. Sequencing was performed using the NextSeq platform (Illumina). Three biological replicates of each sample were sequenced. Data was mapped to the mouse mm10 genome using Subread. ATAC peaks were called using Homer (v4.8.2) with varying FDR cut-offs. Genes that were assigned to overlapping regions had at least 10bps overlap with the gene body. Peaks were called as differentially accessible if they achieved an FDR of 10% with global adjustment and a fold change cut-off of 1.1. Called peaks from all the samples were unified to form a reference annotation then used to summarize reads using the featureCounts in Rsubread. A linear model was fitted to each peak, and empirical Bayes moderated t-statistics was used to assess differences

in accessibility. Pairwise comparisons between the various CD4 T cell subsets were done to determine the extent of chromatin changes.

2.4.6 Chromatin immunoprecipitation (ChIP) sequencing

IRF4 antibody ChIP was performed on flow cytometry purified wildtype Treg cells as previously described (Vasanthakumar, Moro et al. 2015). Sequencing libraries were prepared on the Illumina pipeline. c-Maf ChIP-seq data was downloaded from the GEO database (Ciofani, Madar et al. 2012). Sequence reads were mapped to the mouse genome mm10 using the Subread aligner. IRF4 and c-Maf peaks were called using the Homer program (v4.8.2) with FDRs of 1e-05 and 1e-32 respectively. Overlapping peaks between c-Maf and IRF4 binding sites were identified using bedtools.

2.5 Statistical Analysis

Data were analysed with Prism 8.0 software (GraphPad). Statistical significance of differences was assessed using the two-tailed unpaired Student's t-test. Differences between two populations over time were analysed by ANOVA with Tukey's multiple comparison test. p values of less than 0.05 were considered significant. Error bars on graphs reflect mean $\pm$ S.D, unless stated otherwise.

Chapter Three: Ontogeny of Effector Regulatory T cells

Introduction

A paradigm-shifting discovery in the 1960s first determined that peripheral tissue specific tolerance was programmed in the thymus (Miller 1961), transforming the study of immunology. The earliest evidence of dominant tolerance was reported in the 1980s, when the concept of tolerogenic T cells was demonstrated in a study using wing bud engraftment in the chick-quail system (Ohki, Martin et al. 1987). It was another decade before the discovery of CD25, the first reliable marker to distinguish so-called ‘suppressor’ (regulatory) T cells, that led to intensive studies of this specialised subset of CD4 T cells capable of limiting autoimmunity (Sakaguchi, Sakaguchi et al. 1995, Sakaguchi, Setoguchi et al. 2006, Sakaguchi and Powrie 2007, Sakaguchi, Wing et al. 2007). It was not until the discovery of the lineage-defining transcription factor, forkhead box protein (Foxp)3 linking its expression to regulatory T (Treg) cell differentiation and function, which underscored Treg cells as an indispensable player of the normal immune system (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003). Since then, numerous research groups have established the crucial role of Treg cells in maintaining immunologic self-tolerance and peripheral homeostasis in both mice and humans (Gavin, Rasmussen et al. 2007, Josefowicz, Lu et al. 2012, Liu, Gerner et al. 2015).

The development of Treg cells begins in the thymus through stringent selection processes which are dependent on interactions between the T cell receptor (TCR) and self-peptide/major histocompatibility complex (pMHC) on thymic antigen presenting cells (APCs). These relatively high affinity interactions drive Treg cell differentiation as an alternative negative selection fate to the deletion of these potentially autoreactive cells (Hogquist, Jameson et al. 1994, Klein, Hinterberger et al. 2009). Mature Treg cells leave the thymus and enter the periphery as central (c)Treg cells with a naïve phenotype but a diverse TCR repertoire that is maintained by low affinity self-pMHC interactions. cTreg cells become activated in a TCR-dependent manner, differentiate and acquire functional specialisation, programmed by tissue-specific environmental cues to become effector (e)Treg cells (reviewed in (Teh, Vasanthakumar et al. 2015). Whilst cTreg cells are predominantly found within secondary lymphoid organs, eTreg cells represent the majority of Treg cells identified within non-lymphoid tissues, where their function is tailored to the niche (reviewed

in Cretney, Kallies et al. 2013, Panduro, Benoist et al. 2016). eTreg cells not only maintain immune homeostasis at these sites, but also play an active role in the regulation of tissue metabolism in certain tissues, and participate in tissue repair mechanisms and regeneration (Josefowicz, Lu et al. 2012, Ohkura, Kitagawa et al. 2013, Panduro, Benoist et al. 2016). Cytokines and the expression of various chemokine receptors and adhesion molecules influence the differentiation, homeostasis and migration of Treg cells. Continuous IL-2 signalling is required by Treg cells to maintain their survival and suppressive function (Malek and Castro 2010, Campbell and Koch 2011). However, this dependency on IL-2 signalling does vary depending on the tissue location of eTreg cells. For example, in the visceral adipose tissue, IL-33, but not IL-2, is the critical growth factor for the expansion of eTreg cells (Vasanthakumar, Moro et al. 2015). In addition, eTreg cells also require additional signals through co-stimulation via the inducible costimulator (ICOS) for its maintenance and function (Cretney, Xin et al. 2011, Smigielski, Richards et al. 2014). Accordingly, eTreg cells downregulate the lymphoid-homing receptors CD62L and CCR7 to facilitate their egress from lymphoid organs to gain access to specialized growth factors essential for their adaptation to the local tissue microenvironment (Smigielski, Richards et al. 2014).

As previously detailed in Chapter 1, eTreg cells are characterized by their expression of the transcription factor Blimp-1 (encoded by *Prdm1*), which is essential for the secretion of the immunoregulatory cytokine IL-10 (Cretney, Xin et al. 2011). Blimp-1 expression corresponds with expression of many of the Treg cell signature genes, thus making it a reliable reporter of the eTreg cell phenotype. Prior to the discovery of Blimp-1, eTreg cells were distinguished by the high expression of key effector molecules associated with Treg cell function, including CTLA-4, ICOS and TIGIT (reviewed in (Liston and Gray 2014). Though, Blimp-1 is not strictly required to enforce eTreg cell differentiation, with hypofunctional eTreg cells able to develop following its conditional deletion (Cretney, Xin et al. 2011, Bankoti, Ogawa et al. 2017). Further evidence that other factors are required to enforce the eTreg cell differentiation program includes observations that the expression of specific transcription factors, including IRF4, c-Maf and Myb, are important for the generation of ICOS-expressing eTreg cells (Cretney, Xin et al. 2011, Dias, D'Amico et al. 2017, Neumann, Blume et al. 2019).

IRF4 is a transcription factor that is required for the effector differentiation of multiple cell types (Mittrucker, Matsuyama et al. 1997, Lohoff, Mittrucker et al. 2002, Cretney, Xin et al. 2011, Bollig, Brustle et al. 2012, Ochiai, Maienschein-Cline et al. 2013, Huber and Lohoff 2014). Work in CD8 T cells identified IRF4 to specifically be induced in response to TCR signals and mediating much of the transcriptional response to TCR signalling (Man, Miasari et al. 2013). Initially described to promote the development of Th2-like Treg cells (Zheng, Chaudhry et al. 2009), IRF4 has more recently been demonstrated as critical for eTreg and tissue Treg cell differentiation (Vasanthakumar, Moro et al. 2015). This finding is consistent with data indicating that eTreg cells express high levels of IRF4 relative to other T cell populations (Cretney, Xin et al. 2011) and evidence that Treg cells continue to receive high affinity TCR signals following thymic export (Moran, Holzapfel et al. 2011). Treg cells constitutively express IRF4, which is required for the maintenance of Treg cell identity and stability in concert with Foxp3 (Fu, Ergun et al. 2012). Indeed, IRF4 and Foxp3 colocalize at the *Icos* locus suggesting that IRF4 is central to the Treg cell transcriptional program (Zheng, Chaudhry et al. 2009). Notably, IRF4 expression is a prerequisite to the induction of Blimp-1, further underlining the importance of IRF4 in Treg cell maturation and effector differentiation (Cretney, Xin et al. 2011, Vasanthakumar, Moro et al. 2015, Cretney, Leung et al. 2018).

While a number of transcription factors and signalling pathways have been identified to control Treg cell phenotype and function (Luo and Li 2013), the transcriptional program of eTreg cell differentiation is less well understood. Although an embryonic window of the acquisition of natural tolerance has been described (Yang, Fujikado et al. 2015), it is not clear whether tissue-specific Treg cells emerge from the thymus with their characteristic transcriptional program already predetermined. It has been posited that tissue-specific Treg cells that seed non-lymphoid organs arise from thymocytes generated during the first weeks of life (Kolodin, van Panhuys et al. 2015). Here, Kolodin et al showed that thymectomy at 3-4 weeks of age did not affect the visceral adipose tissue Treg cell compartment, supporting the notion of early seeding of Treg cells with subsequent age-related expansion in non-lymphoid organs. Moreover, Treg cells that have developed early in life appear to be more effective in limiting autoimmune processes and regulating self-tolerance (Yang, Fujikado et al. 2015). Here, using an inducible Treg-cell lineage-tracer system that allowed the labelling of perinatally-derived Treg cells, Yang and colleagues showed that a distinct compartment

of Treg cells that are generated early in life was necessary to prevent autoimmune disease. In support of this, another study found that Treg cell colonization of the skin in adult mice was insufficient to establish tolerance and that this process has to occur during a dedicated period during development to be protective (Scharschmidt, Vasquez et al. 2015).

Although it is evident that commitment to the Treg cell lineage occurs in the thymus, it remains unclear if intrathymic signalling plays any role in the cells' fate to become tissue-seeding eTreg cells. A potential determinant of eTreg cell differentiation is exposure to self-antigens within the thymus. The autoimmune regulator (Aire) is a transcription factor of major importance in this process. Aire is responsible for the promiscuous expression of normally tissue-restricted antigens (e.g. insulin, thyroglobulin) by medullary thymic epithelial cells (mTEC) (Anderson and Takahama 2012). In this way, Aire is involved in shaping the TCR repertoire, directing thymocytes with potentially autoreactive TCRs to die via apoptosis or divert toward the Treg cell lineage (Derbinski, Schulte et al. 2001, Anderson, Venanzi et al. 2002, Aschenbrenner, D'Cruz et al. 2007). As such, Aire is required to maintain self-tolerance (reviewed in (Anderson and Su 2016) and not only impacts the peripheral repertoire of conventional CD4 T cells but also of Treg cells (Liston, Lesage et al. 2003, Hansenne, Louis et al. 2009, Malchow, Leventhal et al. 2013, Yang, Fujikado et al. 2015).

Antigen-specific Treg cells have been found to have enhanced suppressive ability directed against their cognate antigen (Akkaya, Oya et al. 2019), suggesting that TCR specificity is critical for Treg cell function. In line with that, distinct TCR repertoires have been identified amongst Treg cells from different peripheral sites (Lathrop, Santacruz et al. 2008, Burzyn, Kuswanto et al. 2013). This raises the possibility that the thymic education of Treg cells against tissue-restricted antigens may predispose cells to seed those tissues. Although thymic Treg cell development and Treg cell differentiation in the periphery have been extensively researched, it remains to be determined whether eTreg-fated cells may leave the thymus pre-programmed with their effector or tissue-specific fate, or if the eTreg program depends entirely upon stochastically encountered signals in the periphery. Here, we utilized various transgenic mouse models to analyse the perinatal development of eTreg cells in various organ systems, and to examine the contribution of IRF4 and Aire to address these outstanding questions.

Results

3.1 Effector Regulatory T cell development

I) Subclassification of Treg cells based on activation status.

To allow for easy distinction of Treg cell activation state by flow cytometry, we made use of a *Blimp1*^{GFP} reporter mouse, in which green fluorescent protein (GFP) is expressed from the *Prdm1* (which encodes Blimp-1) gene locus (Kallies, Hasbold et al. 2004). Crossing this mouse with the *Foxp3*^{RFP} reporter mouse (Wan and Flavell 2005) yielded *Foxp3*^{RFP}*Blimp1*^{GFP/+} dual reporter mice, which reported *Blimp1* RNA expression. Here, we observed that the expression of CD62L and *Blimp1*^{GFP} reporter distinguished Treg cells into three distinct stages of activation (**Fig. 4a**). Stage 1 or CD62L^{high}*Blimp1*^{GFP} reporter negative central (c)Treg cells, stage 2 or CD62L^{low}*Blimp1*^{GFP} reporter negative activated (a)Treg cells, and stage 3 or CD62L^{low}*Blimp1*^{GFP} reporter positive effector (e)Treg cells (**Fig. 4b**). Confirming this phenotypic scheme, we observed progressive loss of the lymphoid homing receptor CCR7 (**Fig. 4c**), consistent with increased non-lymphoid tissue homing potential as Treg cells further differentiate. Using this system of staging Treg cells based on their activation status, we were able to determine the frequencies of each population in lymphoid and non-lymphoid organs of *Foxp3*^{RFP}*Blimp1*^{GFP/+} reporter mice. We chose to analyse the Treg cell compartments of the liver and small intestine (SI) as both organs are crucial sites for the induction of immune tolerance (Hooper, Littman et al. 2012). In the spleen, the majority of Treg cells consisted of cTreg and aTreg cells (**Fig. 4d**). The liver, exposed to a variety of non-pathogenic antigens through the portal venous system (Breous, Somanathan et al. 2009), interestingly, displayed a slightly different composition of Treg cells compared with the spleen, showing a higher percentage of aTreg cells (**Fig. 4e**). Within the gastrointestinal tract, where there is constant exposure to diverse microbiome and food-derived antigens, the Treg cell compartment predominantly comprised of the terminally differentiated eTreg cells (**Fig. 4f**). Together, our data demonstrate that the stages of Treg cell activation can be determined based on the downregulation of CD62L and subsequent upregulation of *Blimp1*^{GFP} reporter expression, and its organ composition is determined by tissue-specific requirements.

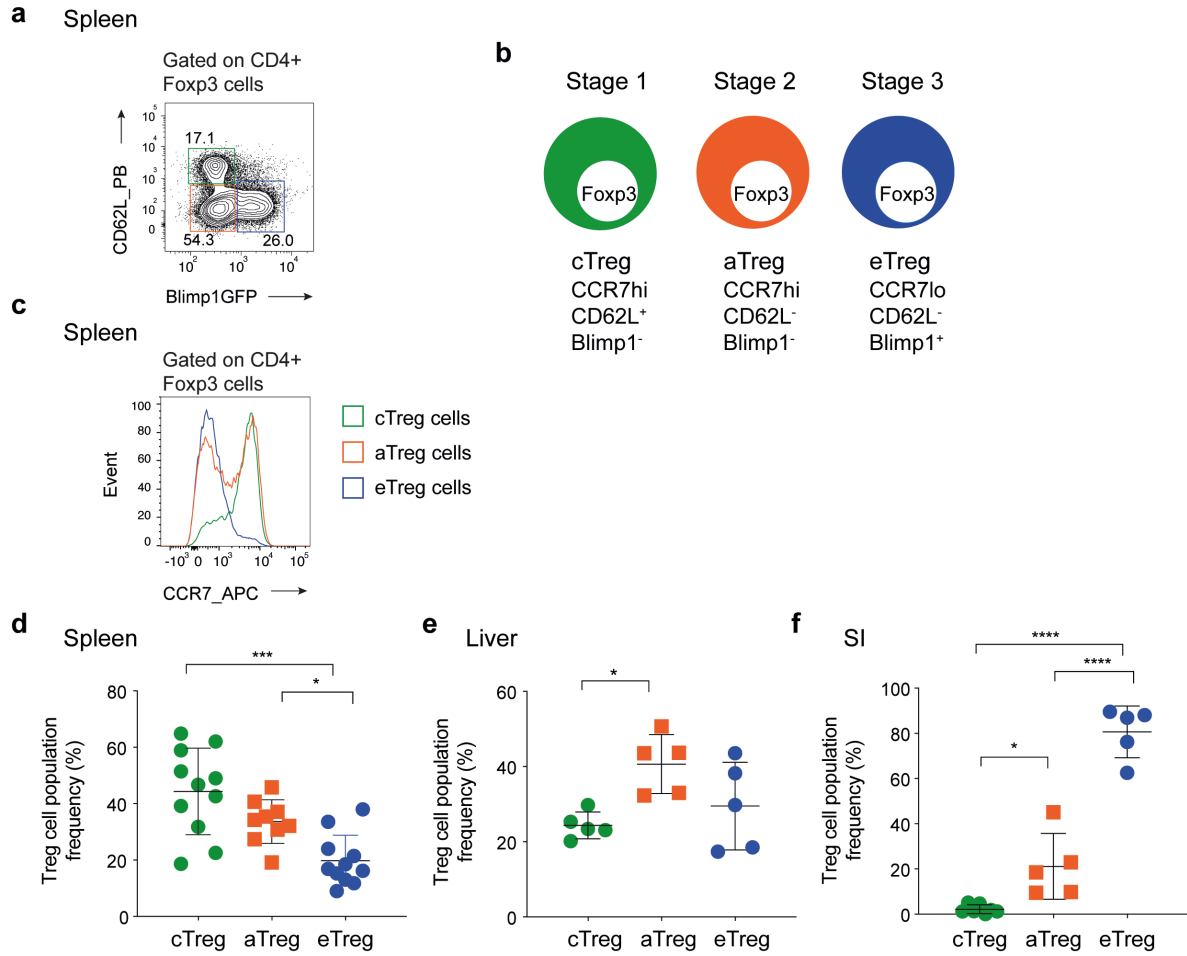


Figure 4. Subclassification of Treg cell based on activation status.

(a) Flow cytometric plot showing the expression of CD62L and *Blimp-1*^{GFP} reporter by splenic Treg cells from *Foxp3*^{RFP}*Blimp-1*^{GFP/+} reporter mice. (b) Graphic summary illustrating the model of Treg cell stages in the periphery. (c) Representative histogram showing the expression of CCR7 by Treg cells based on the 3-stages of activation. (d-f) Graph showing the frequencies of Treg cells in the adult spleen (d), liver (e), and SI (f) based on the 3-stage model.

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Tukey's test for multiple comparisons; non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of three independent experiments with three to four mice per group. aTreg, activated Treg cells; cTreg, central Treg cells; eTreg, effector Treg cells; ns, not significant; SI, small intestine.

II) Ontogeny of Treg cells.

To interrogate the ontogeny of the Treg cells, we used flow cytometry to analyse the T cell compartment of the thymus, spleen, liver and lamina propria of the SI from *Foxp3^{RFP}Blimp1^{GFP/+}* dual reporter mice at various time points after birth. As Treg cells ontogenically begin to appear in the periphery at day 3, we analysed neonatal mice starting from day 3 (Asano, Toda et al. 1996). Mice that were older than 3 months were categorized as adult. **Figure 5a** depicts the gating strategy used to identify thymic Treg cells. In non-lymphoid organs, lack of CD45 expression was also used to exclude non-haematopoietic cells from the analysis. As a first step in comparing the Treg cell compartments, we used percentages to document the relative frequencies of cells within the organs from the same individuals. We observed that global Treg cell frequencies increased from day 3 onwards and peaked at week 2 after birth in the thymus (**Fig. 5b**). Interestingly, there was a modest reduction in Treg cell frequencies at week 4 before returning to the frequency observed at week 2-3 in adulthood (**Fig. 5b**). Treg cell frequencies in the spleen gradually increased after birth from day 3 onward and displayed a similar dip at week 4 to that seen in the thymus (**Fig. 5c**). Next, we analysed the frequencies of Treg cells in the liver and SI from the same individuals. In the first 4 weeks after birth, the liver displayed similar Treg cell frequencies (11-13%) to the spleen (**Fig. 5c-d**). In adult mice, however, the liver Treg cell frequencies decreased to ~7% of all CD4 T cells (**Fig. 5d**). By contrast, Treg cell frequencies in the lamina propria of the SI only increased around the fourth week after birth, correlating with weaning and exposure to novel food antigens (**Fig. 5e**). These observations demonstrate that Treg cell populations arise within lymphoid and non-lymphoid organs at different stages during ontogeny.

III) Effector regulatory T cell kinetics in lymphoid organs during neonatal ontogeny.

In order to elucidate whether these changes reflect alterations in Treg cell subset kinetics, we analysed *Blimp1^{GFP}* reporter expression by thymic and splenic CD4 T cells from *Foxp3^{RFP}Blimp1^{GFP/+}* dual reporter mice during ontogeny. We detected very few *Blimp1^{GFP}* reporter-expressing cells among thymic and splenic Treg cells at day 3 after birth (**Fig. 6a-b**). Notably, thymic *Blimp1^{GFP}* reporter-expressing Treg cells remain at low frequencies until 4 weeks after birth (**Fig. 6a, c**). Though there were few *Blimp1^{GFP}* reporter positive Treg cells in the spleen from day 3 old mice, the *Blimp1^{GFP}* reporter positive Treg cell pool increased

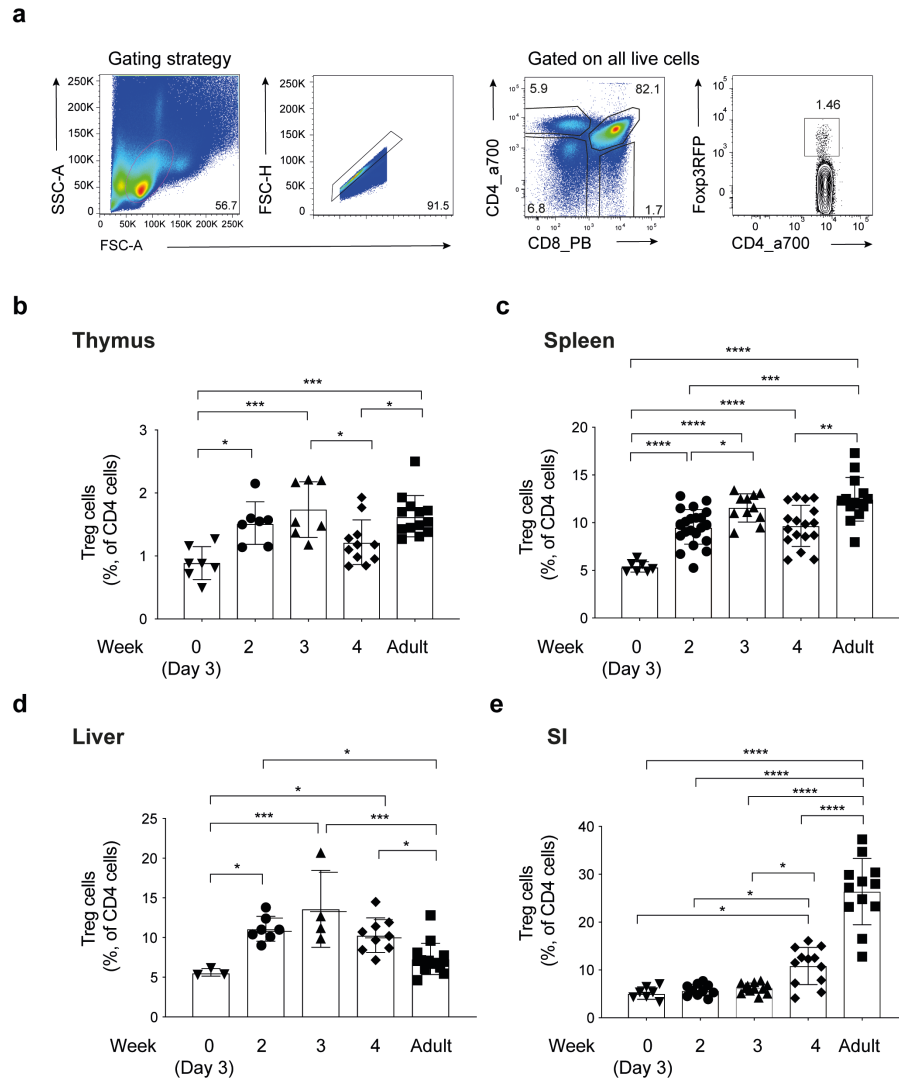


Figure 5. Treg cell kinetics during neonatal ontogeny.

(a) Flow cytometry plots showing the gating strategy of thymic T cells using the *Foxp3^{RFP}Blimp1^{GFP/+}* double reporter mice. (b-e) Quantification of the frequency of *Foxp3^{RFP}* reporter expression by thymic (b), splenic (c), liver (d) and SI (e) Treg cells from *Foxp3^{RFP}Blimp1^{GFP/+}* mice at various ages from day 3 onwards.

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Tukey's test for multiple comparisons; ; non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of 4 – 8 mice analysed per time point. SI, small intestine.

to adult frequencies by 2 weeks of age (**Fig. 6b, d**). The percentage of splenic Treg cells that expressed *Blimp1*^{GFP} reporter remained stable at approximately 15-22% until adulthood (**Fig. 6d**). Together, these results show that *Blimp1*^{GFP} reporter-expressing Treg cells are generated early after birth and remain at a relatively static frequency within the spleen.

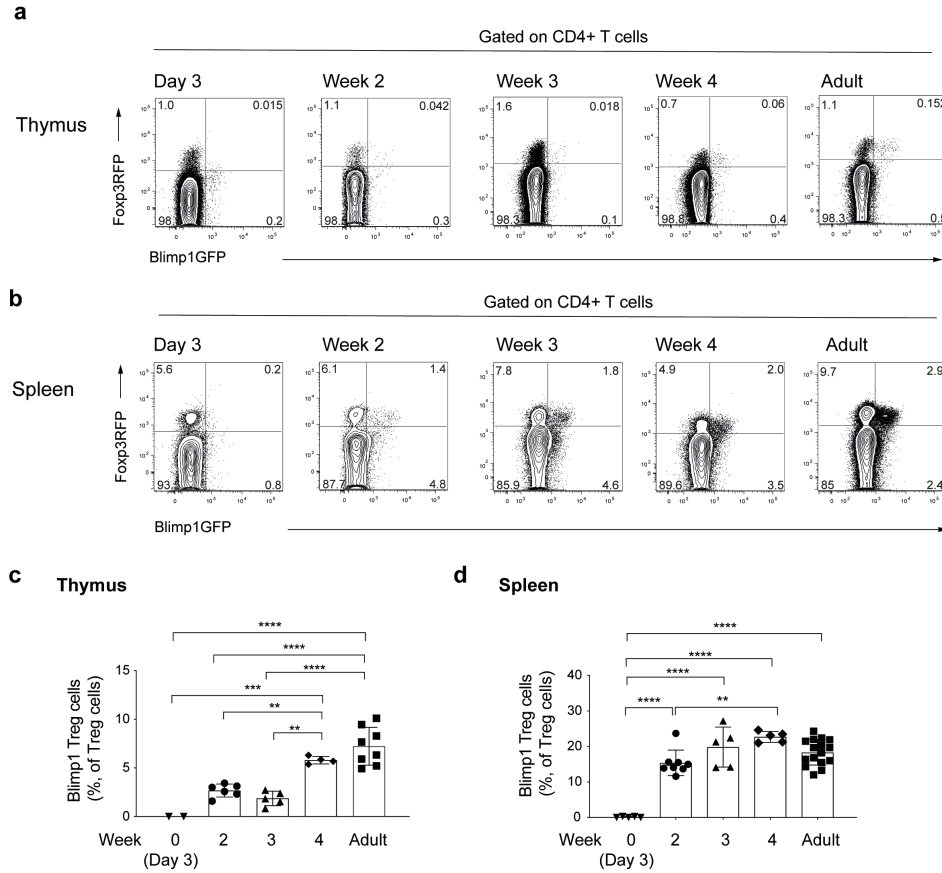


Figure 6. Effector Treg cell kinetics in lymphoid organs during neonatal ontogeny.

(a-b) Representative flow cytometric analysis showing the expression of *Blimp1*^{GFP} reporter (see R2 gate) by thymic (a) and splenic (b) Treg cells of from *Foxp3*^{RFP}*Blimp1*^{GFP/+} dual reporter mice at the indicated ages. All plots are gated on the CD4 single positive population. (c-d) Graphs show the frequencies of *Blimp1*^{GFP} reporter-expressing thymic and splenic Treg cells among CD4+Foxp3+ cells within the same mouse.

The numbers indicate the percentage of cells in each gate. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). *p<0.05, **p<0.01, ****p<0.0001 (one-way ANOVA with Tukey's test for multiple comparisons; non-significant comparisons are unlabelled). Data represents 4 – 8 mice analysed per time point.

IV) Neonatal eTreg cells accumulate in non-lymphoid organs.

To investigate the ontogeny of non-lymphoid organ eTreg cells, we next analysed the Treg cell compartment of the liver from corresponding mice. As the liver is a highly vascular organ, we perfused the liver with PBS during harvest to reduce contamination by circulating haematopoietic cells. Here, we demonstrated that the liver harbours a significantly larger percentage of *Blimp1*^{GFP} reporter Treg cells when compared with the spleens of age-matched neonates (**Fig. 7a-b**). We found that in 2-week old and adult mice, the frequency of *Blimp1*^{GFP} reporter-expressing Treg cells in the liver was significantly higher compared to the spleen (**Fig. 7b**). Interestingly, when compared to the spleen of 2-week-old mice, there were reduced frequencies of cTreg cells in the liver (**Fig. 7c-d**).

To determine whether this Treg cell activation profile was unique to the liver, we analysed the Treg cell compartment from the SI. Treg cells expressing *Blimp1*^{GFP} reporter were detected at low frequencies during the first three weeks after birth (**Fig. 7e-f**). We found a higher *Blimp1*^{GFP} reporter Treg cell percentage in the SI than in the thymus in 4-week old and adult mice (**Fig. 7f**). Compared to the thymus, there was an abrupt accumulation of *Blimp1*^{GFP} reporter-expressing Treg cells in the SI between week 3 to 4 after birth (**Fig. 7f**). Treg cells in the SI of 2-week-old mice displayed a non eTreg cell phenotype, as evidenced by predominantly *Blimp1*^{GFP} negative Treg cells in contrast to adult mice (**Fig. 7g-h**). Accordingly, we observed a higher frequency of cTreg and aTreg cells when compared to the Treg cell compartment in the SI of adult mice (**Fig. 7h**). Specifically analysing the aTreg cell compartment of 2-week-old mice, we saw increased frequencies within the SI compared with the liver and spleen (**Fig. 7i**).

Based on previous studies, non-lymphoid organ Treg cells are predominantly eTreg cells and express high levels of activation-associated molecules including ICOS and KLRG1 (Cretney, Xin et al. 2011, Vasanthakumar, Moro et al. 2015). Whilst fewer in frequencies, compared to SI aTreg cells, SI eTreg cells expressed higher levels of ICOS and KLRG1 (**Fig. 7j**). Notably, a large proportion of aTreg and eTreg cells from the SI of 2-week-old mice expressed the cell surface receptor, neuropilin (Nrp)1 (**Fig. 7k**). Nrp1, often used as a marker of tTreg cells (Weiss, Bilate et al. 2012, Yadav, Louvet et al. 2012), suggests that the population of Treg cells in the SI are tTreg cells, as there are minimal food antigens present in the SI at that time. Collectively, these results show that the majority of Treg cells in the SI compartment of young mice do not express *Blimp1*^{GFP} reporter. However, Treg cells that did

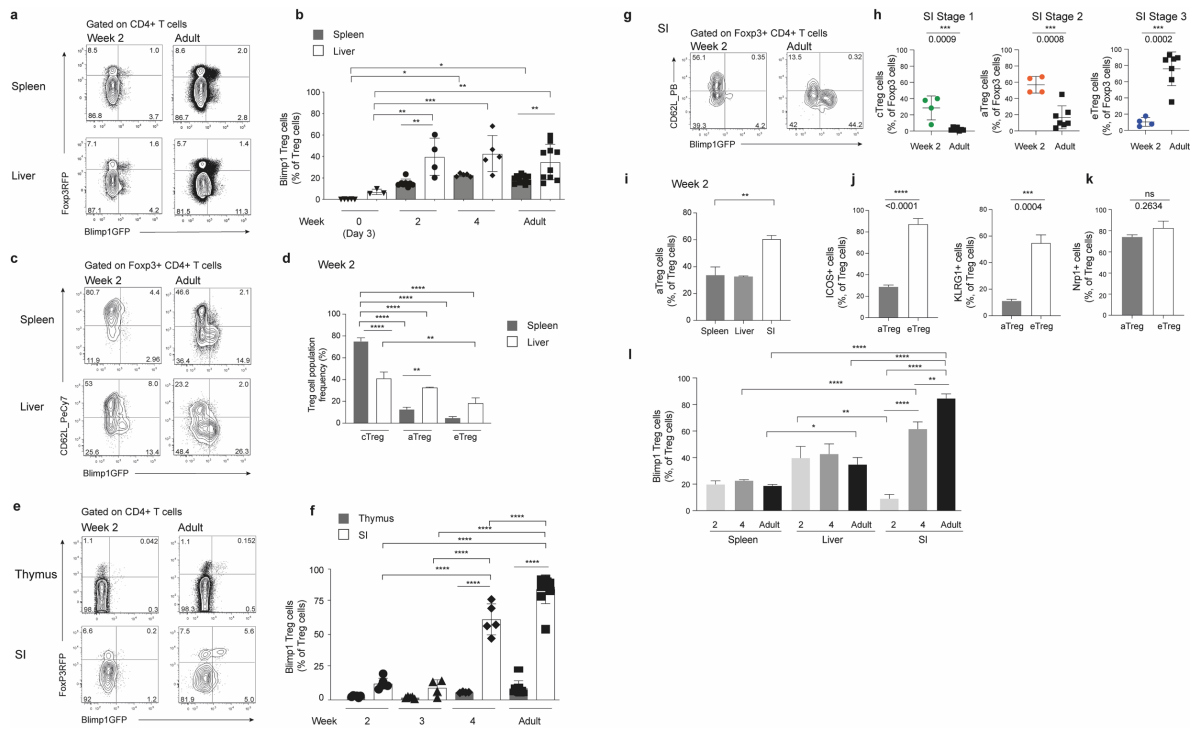


Figure 7. The ontogeny of effector Treg cells in the liver and small intestine.

(a) Flow cytometric analysis of *Blimp1*^{GFP} reporter expression (see R2 gate) by CD4⁺ T cells from the spleen and liver of *Foxp3*^{RFP}*Blimp1*^{GFP/+} reporter mice at week 2 after birth compared with adults. (b) Graph showing the frequency of *Blimp1*^{GFP} reporter-expressing Treg cells in the spleen and liver of mice at the indicated ages. (c) Flow cytometric analysis of CD62L and *Blimp1*^{GFP} reporter expression by Treg cells from the liver of mice in (a). (d) Graph showing the frequencies of cTreg, aTreg and eTreg cells in the spleen and liver of 2-week-old mice. (e) Flow cytometric plots of *Blimp1*^{GFP} reporter expression (see R2 gate) by CD4⁺Foxp3⁺ Treg cells from the thymus and SI of mice as in (a). (f) Bar graph showing the frequency of *Blimp1*^{GFP} reporter-expressing Treg cells in the thymus and SI of mice at the indicated ages. (g) Flow cytometry plot of CD62L and *Blimp1*^{GFP} reporter expression by Treg cells from the SI of mice in (a). (h) Graph showing the frequency of cTreg, aTreg and eTreg cells from the SI of 2-week-old and adult mice. (i) Bar graph comparing the frequency of aTreg cells from the spleen, liver and SI of 2-week-old mice. (j-k) Graphs showing the frequency of ICOS⁺, KLRG1⁺ and Nrp1⁺-expressing aTreg and eTreg cells from the SI of 2-week-old mice. (l) Graph showing the frequency of *Blimp1*^{GFP} reporter Treg cells from the spleen, liver and SI of 2- and 4-week-old, and adult mice. Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using (e,k-l) unpaired Student's t-test and (b,d,j,g,m) one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of 4 – 8 mice analysed per time point. aTreg, activated Treg; cTreg, central Treg; eTreg, effector Treg; SI small intestine.

express the *Blimp1*^{GFP} reporter conform to the eTreg cell phenotype.

Finally, comparing the frequencies of *Blimp1*^{GFP} reporter-expressing Treg cells in the lymphoid versus non-lymphoid organs from week 2 and 4, and adult mice, we observed that the hepatic Treg cell compartment had double the frequencies of *Blimp1*^{GFP} reporter Treg cells compared with the spleen. Within the SI Treg cell compartment, there was an abrupt accumulation of *Blimp1*^{GFP} reporter-expressing Treg cells from week 4 onwards (**Fig. 7m**). Overall, our data demonstrate the variable contribution of different stages of Treg cell activation to the spleen, SI and liver in mice at the indicated ages after birth, suggesting that the local environment influences the composition of Treg cells.

V) Dynamics of ICOS expression during Treg cell differentiation.

Costimulatory receptors such as the inducible T cell co-stimulator, ICOS is expressed by activated T cells (Watanabe, Hara et al. 2005) and its expression parallels with Treg cell suppressive function (Cretney, Xin et al. 2011, Dias, D'Amico et al. 2017). To better understand the kinetics of ICOS expression during the stages of Treg cell activation, we examined the splenic Treg cell compartment of *Foxp3*^{RFP}*Blimp1*^{GFP/+} dual reporter mice by flow cytometry. ICOS was highly expressed by eTreg cells compared to cTreg and aTreg cells (**Fig. 8a**). The frequencies of ICOS-expressing cTreg and eTreg cells were similar irrespective of the age of the mice (**Fig. 8b**). We observed the highest frequencies of ICOS-expressing aTreg cells in the spleen of adult mice (**Fig. 8b**).

In the liver, there was a significant reduction of the frequency of ICOS-expressing cTreg and eTreg cells with age (**Fig. 8c**). Whilst not statistically significant, the frequency of ICOS-expressing aTreg cells showed a similar trend of reduction with age. Interestingly, 2-week-old mice showing the highest frequency of ICOS-expressing eTreg cells when compared to 4-week old and adult mice (**Fig. 8c**).

As shown in **Fig. 4f**, we found a small population of aTreg cells and an almost negligible population of cTreg cells in the SI of adult mice. As expected, we observed only a small percentage of ICOS-expressing cTreg and aTreg cells in the SI of adult mice (**Fig. 8d**). The majority of the eTreg cells within the SI of adult mice expressed ICOS (**Fig. 8d**). Mice at 4-weeks of age had an increased frequency of ICOS-expressing aTreg cells when compared to 2-week-old and adult mice (**Fig. 8d**). Additionally, the frequency of ICOS-expressing eTreg cells in the SI was highest at 4-weeks of age even when compared with adult mice (**Fig. 8d**).

Overall, we observed an increase in the frequency of ICOS-expressing Treg cells with each stage of Treg cell activation (**Fig 8b-d**). The findings from these experiments demonstrate that ICOS expression strongly correlates with Treg cell activation and supports the finding that eTreg cells are the most terminally differentiated and suppressive of all Treg cells (Cretney, Xin et al. 2011).

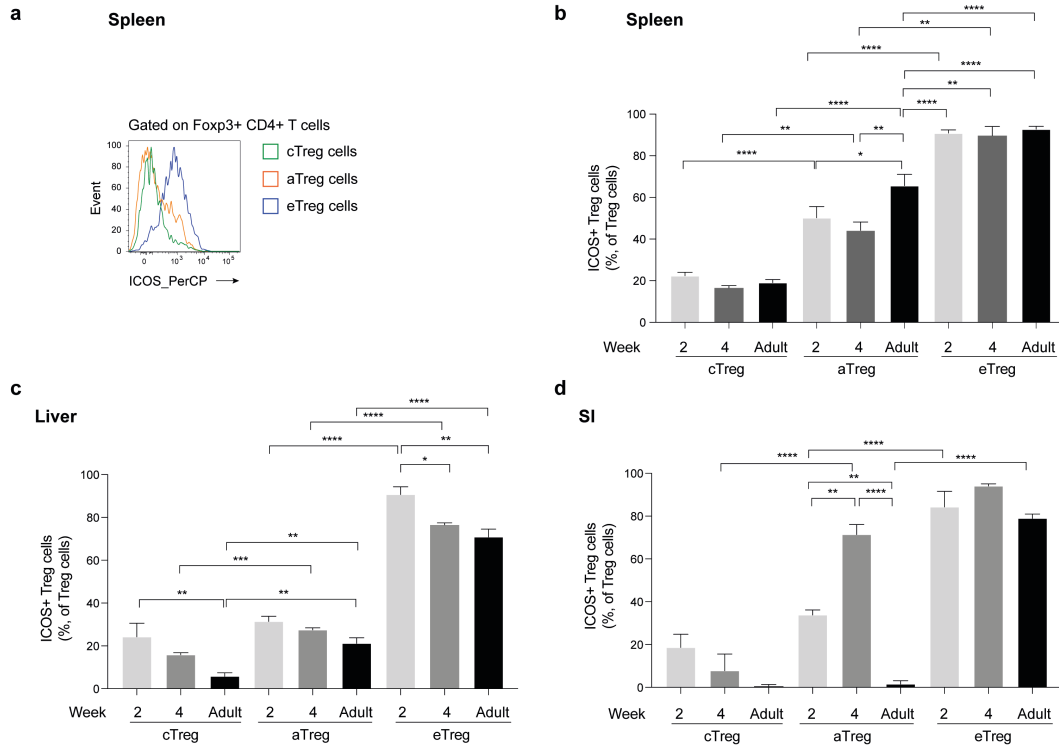


Figure 8. ICOS expression during eTreg cell development.

(a) Representative histogram of ICOS expression by Treg cells from the spleen of an adult *Foxp3^{RFP} Blimp1^{GFP/+}* reporter mouse. (b-d) Bar graphs showing frequencies of ICOS-expressing splenic (b), liver (c) and SI (d) Treg cells from the cTreg, aTreg and eTreg cell populations of 2- and 4-week-old and adult mice.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (two-way ANOVA using Tukey's test for multiple comparisons; non-significant comparisons are unlabelled). Flow cytometric plot and data are representative of 4 – 8 mice analysed per time point. aTreg, activated Treg; cTreg, central Treg; eTreg, effector Treg; SI small intestine.

3.2 The role of AIRE in early effector Treg cell program

I) Ontogeny of neonatal thymic Treg cells and eTreg cells in Aire-deficient mice.

Treg cell generation during the perinatal age window has been shown to be critical for limiting autoimmunity and is dependent on Aire (Yang, Fujikado et al. 2015). Detection of tissue Treg cells in 2-week-old *Foxp3^{RFP}Blimp1^{GFP/+}* dual reporter mice suggest that AIRE-regulated tissue antigens may be involved in the early thymic programming of eTreg cell differentiation, and may be prescriptive of their tissue localization. To test this hypothesis, we crossed mice that were deficient for *Aire* (Anderson, Venanzi et al. 2002) to the *Foxp3^{RFP}Blimp1^{GFP/+}* reporter mice for the identification of eTreg cells. *Aire^{-/-}Foxp3^{RFP}Blimp1^{GFP/+}* mice were healthy at birth compared to wild type littermates and exhibited no overt signs of autoimmune disease throughout the period of observation (up to 12 months). Consistent with a previous publication (Yang, Fujikado et al. 2015), analysis of the T cell compartment from the thymi of 1-2-week-old *Aire*-deficient mice revealed similar frequencies of Treg cells compared to controls (**Fig. 9a-b**). Specifically assessing the expression of *Blimp1^{GFP}* reporter by thymic Treg cells, we saw no differences in the frequencies of *Blimp1^{GFP}* reporter expression in 1-2-week-old *Aire*-deficient mice compared to controls (**Fig. 9c**). However, there was a significant increase in the frequency of *Blimp1^{GFP}* reporter positive Treg cells in adult *Aire*-deficient mice, perhaps in response to the presence of increased autoreactive T cells in the periphery, highlighting the role of Aire in maintaining central tolerance. Our data suggest that *Aire* may be required to promote the early generation of a population of thymic Treg cells.

II) Kinetics of lymphoid and non-lymphoid organ Treg cells in the absence of Aire.

To interrogate a possible role for Aire in the early programming of lymphoid localization and tissue residency of Treg cells, we analysed the eTreg cell compartments of the spleen, liver and SI of *Aire^{-/-}Foxp3^{RFP}Blimp1^{GFP/+}* mice and controls at 2 and 4-weeks of age. In the spleen of 4-week old mice, we saw a 50% reduction in *Blimp1^{GFP}* reporter positive Treg cells in *Aire*-deficient mice compared to controls (**Fig. 10a**). Within the liver, there were no differences in the frequencies of *Blimp1^{GFP}* reporter-expressing Treg cells in *Aire*-sufficient and -deficient mice of both age groups (**Fig. 10b**). In the lamina propria of the SI, whilst there was no difference seen in the frequency of *Blimp1^{GFP}* reporter-expressing Treg cells in 2-

week-old *Aire*-deficient mice compared to controls, we observed a significant increase in the frequencies of *Blimp1*^{GFP} reporter Treg cells from 4-week-old *Aire*-deficient mice (**Fig. 10c**). This aligned with a reciprocal dip of *Blimp1*^{GFP} reporter-expressing Treg cells in the splenic compartment (**Fig. 10a and c**), suggestive of a possible role of *Aire* in the perinatal regulation of eTreg cell localization in the periphery, which prompted us toward closer examination of the phenotype of eTreg cells in adult *Aire*^{-/-} mice.

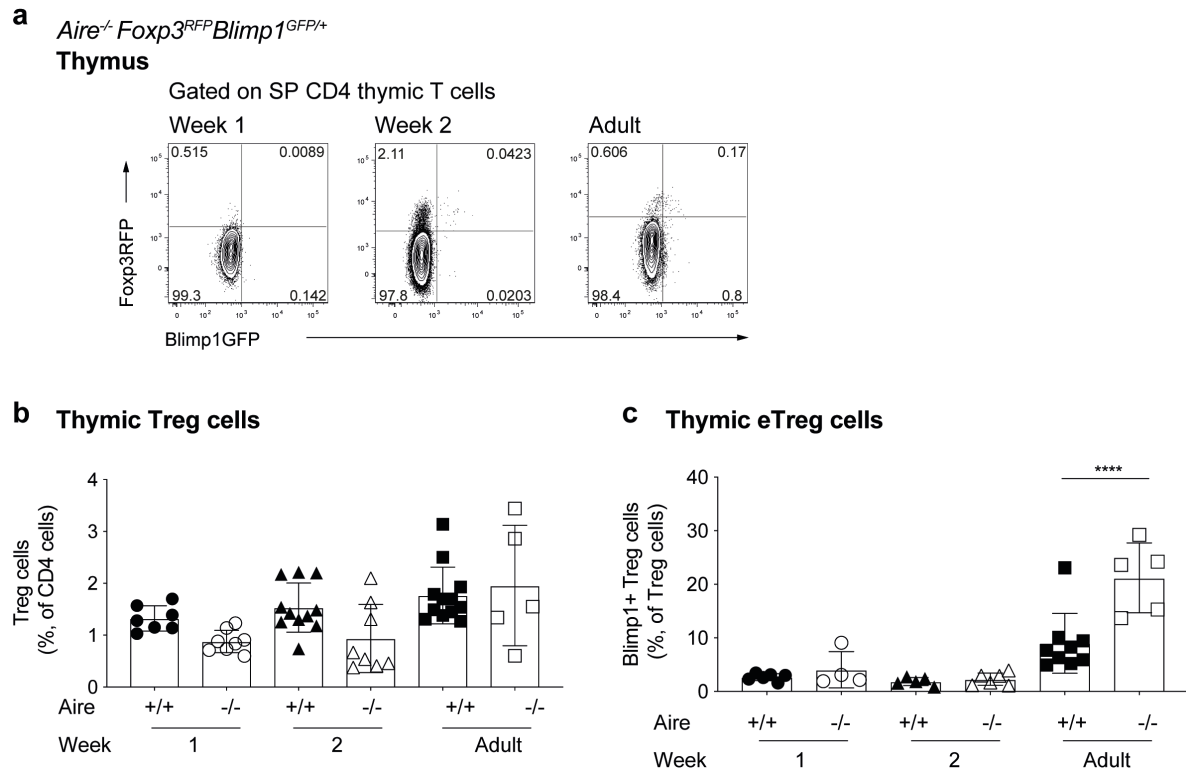


Figure 9. Ontogeny of Treg cells in *Aire*-deficient mice.

(a) Representative flow cytometric plots showing *Blimp1*^{GFP} reporter expression by CD4 single positive thymocytes from *Aire*^{-/-} *Foxp3*^{RFP} *Blimp1*^{GFP/+} (*Aire*^{-/-}) mice of indicated ages. (b) Bar graph showing the frequency of thymic Treg cells from *Aire*^{-/-} mice as in (a) compared to *Aire*^{+/+} mice. (c) Bar graph depicting the frequency of *Blimp1*^{GFP} reporter-expressing Treg cells from the thymi of *Aire*^{-/-} mice and controls.

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). *** $p < 0.001$ (one-way ANOVA with Tukey's test for multiple comparisons; non-significant comparisons are unlabelled). Data are representative of 3 – 8 mice analysed per time point.

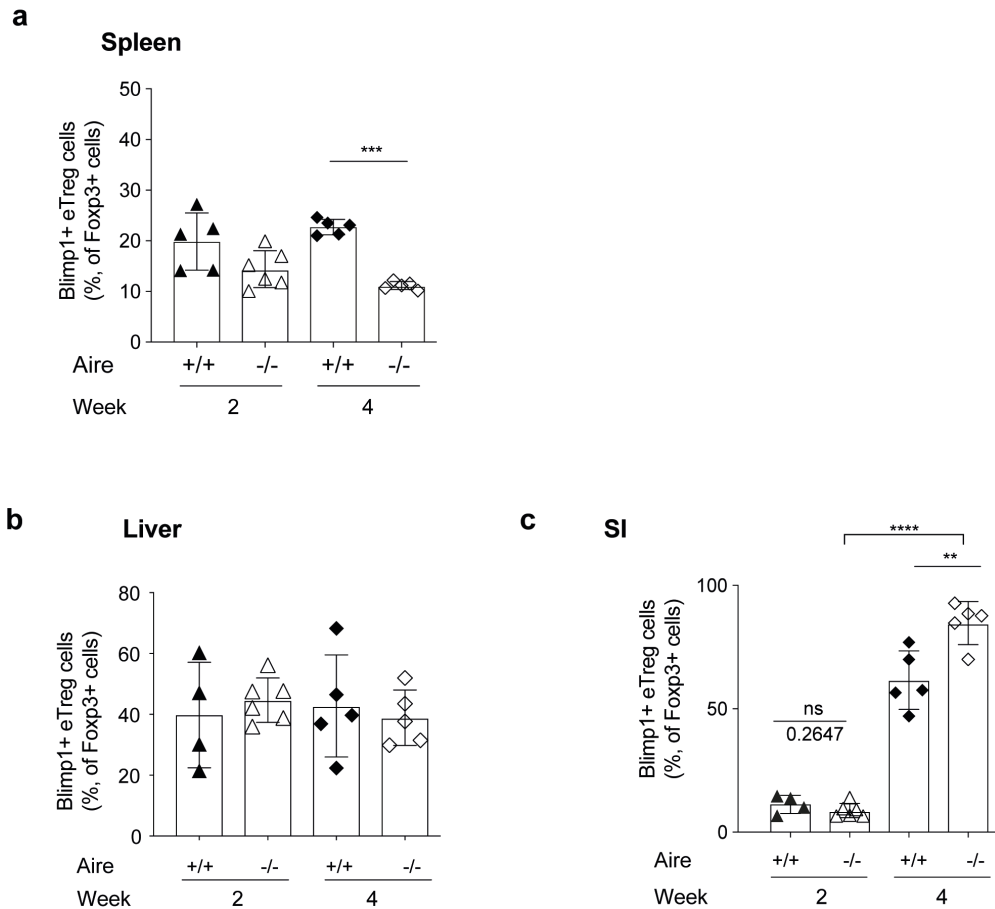


Figure 10. Kinetics of lymphoid and non-lymphoid organ Treg cells in the absence of Aire.

(a) Bar graph depicting the frequency of *Blimp1*^{GFP} reporter-expressing Treg cells from the spleen of *Aire*^{+/+}*Foxp3*^{RFP}*Blimp1*^{GFP/+} (*Aire*^{+/+}) and *Aire*^{-/-}*Foxp3*^{RFP}*Blimp1*^{GFP/+} (*Aire*^{-/-}) mice. (b-c) Bar graphs showing the frequency of *Blimp1*^{GFP} reporter-expressing Treg cells from the liver (b) and SI (c) of mice as in (a).

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). ** $p < 0.01$, **** $p < 0.0001$ (ANOVA with Tukey's test for multiple comparisons; non-significant comparisons are unlabelled). Data are representative of 3 – 8 mice analysed per time point. SI, small intestine.

III) The requirement of Aire in the regulation of splenic eTreg cell compartment.

To examine whether Aire contributes to the regulation of eTreg cell differentiation and localization, separate from ontogeny, we first analysed the spleens of *Aire*-deficient adult mice compared to controls. Here, we saw that *Aire*-deficient mice had similar frequencies of conventional CD4 T cells but increased Treg cell frequencies in the spleen compared to age-matched controls (**Fig. 11a-b**). Along with this, we also saw an increase in *Blimp1*^{GFP} reporter expression by Treg cells of mice that were deficient for *Aire* (**Fig. 11a-b**). There were, however, no differences in the naïve and antigen-experienced Treg cells as determined by the expression of CD44 and CD62L (**Fig. 11c**). Using our 3-stage model of Treg cell activation based on the expression of CD62L and *Blimp1*^{GFP} reporter, we also found no differences in the frequency of cTreg, aTreg and eTreg cells between *Aire*-sufficient and -deficient mice (**Fig. 11d**). There were also no observable differences in the frequency of ICOS-expressing Treg cells based on the stages of activation compared to controls (**Fig. 11e**). *Blimp1*^{GFP} reporter positive Treg cells isolated from the spleen of *Aire*-deficient mice were equally able to upregulate activation-associated molecules such as Nr1 and TIGIT, similar to control Treg cells (**Fig. 11f**). *Aire*-deficiency did not alter the capacity of eTreg cells to downregulate CCR7 (**Fig. 11f**). Taken together, our data suggests that *Aire* does not impair splenic Treg cell activation nor regulate migration.

IV) AIRE and eTreg cells in the SI.

Given the observation of a higher frequency of eTreg cells in the SI of 4-week-old *Aire*-deficient mice (**Fig. 10c**), we were interested to determine if the absence of *Aire* results in an altered tissue Treg cell compartment. As such, we closely examined the T cell compartment from the lamina propria of SI of adult *Aire*-deficient and control mice. Whilst the frequencies of conventional CD4 T were similar in *Aire*-deficient mice compared to controls, there was a slight increase in the frequencies of total Treg cells (**Fig. 12a**). Despite the differences seen in the frequencies of *Blimp1*^{GFP} reporter expression by Treg cells from the SI compartment of *Aire*-deficient and -sufficient mice of week-4-old mice (**Fig. 10c**), this was not reflected in the SI of adult mice (**Fig. 12b**). Based on the 3-stage model of Treg cell activation, there were overall no differences seen in the Treg cell frequencies of *Aire*-deficient mice compared to controls (**Fig. 12c**). Furthermore, the frequency of ICOS-expressing eTreg cells from *Aire*-deficient mice was similar to that of the controls (**Fig. 12d**).

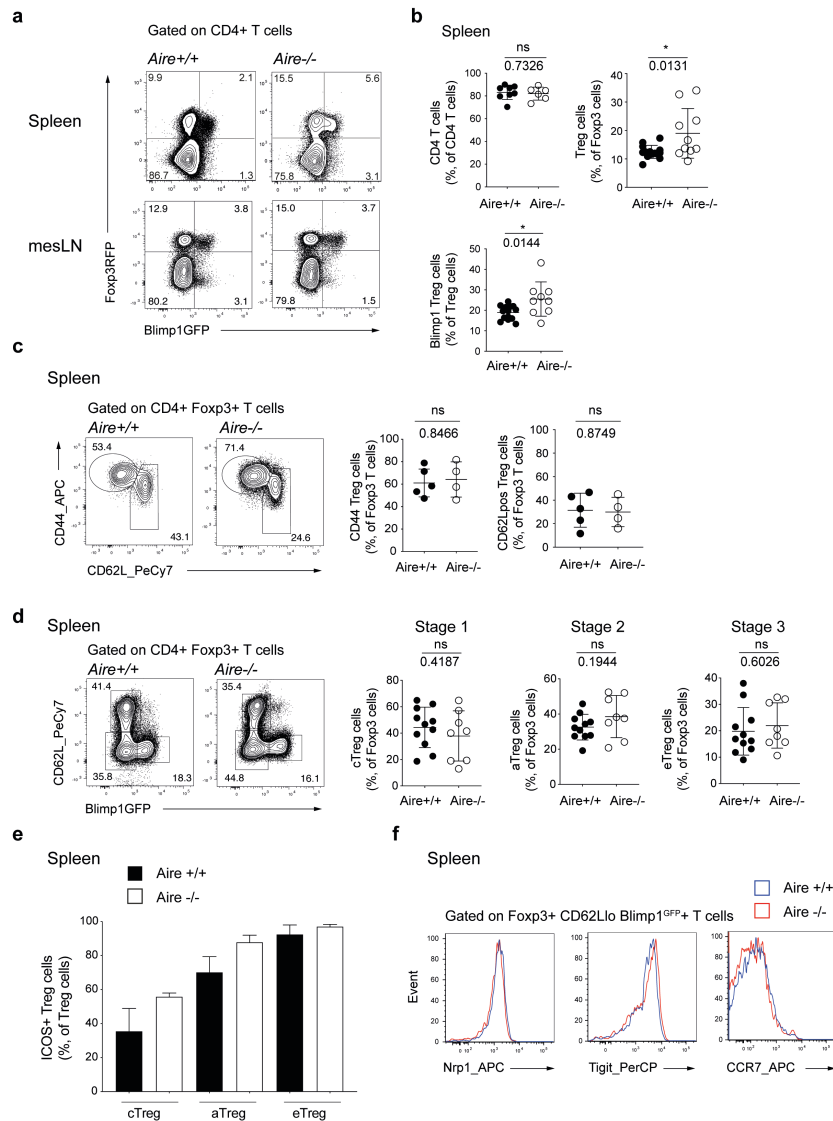


Figure 11. Phenotype of lymphoid organs eTreg cells from adult Aire-deficient mice.

(a) Flow cytometric plots showing *Blimp1*^{GFP} reporter expression by splenic and medLN CD4 T cells from *Aire*^{-/-}*Foxp3*^{RFP}*Blimp1*^{GFP/+} (*Aire*^{-/-}) and control mice. (b) Graphs showing frequencies of CD4 T, Treg and *Blimp1*^{GFP} reporter-expressing Treg cells in mice as in (a). (c) Flow cytometric plots (left) and quantification (right) of the activation status of Treg cells from mice as in (a). (d) Expression of CD62L and *Blimp1*^{GFP} reporter by Treg cells from mice as in (a). Right, quantification. (e) Bar graphs showing the expression of ICOS by splenic Treg cells based on the 3-stage model of activation from mice as in (a). (f) Representative histogram showing the expression of Nrp-1, Tigit and CCR7 by eTreg cells (Foxp3⁺CD62L^{low}Blimp1^{GFP+}) from mice as in (a).

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using (b-d) unpaired Student's t-test and (e) one-way ANOVA (*p<0.05, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of 2 independent experiments with 2-4 mice per group. aTreg, activated Treg; cTreg, central Treg; eTreg, effector Treg.

Collectively, the data from this section indicate that effector programming of Treg cells may occur in the thymus, but Treg cell activation and localization did not depend upon the expression of tissue-restricted antigen by AIRE.

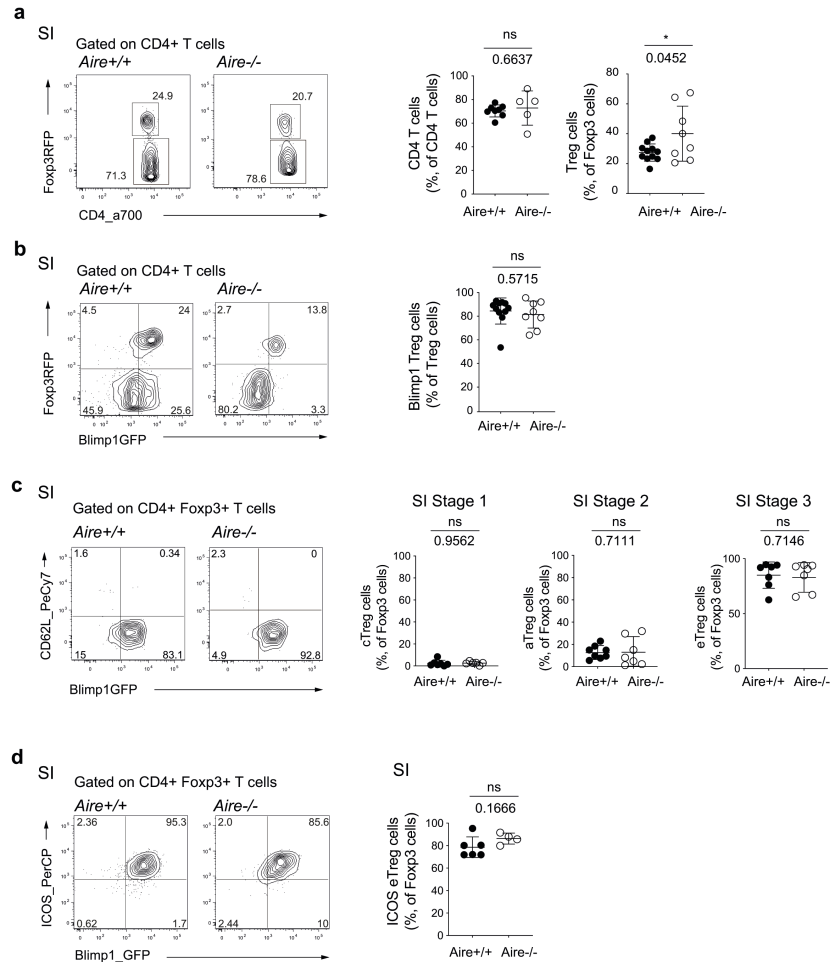


Figure 12. Phenotype of eTreg cells within the lamina propria of the SI.

(a) Flow cytometric plots showing the frequency of conventional CD4 T and Treg cells in the lamina propria of the SI of *Aire*^{-/-} mice and controls. Right, quantification. (b) Flow cytometric plots (left) and quantification (right) showing the frequencies of *Blimp1*^{GFP} reporter-expressing Treg cells in the lamina propria of the SI from mice as in (a). (c) Expression of CD62L and *Blimp1*^{GFP} reporter by Treg cells. Right, quantification. (d) Flow cytometric plots showing the co-expression of ICOS and *Blimp1*^{GFP} reporter by SI Treg cells of *Aire*^{-/-} mice and controls. Right, quantification.

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). * $p < 0.05$ (unpaired Student's t-test). Flow cytometric plots and data are representative of two independent experiments with three mice per group. aTreg, activated Treg; cTreg, central Treg; eTreg, effector Treg; ns, not significant; SI, small intestine.

V) AIRE does not affect the generation of a subset of Treg cells during influenza infection.

Based on the data presented above, we observed that the impact of the absence of *Aire* was not seen in steady-state conditions. As *Aire* has been shown to be critical for the regulation of organ-specific immune tolerance, such as the pancreas and prostate (Liston, Lesage et al. 2003, Malchow, Leventhal et al. 2013), we asked if the effector programming of a specialised subset of eTreg cells, called the T follicular regulatory (T_{FR}) cells, is dependent on the expression of tissue-restricted antigens by *Aire*. To this end, we infected *Aire*^{-/-} *Foxp3*^{RFP} *Blimp1*^{GFP/+} and control mice with influenza virus and used T_{FR} cells as a readout of eTreg cells. Firstly, when we analysed the Treg cell compartment from the spleen and mediastinal lymph node (medLN), *Aire*-deficient mice showed a similar pattern of Treg cell accumulation when compared to controls at day 9 after infection (**Fig. 13a-b**). There were no differences in *Blimp1*^{GFP} reporter-expressing Treg cells in the spleens or medLN from both groups of mice (**Fig. 13c**). Analysis of the splenic Treg cell compartment based on CD62L and *Blimp1*^{GFP} reporter expression revealed similar frequencies of Treg cell activation in all 3 stages from *Aire*-sufficient and -deficient mice (**Fig. 13d**). There was, however, a reduced frequency of ICOS-expressing eTreg cells from *Aire*-deficient mice compared to controls (**Fig. 13e**). Nonetheless, the reduction in ICOS expression did not affect the capacity of Treg cells from *Aire*-deficient mice to further differentiate to T_{FR} cells. Analysis of the T_{FR} cell population (gated as CD19⁻CD4⁺Foxp3⁺CXCR5^{high}PD1^{high} cells) from both the spleen and medLN revealed no differences (**Fig. 13f**). The frequency of *Blimp1*^{GFP} reporter-expressing T_{FR} cells was also comparable to controls (**Fig. 13g**). Furthermore, the expression of ICOS by both *Blimp1*^{GFP} reporter positive and negative T_{FR} cells was not significantly altered in *Aire*-deficient mice (**Fig. 13h**). Compared to *Aire*-sufficient mice, there were similar frequencies of T_{FH} cells in *Aire*-deficient mice (**Fig. 13i**). Finally, there were no differences in germinal centre B cells (as gated on G17⁺Fas⁺CD19⁺ cells) nor plasma cells (gated on CD138⁺ cells) in the *Aire*-deficient mice (**Fig. 13j**). Collectively, these observations indicate that *Aire* does not play a role in the generation of T_{FR} cells after influenza infection.

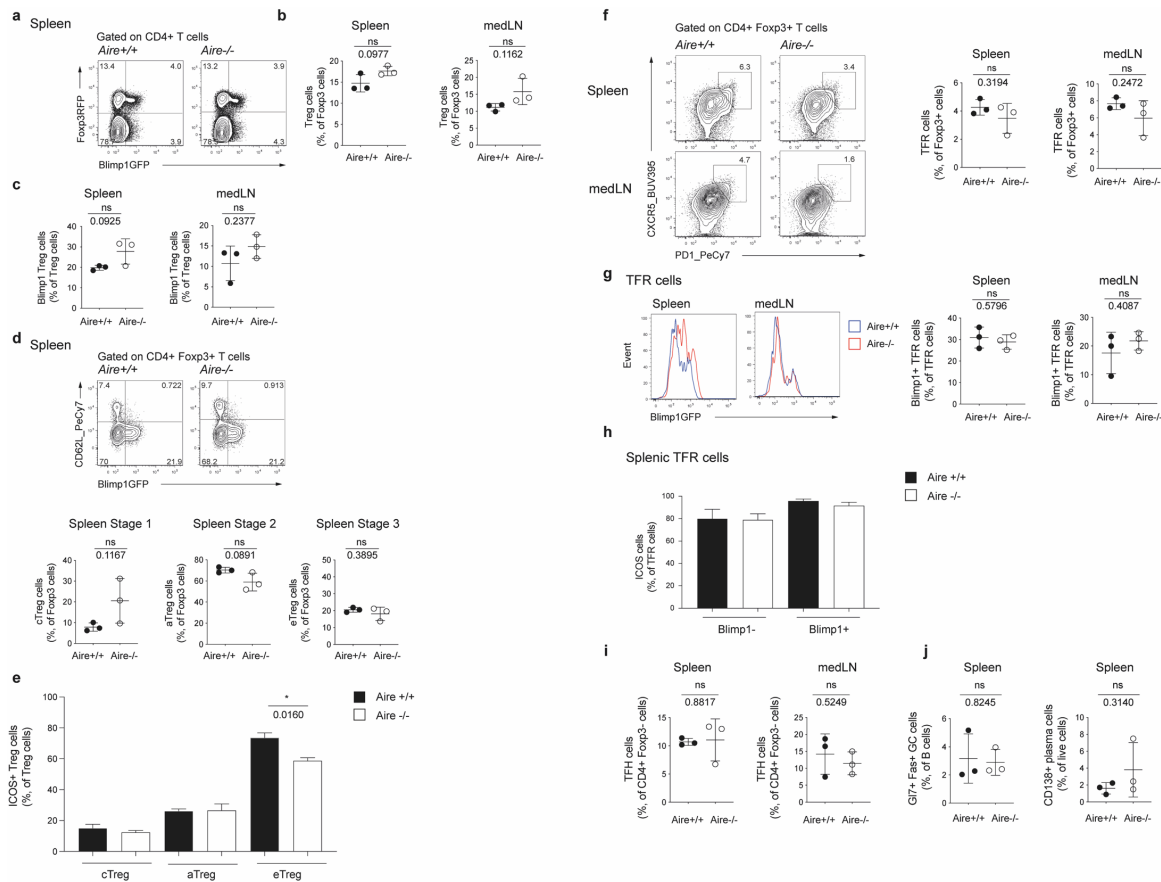


Figure 13. Aire-deficiency does not affect T_{FR} cell differentiation.

(a) Flow cytometric plots showing *Blimp1*^{GFP} reporter expression by splenic Treg cells in *Aire*^{-/-} *Foxp3*^{RFP} *Blimp1*^{GFP/+} (*Aire*^{-/-}) and wild type control mice 9 days after influenza infection. (b) Graph showing Treg cell frequencies in the spleen and medLN of mice from (a). (c) Graph showing the frequencies of *Blimp1*^{GFP} reporter-expressing Treg cells from the spleen and medLN of mice from (a). (d) Expression of CD62L and *Blimp1*^{GFP} reporter by Treg cells from the spleen. Below, quantification. (e) Graph comparing the frequency of ICOS expression by Treg cells based on the 3-stage model of activation from *Aire*^{-/-} mice and controls. (f) Flow cytometric plots (left) and quantification (right) of splenic and medLN T_{FR} cells from *Aire*^{-/-} and control mice 9 days after influenza infection. (g) Representative histogram (left) and quantification (right) showing the expression of *Blimp1*^{GFP} reporter by T_{FR} cells from the spleen and medLN of *Aire*^{-/-} and control mice. (h) Graph showing the expression of ICOS and *Blimp1*^{GFP} reporter by T_{FR} cells from the spleen of mice from (a). (i) Graphs showing the frequencies of T_{FH} cells in the spleen and medLN of mice from (a). (j) Graphs showing the frequencies of Gl7⁺Fas⁺CD19⁺ GC B cells and CD138⁺ plasma cells from the spleen of mice as in (a).

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using (b-d, f-g, i-j) unpaired Student's t-test and (e, h) ANOVA (*p<0.05, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of 2 independent experiments with 3 mice per group. aTreg, activated Treg; cTreg, central Treg; eTreg, effector Treg; GC, germinal centre; medLN, mediastinal lymph node; ns, not significant; T_{FH}, T follicular helper; T_{FR}, follicular Treg.

3.3 The expression of IRF4 in Treg cells

I) Generation and validation of a novel *Irf4* reporter mouse.

To better understand the sequence of eTreg cell differentiation in the context of the ontogeny observed above, we aimed to resolve the expression of IRF4 by Treg cells at different stages after birth. IRF4, a TCR-driven transcription factor, is required for T cell effector function and is critical for eTreg cell development and function (Cretney, Xin et al. 2011, Huber and Lohoff 2014). However, tools for assessing its expression on a single-cell basis are limited. Divergence between the levels of *Irf4* transcript and protein (unpublished observations, Kallies lab) represent a further level of complication when assessing IRF4 expression. To overcome these challenges, we generated a novel reporter mouse to allow direct assessment of both *Irf4* mRNA and protein in cells of interest. To this end, a tandem tomato (tdTomato) cassette was inserted immediately downstream of the last exon of *Irf4* to produce an IRF4-tdTomato fusion protein that reports IRF4 protein levels. Downstream of the tdTomato cassette we included another copy of the final *Irf4* exon, followed by an IRES and an enhanced (e)GFP cassette to report levels of transcription. Flanking of each reporter cassette with specific recombinase recognition sequences allows the initial construct, once successfully inserted to the genome, to be recombined to produce single IRF4-tdTomato protein or *Irf4*-eGFP mRNA reporter mouse lines (**Fig. 14a**). These novel reporter lines represent a powerful tool for visualization of IRF4 expression in any cell of interest (Man et al. Immunity 2017, and Kallies et al. unpublished). For the purpose of this thesis, I have utilized the single IRF4 reporter strains either as *Irf4^{tdTom/+}* or *Irf4^{GFP}*. Of note, the tdTomato reporter expression is preserved after intracellular fixation, which was compatible with the identification of Foxp3⁺ Treg cells.

In order to validate these new tools, we assessed whether the patterns of *Irf4* reporter expression were consistent with those previously described by Western blot (Man, Miasari et al. 2013). To this end, we infected *Irf4^{tdTom/+}* reporter mice with influenza virus and assessed *Irf4^{tdTom}* reporter expression by lymphoid lineage cells nine days later. Consistent with published observations (Ochiai, Maienschein-Cline et al. 2013, Willis, Good-Jacobson et al. 2014), plasma cells expressed the highest levels of *Irf4^{tdTom}* reporter (**Fig. 14b**), while CD4⁺ T cells displayed varying levels of reporter expression. Gating on the CD4⁺ population, we identified that Treg cells constitutively expressed high levels of *Irf4* reporter relative to

Foxp3⁻ conventional CD4 T cells (**Fig. 14c**). Within the Treg cell pool, we further observed antigen-experience (CD62L^{low}) cells to express higher levels of *Irf4* reporter than cTreg cells (**Fig. 14d**). Finally, we used western blotting to detect the TCR-dependent induction of IRF4 expression by wild type and *Irf4*^{tdTom/+} CD4 T cells. IRF4 was absent from unstimulated cells, while reporter CD4 T cells displayed appropriate and similar induction of both endogenous and fusion IRF4 protein bands attributed to the use of the hemizygous reporter mouse (**Fig. 14e**). Notably, the tdTomato fusion protein was more abundant compared to the endogenous IRF4 protein suggesting that the fusion protein has a longer half-life. These data validate this new mouse strain as a sensitive reporter of IRF4 expression. To understand when and where during Treg cell ontogeny IRF4 is induced, we generated double reporter mice by crossing the *Irf4*^{GFP} reporter mice with mice carrying the *Foxp3*^{RFP} allele. These double reporter mice enabled the identification of Foxp3⁺ Treg cells within lymphoid and non-lymphoid organs described in the following section.

II) High *Irf4* transcription in thymic Treg cells.

To determine the expression of *Irf4*^{GFP} reporter in dual *Irf4*^{GFP}*Foxp3*^{RFP} reporter mice, we first took the thymi from the neonate (day 4-old), young (3-week-old) and adult mice and analysed the T cell compartments by flow cytometry (gating strategy see **Fig. 15a**). The expression of *Irf4*^{GFP} reporter by Treg cells is detected as early as day 4 after birth and increases as mice age (**Fig. 15b**). In the neonates, we observed Foxp3⁺ CD4 thymocytes to express the highest levels of *Irf4*^{GFP} reporter expression when compared with CD4⁻CD8⁻, CD4⁺ CD8⁺, CD8 or CD4 single positive thymocytes (**Fig. 15c**). This expression pattern was also observed in the thymi of adult mice (**Fig. 15d**). Next, we wanted to determine whether the global expression of *Irf4*^{GFP} mRNA in the thymus was different in neonates compared with adult mice. To this end, we gated all *Irf4*^{GFP} reporter positive thymocytes in both adult and neonates (**Fig. 15e**). The majority of the *Irf4*^{GFP} reporter positive thymocytes in both the neonates and adult mice were CD4 single positive thymocytes (**Fig. 15e**). When compared to *Irf4*^{GFP} reporter negative CD4 single positive thymocytes, *Irf4*^{GFP} reporter positive CD4 single positive thymocytes showed higher expression of TCRβ regardless of age (**Fig. 15f**), in line with a previous publication that IRF4 expression is induced by TCR (Man, Miasari et al. 2013). We observed that in both the neonates and adult mice, a proportion of CD4 single positive thymocytes that did not express Foxp3 also expressed *Irf4*^{GFP} reporter (**Fig. 15g**).

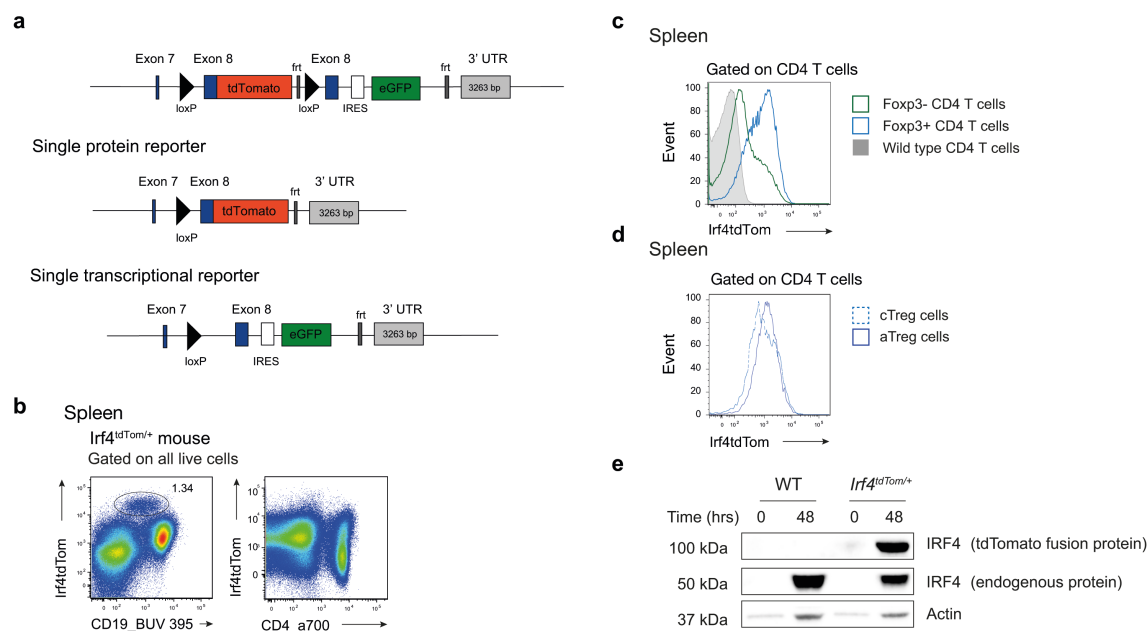


Figure 14. Generation and validation of novel *Irf4* reporter mice.

(a) Schematic representation of the *Irf4^{tdTom/GFP}* dual reporter transgenic mouse construct. A tandem tomato (tdTomato) cassette flanked by two loxP sites was inserted downstream of exon 8 of *Irf4*. Another copy of the final exon of *Irf4* and an IRES-eGFR gene cassette was inserted downstream of exon 8. Removal of the flp flanked eGFP results in a single protein reporter, whilst removal of the loxp flanked exon 8 and tdTomato reporter restores exon 8 resulting in a single transcriptional reporter. (b) Representative flow cytometric plot of *Irf4^{tdTom}* reporter expression by CD4 and CD19 cells in the spleen of *Irf4^{tdTom/+}* reporter mice infected with influenza virus at day 9. (c) Representative histogram showing the expression of *Irf4^{tdTom}* reporter in conventional and regulatory CD4 T cells. Shaded area represents *Irf4^{tdTom}* reporter expression by wild type conventional CD4 T cells from non-reporter mice. (d) Representative histogram showing the expression of *Irf4^{tdTom}* reporter by cTreg and eTreg cells. (e) Western blot of purified naïve CD4 T cells from wild type and *Irf4^{tdTom/+}* reporter mice showing *Irf4^{tdTom}* reporter expression before and after *in vitro* activation with plate bound α CD3, soluble α CD28 and IL-2 at day 2.

Number adjacent to gated areas indicate percent cells. Flow cytometric plots and data in (b–d) are representative of two independent experiments with three mice per group. Data in e is representative of two independent experiments. cTreg, central Treg; eTreg, effector Treg; eGFP, enhanced green fluorescence protein; IRES, internal ribosome entry site.

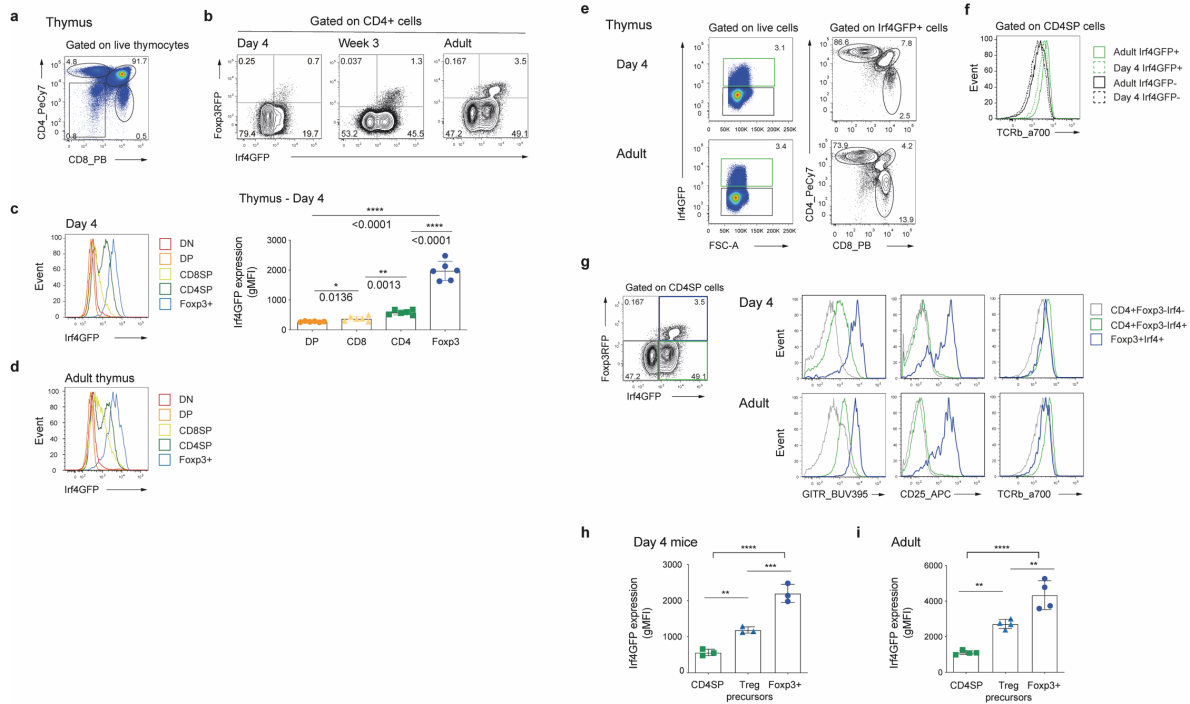


Figure 15. Thymic Treg cells express high levels of *Ir4*.

(a-b) Flow cytometric plots of showing the gating strategy of CD4 T cells from thymi of *Ir4*^{GFP/+} *Fxp3*^{RFP} mice (a) and *Ir4*^{GFP} reporter expression at week 1 and 3 after birth and in adult mice (b). (c) Representative histogram (left) and quantification (right) showing the expression of *Ir4*^{GFP} reporter by DN, DP, CD8SP, CD4SP and Fxp3+ thymic T cells in day 4 old mice. (d) Representative histogram showing *Ir4*^{GFP} reporter expression by thymic T cell subsets from adult mice. (e) Representative flow cytometric plots showing the expression of *Ir4*^{GFP} reporter by all live cells and the gating strategy of *Ir4*^{GFP} reporter positive thymic T cell subsets from day-4-old and adult mice. Representative histogram on the right showing the TCRβ expression by CD4SP thymocytes from day-4-old and adult mice based on the expression of *Ir4*^{GFP} reporter. (g) Flow cytometric plot and representative histograms showing the expression of GITR, CD25 and TCRβ by *Ir4*^{GFP} reporter negative CD4SP T cells, *Ir4*^{GFP} reporter positive CD4SP and regulatory T cells. (h-i) Bar graphs showing *Ir4*^{GFP} reporter expression by CD4SP, Treg cell precursors (gated on CD4+Fxp3-GITR+CD25+ cells) and Treg cells from day-4-old (h) and adult (i) mice.

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Flow cytometric plots and data are representative of 3 – 8 mice analysed per time point. CD4SP, CD4 single positive; CD8SP, CD8 single positive; DN, double negative; DP, double positive; gMFI, geometric mean fluorescence index.

This population is enriched for cells expressing intermediate levels of the glucocorticoid-induced tumour necrosis factor receptor (GITR) but not CD25 when compared with $\text{Foxp3}^+\text{Irf4}^{\text{GFP}+}$ thymocytes (**Fig. 15g**). TCR β expression by these $\text{CD4}^+\text{Foxp3}^-\text{Irf4}^{\text{GFP}+}$ thymocytes was higher when compared to $\text{CD4}^+\text{Foxp3}^-\text{Irf4}^{\text{GFP}-}$ and $\text{Foxp3}^+\text{Irf4}^{\text{GFP}+}$ thymocytes, indicating that these could be Treg cell precursors. Next, we analysed the kinetics of Irf4^{GFP} reporter expression by thymic T cells of neonates and adult mice by flow cytometry. In the CD4^+ single positive Foxp3^- compartment, Irf4^{GFP} reporter was expressed at low levels compared with thymic Treg precursors and thymic Treg cells (gated on $\text{CD4}^+\text{Foxp3}^-\text{GITR}^+\text{CD25}^+$) irrespective of the age of the mouse (**Fig. 15h**). Overall the expression Irf4^{GFP} reporter by thymic T cell subsets was higher in adult mice compared with neonates (**Fig. 15h-i**). Taken together, our data demonstrate that Irf4^{GFP} reporter expression by thymic Treg cells and its precursors increased with age, and as such, implicates the requirement for *Irf4* in the different stages of Treg cell generation.

III) *Irf4* transcription in splenic Treg cells.

Finally, we analysed the splenic T cell compartment from the same $\text{Irf4}^{\text{GFP}}\text{Foxp3}^{\text{RFP}}$ reporter mice and observed that Irf4^{GFP} reporter expression is also detected early after birth. Similar to our observations in the thymi, the majority of Foxp3^+ -expressing CD4^+ T cells from the spleen of neonates, young and adult mice expressed Irf4^{GFP} reporter (**Fig. 16a**). Foxp3^+ Treg cells expressed the highest levels of Irf4^{GFP} reporter, when compared to CD4^+ and CD8^+ T cells in both the neonates and adult mice (**Fig. 16b**). In the absence of $\text{Blimp1}^{\text{GFP}}$ reporter, we were limited in our ability to define eTreg cells. As such, we distinguished activated splenic Treg cells based on CD62L expression. Here, we observed highest levels of Irf4^{GFP} reporter expression by Treg cells that were antigen-experienced (**Fig. 16c**). Our data demonstrate that splenic T cells from neonates, young and adult mice express distinct patterns of Irf4^{GFP} , with antigen-experienced Treg cells expressing the highest levels, suggesting that IRF4, a downstream target of TCR stimulation, is required in the periphery to promote Treg cell activation.

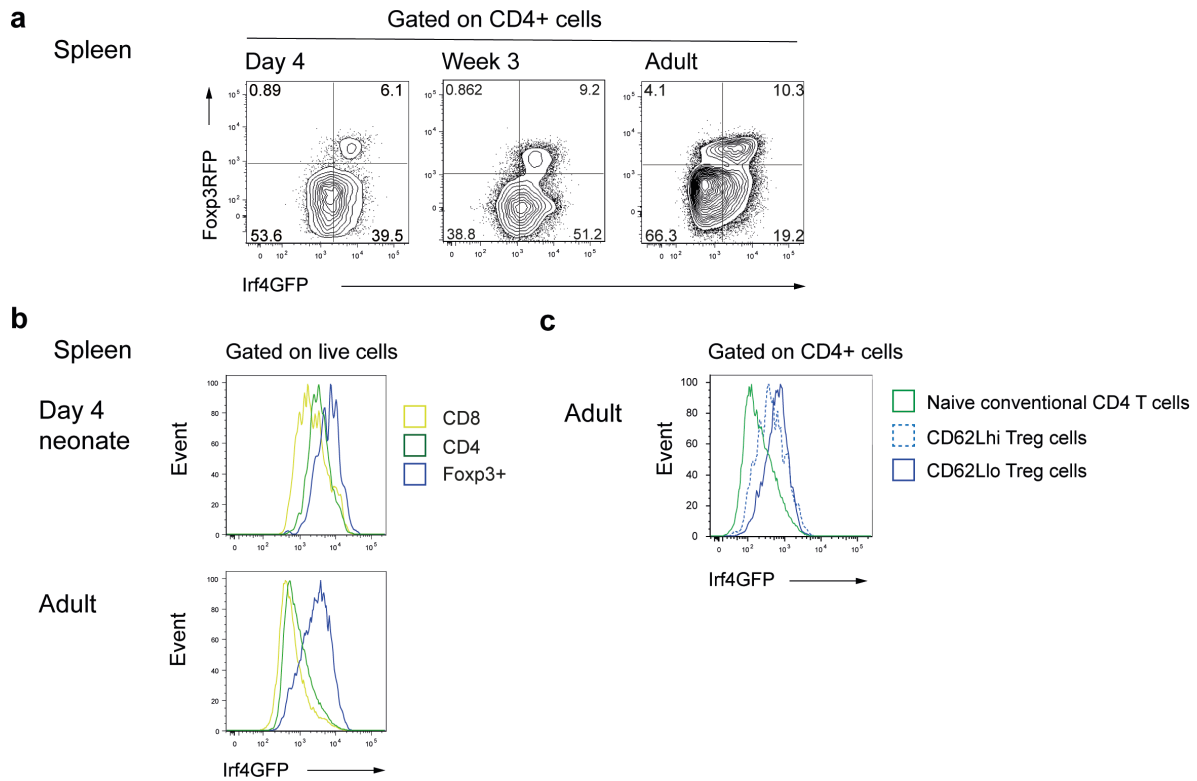


Figure 16. *Irf4* transcription in splenic Treg cells.

(a) Flow cytometric plots of CD4 T cells from the spleen of mice at day 4 and week 3 after birth and adult *Irf4^{GFP/+}Foxp3^{RFP}* dual reporter mice. (b) Representative histogram showing *Irf4^{GFP}* reporter expression by CD8, CD4 and Foxp3-expressing Treg cells from the spleen of day-4-old and adult mice. (c) Representative histogram showing *Irf4^{GFP}* reporter expression by naïve conventional CD4 T, cTreg and eTreg cells. Numbers adjacent to gated areas indicate percent cells. Flow cytometric plots and data are representative of 3 – 8 mice analysed per time point. cTreg, central Treg; eTreg, effector Treg.

Discussion

In the first results chapter of this thesis, we mapped the ontogeny of eTreg cells from thymic development to tissue population using the *Foxp3^{RFP}Blimp1^{GFP/+}* double reporter mice at various ages. We documented a way to distinguish the activation status of Treg cells based on their expression of CD62L and *Blimp1^{GFP/+}*, and identified a 3-stage model. Stage 1 Treg cells or cTreg cells have a naïve phenotype and express high levels of CD62L. Stage 2 Treg cells or aTreg cells are antigen-experienced and have downregulated CD62L. Stage 3 Treg or eTreg cells are characterized by the expression of *Blimp-1*, and are the most suppressive of all Treg cells. This staging model enabled the in-depth characterisation of Treg cells that seed non-lymphoid organs. Moreover, we demonstrated that the transcription factor responsible for the promiscuous gene expression of tissue-restricted antigen, *Aire* is not specifically required for the generation of eTreg cells that populate the spleen or liver in the adult mice; though *Aire* did appear to influence the frequencies of eTreg cells in the perinatal period within the SI Treg cell compartment. Additionally, we found the expression of the TCR-induced transcription factor, IRF4, to be highest in neonatal and adult Treg cells implicating a role for IRF4 in Treg cell development in the thymus and the periphery.

Ontogeny of effector regulatory T cell

Neonatal Treg cells play critical roles in maintaining tolerance to self-antigens as well as microbial antigens, as exemplified by the development of autoimmune disease in thymectomised neonates (Sakaguchi 2004). Treg cell development occurs in the thymus and critically depends on signals received through the TCR. We observed two waves of Treg cell proliferation in the thymus of developing mice, suggesting a stepwise manner of thymic Treg cell generation. During the second week after birth, we observed the presence of eTreg cells, marked by *Blimp1^{GFP}* expression in the thymus. This may reflect an early ‘wave’ of commitment to the eTreg cell program with subsequent export to lymphoid and non-lymphoid organs. As an alternative explanation, this *Blimp1^{GFP}* Treg cell population may contain a pool of recirculating Treg cells required to control the selection of thymic T cells. Similarly, the second peak of thymic eTreg cells seen at week 4 after birth could also be enriched for a population of recirculating eTreg cells required to regulate the pool of developing Treg cells (Thiault, Darrigues et al. 2015).

During the first few weeks after birth, we observed distinct patterns of Treg cell activation and tissue population of Treg cells in the spleen, liver and lamina propria of SI. Similar to what we saw in the thymus, Treg cells, including those that express *Blimp1*^{GFP}, were readily detected in the spleen, liver and SI of mice in the first week (days 3 – 7) after birth. Whilst there were similar frequencies of splenic and hepatic Treg cells at week 2 and 3 after birth, there was a significant reduction in splenic Treg cells at week 4. Notably, this reduction coincided with an abrupt accumulation of Treg cells within the SI. This observation can be attributed to the sizeable requirement of Treg cells to fill the SI niche at a time of exposure to novel food antigens, which reflects the plasticity of the immune system in its response to increased demands. This is reminiscent of the paradigm described by Dunon where thymocytes with distinct TCR usage during T cell development colonize non-lymphoid organs in discrete waves (Dunon, Courtois et al. 1997).

The acquisition of the eTreg cell phenotype and Treg cell migration to non-lymphoid organs, in particular the SI, was of interest to us. Over the last decade, there has been extensive research on the influence of commensal and pathogenic microbiota on the host immune system (reviewed in (Belkaid and Hand 2014)). Treg cells are essential to regulate the inflammatory responses of CD4⁺ effector T cells in the SI to microbiota (Pandiyana, Zheng et al. 2007, Cebula, Seweryn et al. 2013). Fascinatingly, consistent with a previous publication (Kim, Hong et al. 2016), we found a notable increase in Treg cells in the lamina propria of the SI at week 4 after birth, in line with exposure to dietary antigens from solid food. This phenomenon correlated with the dip of Treg cells in both the thymus and spleen, supporting Treg cell egress to the SI. A significant proportion of these Treg cells expressed *Blimp1*^{GFP}, characteristic of Treg cells with an effector phenotype (Cretney, Xin et al. 2011). Even when compared with age-matched splenic and liver Treg cells, the adult SI Treg compartment expressed the highest frequency of *Blimp1*^{GFP} Treg cells, likely reflecting the substantial antigenic load within the SI. These SI *Blimp1*^{GFP} Treg cells are likely to be important for the secretion of the immunosuppressor cytokine, IL-10, essential for the maintenance of immune tolerance at mucosal sites (Rubtsov, Rasmussen et al. 2008).

Furthermore, the detection of cTreg cells within the liver and SI in the first 2 weeks after birth suggests that a subset of Treg cells leave the thymus as naïve Treg cells and populate tissues prior to co-opting transcription factors and signalling pathways characteristic of the target tissue. These observations are in line with our postulate that tissue-specific cTreg cells

undergo stimulus-specific differentiation, whilst the intermediate and terminally differentiated a- and eTreg cells clonally expand to allow for an appropriate range of Treg cell responses to exogenous antigens. Notably, SI Treg cells showed high levels of Nrpl expression, which in some studies (Weiss, Bilate et al. 2012, Yadav, Louvet et al. 2012) but not all (Bruder, Probst-Keppler et al. 2004) identify Treg cells of thymic origin. As the *Blimp1*^{GFP} reporter expression is quenched by intracellular fixation, we were unable to use gut Treg cell-specific transcription factors, such as GATA3 and ROR-γt, or Helios to distinguish peripheral (p)Treg cells, which are often found at mucosal sites (Rudensky 2011, Ohkura, Kitagawa et al. 2013).

Co-stimulation via ICOS has been demonstrated as necessary for the suppressive function of Treg cells (Herman, Freeman et al. 2004, Ito, Hanabuchi et al. 2008). We demonstrated that upregulation of ICOS expression on Treg cells paralleled the activation state of Treg cells, with eTreg cells expressing the highest frequency of ICOS in both lymphoid and non-lymphoid organs, conforming with the phenotype and function of eTreg cells. The Treg cell compartment from the SI of adult mice consisted predominantly of ICOS⁺ eTreg cells, likely essential for the role of eTreg cells in maintaining intestinal homeostasis (Landuyt, Klocke et al. 2019, Neumann, Blume et al. 2019). Our data demonstrated that the SI Treg cell compartment comprised of a combination of eTreg cells with their cell-fate pre-programmed in the thymus, eTreg cells that differentiate from cTreg cells in the periphery, and pTreg cells highlighting the contribution of both the instructive and stochastic models in Treg cell development and differentiation.

***Aire* limits tissue-specific inflammation**

It is well described that Aire is essential to promote immune tolerance through the induction of tissue-specific antigen expression by mTECs (Mathis and Benoist 2009, Metzger and Anderson 2011). The absence of Aire results in various pathological outcomes both in humans and mice, supporting its importance for the prevention of autoimmune disease (Anderson, Venanzi et al. 2002, Anderson, Venanzi et al. 2005, Kekalainen, Tuovinen et al. 2007, Malchow, Leventhal et al. 2013, Anderson and Su 2016). The observation of a lower Treg cell frequency in 1- and 2-week old mice in *Aire*-deficient mice suggests that the generation of a proportion of thymic Treg cells is *Aire*-dependent. Consistent with a previous publication (Anderson, Venanzi et al. 2002), we observed similar frequencies of Treg cells

in the thymi of adult *Aire*-deficient mice compared to controls. However, there was a higher frequency of *Blimp1*^{GFP} reporter-expressing Treg cells in the thymi these mice, which suggests an increased propensity for eTreg cell generation in *Aire*-deficient mice, perhaps in response to the presence of autoreactive T cells in the periphery. This is supported by the observation of an increased splenic eTreg cell population in the absence of *Aire*, which in turn may lead to thymic recirculation of Treg cells.

We also observed a significant increase of eTreg cells in the SI of 4-week old *Aire*-deficient mice, likely required to control effector CD4 T cell homeostasis. This finding is in keeping with the known role of *Aire* in limiting tissue-specific autoimmune manifestations (Liston, Gray et al. 2004, Mathis and Benoist 2009), in particular at a time of high antigenic exposure. Alternatively, this could also be explained by an increase in pTreg cell generation in the absence of sufficient thymic Treg cells to fill the niche. This observation is supported by prior studies that have demonstrated the variability of disease severity in *Aire*-deficiency that is also organ-specific, such as the pancreas, adrenal glands, prostate and retina (Anderson, Venanzi et al. 2002, Ramsey, Winqvist et al. 2002, Liston, Lesage et al. 2003, Malchow, Leventhal et al. 2013). Overall, our data demonstrates that there are subtle differences detected in organ-specific eTreg cell differentiation implicating the likely role of *Aire* in mediating the recirculation of antigen-experienced Treg cells to the thymus as an attempt to regain control of central tolerance mechanisms.

***Irf4* in early Treg cell development**

Treg cells undergo functional diversification in response to a variety of stimuli, which is driven by transcription factors other than Foxp3 (Luo and Li 2013). IRF4 is known to support eTreg cell differentiation (Cretney, Xin et al. 2011) and as such, the generation of a novel *Irf4*^{GFP} fluorescent reporter mouse enabled the detailed investigation of *Irf4* expression during Treg cell ontogeny and development. We found that *Irf4* is expressed by the majority of thymic T cells, from the single positive stage to Foxp3-expressing Treg cells early after birth. This expression pattern is consistent with the finding that IRF4 is induced in response to TCR engagement (Man, Miasari et al. 2013). We also observed thymic Treg cells to express the highest levels of *Irf4*^{GFP} reporter when compared to Foxp3 negative or thymic Treg cell precursors, which supports the role of high-affinity TCR signalling required for thymic Treg cell development (Jordan, Boesteanu et al. 2001). This pattern of *Irf4*^{GFP}

expression in response to TCR signalling is reminiscent of the expression pattern of *Nur77^{GFP}* in reporter mice that demonstrated increased TCR signal strengths perceived by Treg cells (Moran, Holzapfel et al. 2011).

Despite elevated expression levels of *Irf4^{GFP}* reporter in almost all Foxp3-expressing thymocytes, irrespective of age, notably not all mature Treg cells in the periphery of these mice have the eTreg cell phenotype. The detection of CD62L^{high} antigen-inexperience cells in the splenic Treg cell compartment suggests that the acquisition of an effector phenotype and functional specialization requires more than just TCR engagement. Unfortunately, the *Irf4^{GFP}* reporter mouse did not allow for direct assessment of TCR-ligand affinity, which may influence eTreg cell differentiation.

Neonatal splenic naive conventional CD4 T cells expressed similar levels of *Irf4^{GFP}* reporter compared with adult Treg cells, suggesting that high TCR signalling is necessary in early T cell differentiation compared to the tonic signalling experienced by mature T cells. Additionally, our data showed that splenic Treg cells expressed the highest level of *Irf4^{GFP}* reporter, in keeping with the finding that continuous TCR signalling is necessary both for the expression of key Treg cell signature genes and to maintain a functional Treg cell pool (Moran, Holzapfel et al. 2011, Levine, Arvey et al. 2014, Vahl, Drees et al. 2014).

Having identified that both *Blimp1* and *Irf4* are expressed early in eTreg cell ontogeny, we wanted to determine if these transcription factors were also required for the differentiation, function and maintenance of a specific subset of eTreg cells. In the following chapter, we utilize T_{FR} cells to further understand the molecular determinants and transcriptional network required to shape the eTreg cell program specifically during the germinal centre response.

Chapter Four: T Follicular Regulatory cell differentiation is dependent on the Transcription Factor IRF4

Introduction

Infection and immunization generate the formation of highly specialized structures within secondary lymphoid organs called germinal centres. The germinal centre is a distinct and organized but transient structure found within B cell follicles (Stebegg, Kumar et al. 2018). It is in this structure where B cells undergo class switching and affinity maturation to generate long lasting antibody responses (Mesin, Ersching et al. 2016). CD4 T cell help is provided by a subset of CD4⁺ T cells called follicular T helper (T_{FH}). T_{FH} cell help is critical not only for the selection of high affinity clones during the germinal centre reaction but also for the formation and maintenance of the germinal centre (Victora, Schwickert et al. 2010, Crotty 2014). T_{FH} cells interact with cognate B cells and supply signals by way of costimulatory molecules such as CD40L and cytokines, such as interleukin (IL)-4 and IL-21 to promote B cell responses (reviewed in Crotty 2014). This interaction is essential for the provision of survival and differentiation signals to germinal centre B cells (Deenick, Ma et al. 2011). Overproduction of T_{FH} cells is associated with severe autoimmune disease (Vinuesa, Cook et al. 2005, Linterman, Rigby et al. 2009), and as such, the regulation of T_{FH} cell generation and function is fundamentally required to limit autoimmunity.

Follicular T helper (T_{FH}) cell generation

T_{FH} cells constitute a subset of effector CD4 T cells that differentiate in response to transcriptional cues that are distinct to that of helper T (Th)1, Th2 and Th17 cells (reviewed by Yu, Batten et al. 2009). Initially termed the follicular B helper T cells, for its role in helping B cells differentiate, T_{FH} cell differentiation involves numerous signals including the induction of various transcription factors via T cell receptor (TCR) signalling, co-stimulation, migration signals and cytokines (reviewed in (Vinuesa, Linterman et al. 2016). Early T_{FH} cell generation is dependent on priming by dendritic cells (DC) located in T cell zones (Deenick, Chan et al. 2010). DCs produce cytokines such as IL-6, IL-12 and IL-27 that are important for the instruction of CD4⁺ T cells to acquire the T_{FH} cell phenotype (Goenka, Barnett et al. 2011). Ongoing T_{FH} cell differentiation also relies on T-B cell interactions and thus T_{FH} cells preferentially localize to the germinal centre of secondary lymphoid organs (Nutt and

Tarlinton 2011). These interactions result in the upregulation of the transcription factor, B cell lymphoma (Bcl)6 (Baumjohann, Okada et al. 2011). Furthermore, the expression of SLAM-associated protein (SAP – encoded by *Sh2d1a*) is critical to establish stable T-B cell interactions and thus critical for the development of T_{FH} cells (Qi, Cannons et al. 2008, Linterman, Rigby et al. 2009, Cannons, Wu et al. 2010, Deenick, Chan et al. 2010).

Phenotypic characteristics of follicular T helper cells

T_{FH} cells express key chemokine receptors to facilitate their migration towards the B cell follicles and can be characterized by the co-expression of the CXC chemokine receptor 5 (CXCR5), inducible costimulator (ICOS) and programmed death 1, PD-1 (encoded by *Pdcd1*) (Schaerli, Willimann et al. 2000, Akiba, Takeda et al. 2005, Fazilleau, Mark et al. 2009). The B lymphocyte chemoattractant CX chemokine ligand (CXCL)13 is highly expressed in the follicles of the spleen, lymph nodes and Peyer's patches, for which CXCR5 is the corresponding chemokine receptor (Ansel, Ngo et al. 2000). The gradient of CXCL13 directs follicular T cells into the germinal centre (Xu, Li et al. 2013). As such, T_{FH} cells express high levels of CXCR5 to facilitate their migration from the T cell zone to the B cell follicles (Breitfeld, Ohl et al. 2000). In T_{FH} cells, the helix-loop-helix factor achaete-scute homolog 2 (*Ascl2*) binds to E-box proteins in the *Cxcr5* promoter to promote its expression (Liu, Chen et al. 2014). The expression of ICOS by T_{FH} cells acts both as a costimulatory molecule and a migration receptor (Xu, Li et al. 2013, Pedros, Zhang et al. 2016). The downregulation of certain molecules such as the lymphoid chemokine receptor, CCR7 (Haynes, Allen et al. 2007) and the trafficking receptor, S1PR1 (sphingosine-1-phosphate receptor-1) is also essential for T_{FH} cell migration towards the interfollicular zone and T-B border (Matloubian, Lo et al. 2004). Mechanistically, this is controlled by direct binding of KLF2 (Krüppel-like Factor 2), which also induces the expression CD62L (encoded by *Sell*) (Carlson, Endrizzi et al. 2006, Lee, Skon et al. 2015). ICOS co-stimulation was also found to regulate Klf2 expression to maintain the T_{FH} cell phenotype (Weber, Fuhrmann et al. 2015).

Cytokines essential for the generation of follicular T helper cells

In addition to cell surface ligand-receptor interactions, cytokine signalling is essential for T_{FH} cell proliferation, differentiation and survival (Crotty 2015). Various cytokines contribute to

the germinal centre milieu, in particular those that induce the signal transducer and activator of transcription (STAT)3 pathway, IL-6 and IL-21 (Eddahri, Denanglaire et al. 2009, Wan, Andraski et al. 2015). Unlike other Th cell subsets, T_{FH} cell differentiation is negatively regulated by IL-2 (Ballesteros-Tato, Leon et al. 2012), as IL-2 induces the expression of Blimp-1 and activity of STAT5 (Johnston, Choi et al. 2012). Though, in a more recent publication, IL-2 production was shown to be an early marker for T_{FH} cell commitment (DiToro, Winstead et al. 2018). IL-6, produced by monocytes and B cells, is an important cytokine in the germinal centre environment (Karnowski, Chevrier et al. 2012, Chakarov and Fazilleau 2014). IL-6 has not only been shown to transiently upregulate Bcl6 expression in activated CD4 T cells (Nurieva, Chung et al. 2009), but it also enhances the expression of the pleiotropic cytokine, IL-21. IL-21, produced by a variety of immune cells including T_{FH} cells (Nurieva, Chung et al. 2008, Vogelzang, McGuire et al. 2008) is essential not only for the proliferation of germinal centre B cells but also the development of extra-follicular antibody producing cells (Ozaki, Spolski et al. 2002, Linterman, Beaton et al. 2010, Zotos, Coquet et al. 2010, Eto, Lao et al. 2011). Moreover, the induction of long-lived plasma cells relies on IL-21, which is required to induce Blimp-1 expression in early plasma cells (Krautler, Suan et al. 2017, Ise, Fujii et al. 2018). Interestingly, despite the requirement of IL-21 for efficient antibody responses, only a fraction of T_{FH} cells express IL-21 (Luthje, Kallies et al. 2012). Several other cytokines such as IL-4 and IL-10 are also produced by T_{FH} cells, which stimulate the production of antibodies by B cells (Reinhardt, Liang et al. 2009, Yusuf, Kageyama et al. 2010, Weinstein, Herman et al. 2016).

Transcription factors required for follicular T helper cell generation

Bcl6 is the master transcription factor responsible for the development, maintenance and function of cells involved in the germinal centre reaction (De Silva and Klein 2015). Bcl6 is highly expressed by germinal centre B cells and regulates their survival, proliferation and somatic hypermutation (Dent, Shaffer et al. 1997, Basso and Dalla-Favera 2010, Basso and Dalla-Favera 2012). Its expression is induced by IL-6 and TCF-1 (Nurieva, Chung et al. 2009, Choi, Gullicksrud et al. 2015). In T cells, activation of STAT3 promotes the upregulation of Bcl6 and a commitment towards a T_{FH} cell differentiation pathway (Johnston, Poholek et al. 2009, Nurieva, Chung et al. 2009, Choi, Yang et al. 2013). Specifically, Bcl6 represses the expression of T-bet (encoded by *Tbx21*) and ROR- γ t (encoded by *Rorc*) to limit

induction of the Th1 and Th17 transcriptional programs (Yu, Rao et al. 2009). Bcl6 expression results in the upregulation of many molecules that define the core characteristics of T_{FH} cells such as *Cxcr5*, *Pdcd1*, *Icos* and *Sh2d1a* (Bauquet, Jin et al. 2009, Choi, Kageyama et al. 2011, Kroenke, Eto et al. 2012). Following immunization, mice that had a specific deletion of Bcl6 in T cells, *Bcl6^{fl/fl}CD4^{Cre}* lacked not only T_{FH} cells but also showed a complete absence of germinal centre B cells confirming the essential role of Bcl6 in germinal centre development and maintenance (Hollister, Kusam et al. 2013). Bcl6 has also been shown to regulate IL-21 expression in T_{FH} cells (Linterman, Beaton et al. 2010).

In addition to Bcl6, other T cell activation transcription factors such as Blimp-1, E proteins, IRF4, STATs, and Maf are required to drive the T_{FH} cell program (Lohoff, Mittrucker et al. 2002, Bauquet, Jin et al. 2009, Bollig, Brustle et al. 2012). In B cells, IRF4 has been shown to repress Bcl6 expression (Saito, Gao et al. 2007). IRF4 and STAT functionally cooperate to promote the development of effector CD4 T cells (Kwon, Thierry-Mieg et al. 2009, Bollig, Brustle et al. 2012). Furthermore, concomitant downregulation of the transcriptional repressor Blimp-1 is required for the commitment to T_{FH} cell differentiation (Johnston, Poholek et al. 2009). The induction of Blimp-1 via KLF2 can regulate T_{FH} cell differentiation (Lee, Skon et al. 2015). E protein activity drives the expression of CXCR5, whose activity is inhibited by Id2, which in turn is repressed by Bcl6 (Shaw, Belanger et al. 2016). The generation of T_{FH} cells is a multistep process governed by various cytokines and transcription factors.

Discovery of T Follicular Regulatory cells

Initial studies on Foxp3⁺ Treg cells have shown that in the absence of a functional Treg cell compartment, mice had dysregulated germinal centre responses and as such, production of pathogenic auto-antibodies (Lin, Truong et al. 2005, Sakaguchi, Yamaguchi et al. 2008). Subsequently, in 2011, 3 seminal papers described a unique population of Treg cells that express characteristic T_{FH} cell markers that were identified in the germinal centre of secondary lymphoid organs, termed T follicular regulatory (T_{FR}) cells (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011, Wollenberg, Agua-Doce et al. 2011). Accumulating evidence suggests that T_{FR} cells are a highly important Treg cell subset essential for the regulation of T_{FH} cells and antibody secretion through direct and indirect ways of limiting

germinal centre responses (Sage, Ron-Harel et al. 2016, Fu, Liu et al. 2018, Clement, Daccache et al. 2019, Wang, Shen et al. 2019).

Key T_{FR} cell characteristics

T_{FR} cells are a subset of thymic-derived Treg cells that express the Treg cell lineage-defining transcription factor, Foxp3 and have an effector function (Linterman, Pierson et al. 2011). T_{FR} cells are generally detectable at low levels in uninfected mice, but rapidly expand after the germinal centre reaction has developed. Phenotypically, T_{FR} cells express similar cell surface markers to the cells that they suppress, the T_{FH} cells. The balance of both cell types allows for dynamic regulation of the germinal centre, essential for normal immune function and also to prevent autoimmune disease. *In vivo* studies have shown that T_{FR} cells constrain the help provided by T_{FH} cells to B cells to limit germinal centre reactions (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011, Wollenberg, Agua-Doce et al. 2011, Sage, Paterson et al. 2014). T_{FR} cells also regulate the production of effector cytokines by T_{FH} cells (Sage, Alvarez et al. 2014). Dysregulated germinal centre reactions through excessive cytokine secretion by T_{FH} cells in the absence of T_{FR} cells resulted in an abnormal antibody response (reviewed in Sage and Sharpe 2016). More recently, T_{FR} cell have been shown to limit the quantity of antigen-specific antibody production and promote affinity during the memory response (Clement, Daccache et al. 2019). Interestingly, T_{FR} cells can also regulate the B cell metabolic responses through the inhibition of glycolysis (Sage, Ron-Harel et al. 2016).

Like T_{FH} cells, T_{FR} cells are characterized based on the surface expression of CXCR5, PD-1, and ICOS (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011, Wollenberg, Agua-Doce et al. 2011). T_{FR} cells express high levels of CXCR5 required for their location, differentiation and maintenance within the germinal centre. Unlike T_{FH} cells, the upregulation of CXCR5 expression in T_{FR} cells is not regulated by Ascl2, but instead, nuclear factor of activated T cells (NFAT)2 (encoded by *Nfatc1*) (Vaeth, Muller et al. 2014). In addition, T_{FR} cells express canonical Treg cell activation-associated markers such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), ICOS, neuropilin-1 (Nrp1), killer-cell lectin like receptor G1 (KLRG1), and glucocorticoid-induced TNFR family related protein (GITR encoded by *Tnfrsf18*) (Linterman, Pierson et al. 2011).

T_{FR} cell differentiation and function

Like T_{FH} cells, T_{FR} cells undergo a multistage differentiation process that is dependent on positive co-stimulation via CD28, ICOS and SAP (Linterman, Pierson et al. 2011, Sage, Francisco et al. 2013). The generation of T_{FR} cell also requires sustained interaction with antigen presenting cells (APCs), such as DC or B cells (Linterman, Pierson et al. 2011, Aloulou, Carr et al. 2016). Following immunization, mice deficient for *Cd28* have reduced frequencies of splenic, peripheral lymph node (LN) and blood T_{FR} cells (Yoshinaga, Whoriskey et al. 1999, Linterman, Pierson et al. 2011). ICOS, a potent co-receptor found on chromosome 1, is expressed on activated T cells (Hutloff, Dittrich et al. 1999). The expression of ICOS is not only essential for the function of Treg cells but promotes the generation and maintenance of T_{FR} cells (Herman, Freeman et al. 2004, Burmeister, Lischke et al. 2008, Busse, Krech et al. 2012, Sage and Sharpe 2015). In contrast, PD-1 expression is inhibitory towards both T_{FR} cell differentiation and function. Following immunization with antigen in CFA (Complete Freund's Adjuvant), mice deficient for PD-1 had increased frequencies of T_{FR} cells (Sage, Francisco et al. 2013).

The cytokine, IL-2 plays a central role in Treg cell biology. Whilst T_{FR} cells are functionally similar to eTreg cells, the development and maintenance of T_{FR} cells are inhibited by IL-2 (Botta, Fuller et al. 2017). Instead, the differentiation of T_{FR} cells depend on cytokines that are available in the germinal centre milieu, such as IL-6 and IL-21 (Jandl, Liu et al. 2017). In contrast to T_{FH} cells, T_{FR} cells do not express the CD40L or helper cytokines IL-4 or IL-21 (Linterman, Pierson et al. 2011). Similar to eTreg cells, T_{FR} cells have recently been shown to secrete IL-10, essential for the proliferation of B cells and its entry into the germinal centre (Laidlaw, Lu et al. 2017).

In the literature, T_{FR} cells have mainly been described to be suppressors of the germinal centre reaction through the regulation of T_{FH} cells and germinal centre B cells, thus limiting the generation of antibody secreting cells (Wing, Ise et al. 2014, Sage and Sharpe 2015, Sage and Sharpe 2016, Botta, Fuller et al. 2017, Fu, Liu et al. 2018, Clement, Daccache et al. 2019). As such, the tight control of germinal centre reactions after immunization or infection relies critically on interactions between T_{FH} and T_{FR} cells. Similar to Treg cells, multiple mechanisms have been proposed to account for the immunosuppressive properties of T_{FR} cells. Treg cells confer their suppressive activity via the expression of CTLA-4 (Wing, Ise et al. 2014). CTLA-4 or CD152 negatively regulates cellular immunity through the binding of

CD80 and CD86 present on APCs (Oderup, Cederbom et al. 2006, Qureshi, Zheng et al. 2011). Treg cell expression of CTLA-4 can directly control CD28 signalling in conventional CD4 T cells by restricting CD28 ligand availability (Rowshanravan, Halliday et al. 2018). Accordingly, T_{FR} cells express high levels of the inhibitory molecule, CTLA-4 (Linterman, Pierson et al. 2011, Sage, Paterson et al. 2014). As T_{FR} cells arise from Treg cells, it is not unreasonable to assume that T_{FR} cells induce their suppressive function in a similar manner to Treg cells, through cell-to-cell contact and the production of soluble factors including the cytokines TGF- β and IL-10 (Saraiva and O'Garra 2010, Rutz and Ouyang 2016). Though, in T_{FR} cells, IL-10 has also been shown to promote the production of plasma cells (Laidlaw, Lu et al. 2017). TGF- β signalling has been shown to potently inhibit the effector functions of activated T cells via the inhibition of various transcription factors that are fundamental for Th cell differentiation (reviewed in Gorelik and Flavell 2002). One previous study showed that TGF- β signalling plays an essential role in the regulation of T_{FH} cells during influenza virus infection (Marshall, Ray et al. 2015). In Treg cells, direct TGF- β signals are important for Treg cell function and homeostasis in the periphery (Li, Wan et al. 2006, Marie, Liggitt et al. 2006). Additionally, mouse models have shown that T_{FR} cells express high levels of TIGIT (T cell immunoreceptor with Ig and ITIM domains), which contribute to their suppressive function (Wu, Chen et al. 2016). More recently, T_{FR} cells have also been shown to suppress the production of granzyme B secreting T_{FH} cells (Xie, Fang et al. 2019).

Transcriptional regulation of T_{FR} cell differentiation

Transcription factors regulate gene expression and thereby control survival, proliferation, differentiation, and cell and location-specific function of immune cells. Mature naïve Treg cells can give rise to activated and effector Treg cells in the presence of specific cytokines and adopt the expression of various transcription factors in addition to Foxp3 in order to adapt to diverse tissue environments. For example, Th1 type Treg cells upregulate the transcription factor T-bet, a proportion of intestinal Treg cells upregulate the expression of ROR- γ t and visceral adipose tissue Treg cells express PPAR γ (Koch, Tucker-Heard et al. 2009, Cipolletta, Kolodin et al. 2011, Sefik, Geva-Zatorsky et al. 2015). Similarly, Treg cells that are found within the germinal centre upregulate the expression of Bcl6, essential for T_{FR} cell differentiation (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011). In Treg cells, Bcl6 has been shown to inhibit GATA3, thereby repressing Th2-type Treg cell responses

(Sawant, Sehra et al. 2012). In an adoptive transfer experiment, following immunization with T-dependent antigen, NP-KLH (4-hydroxy 3-nitrophenyl keyhole limpet hemocyanin), *Tcr β* ^{-/-} mice that received *Bcl6*^{-/-} CD25^{high}CD4⁺ T cells showed increased antibody production compared to those that received wild type Treg cells (Chung, Tanaka et al. 2011). Following immunization with NP-KLH, mice with a Treg cell specific deletion of Bcl6 showed reduced expression of T_{FR} cells and succumbed to autoimmune disease (Fu, Liu et al. 2018).

In T cells, the induction of Blimp-1 expression in activated CD4 and CD8 T cells is essential for the acquisition of an effector or memory phenotype (Martins and Calame 2008, Kallies, Xin et al. 2009). Blimp-1 drives the terminal effector differentiation of Treg cells (Cretney, Xin et al. 2011) yet the master transcription factor for T_{FH} and T_{FR} cells, Bcl6 and Blimp-1 reciprocally repress expression of the other (Johnston, Poholek et al. 2009, Xie, Koh et al. 2017). Unlike T_{FH} cells, T_{FR} cell differentiation is regulated by Blimp-1, which is independent of Bcl6 (Xie, Koh et al. 2017).

Recently, three groups had demonstrated that in mice with a specific loss of Blimp-1 in Treg cells, there was an expansion of T_{FR} cell frequencies (Yang, Yang et al. 2018, Wang, Shen et al. 2019, Xie, Fang et al. 2019). In these mice, Treg cells are present and capable of expressing activation-induced molecules, though the suppressive function of T_{FR} cells were reduced (Yang, Yang et al. 2018). Xie et al showed that whilst there was augmented T_{FR} cell development, these mice displayed a 3 fold increase in germinal centre B cell responses compared to wild-type mice (Xie, Koh et al. 2017). The study by Wang et al demonstrated elevated IgE levels and dysregulated antibody production in the absence of Blimp-1 (Wang, Shen et al. 2019).

In addition to Bcl6 and Blimp-1, IRF4 has been shown to positively regulate T_{FH} cell generation (Bollig, Brustle et al. 2012). The expression of IRF4 is crucial for the terminal differentiation of eTreg cells (Cretney, Xin et al. 2011) and cooperates with numerous transcription factors to regulate gene expression (Ciofani, Madar et al. 2012, Li, Spolski et al. 2012). However, the role of IRF4 in T_{FR} cell development has not been fully elucidated.

Other transcription factors have also been implicated in T_{FR} cell generation. In particular, the transcription factor, forkhead box protein O1 (Foxo1), which belongs to the family of forkhead box proteins, is essential at multiple stages of B and T cell development (Dengler, Baracho et al. 2008, Dejean, Hedrick et al. 2011, Hawse, Sheehan et al. 2015). Foxo1 is found in the nucleus of quiescent T cells and translocates into the cytoplasm when inactivated by

phosphorylation through the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway (Coffer and Burgering 2004, Calnan and Brunet 2008). Conversely, cellular stress and cytokine withdrawal results in nuclear relocalization of Foxo proteins (Fabre, Lang et al. 2005). Reduced expression of key CD4 T cell migration molecules such as *Ccr7*, *Sell*, *Klf2* and *Slpr1* in the absence of Foxo1 resulted in impaired T cell homing (Gubbels Bupp, Edwards et al. 2009, Kerdiles, Beisner et al. 2009). Emerging evidence has identified that Treg cells require the expression of Foxo transcription factors for their development and function (Friedline, Brown et al. 2009, Ouyang, Liao et al. 2012). Foxo1 deficiency in T cells resulted in dysfunctional Foxp3⁺ Treg cells (Kerdiles, Stone et al. 2010, Ouyang, Liao et al. 2012). Mice that had a T cell specific deletion of Foxo1 demonstrated autoimmunity with T cell organ infiltration and accumulation of a large population of T_{FH} cells (Kerdiles, Stone et al. 2010). This phenotype was exaggerated by the additional loss of Foxo3 (Kerdiles, Stone et al. 2010). Both FOXO1 and FOXO3 bind to the promoter regions of not only Foxp3 but of functional Treg cell molecules such as CTLA-4, regulating Treg cell development and function (Ouyang, Beckett et al. 2010, Ouyang, Liao et al. 2012). In addition, inactivation of Foxo1 through ICOS signalling promotes T_{FH} cell differentiation (Stone, Pepper et al. 2015). It is however not known if the differentiation of T_{FR} cells is regulated by Foxo1.

Whilst it is well known that the precise control of the germinal centre reaction is determined by the complex interactions between T_{FH} and T_{FR} cells (Vinuesa, Sanz et al. 2009, Crotty 2011), the exact mechanisms that control T_{FR} cell development and differentiation are still not fully understood. Until recently, the exact location of T_{FR} cells within the germinal centre had not been examined (Sayin, Radtke et al. 2018). In this chapter, we sought to further understand the role of transcription factors, in particular IRF4 and its targets, in T_{FR} cell differentiation and to unravel the transcriptional network of the eTreg cell of the germinal centre.

Results

4.1 Kinetics of the germinal centre response to influenza infection.

Influenza virus infection reproducibly and robustly induces cellular and humoral immunity at the site of infection and secondary lymphoid organs. As such, we performed a time course experiment to identify the optimal point to analyse follicular T cell and germinal centre populations in the spleen, and the draining lymph nodes of the lungs, the mediastinal lymph

nodes (medLN). Here, we used the HKx31 influenza virus to induce T_{FH} and sustained germinal centre responses within the spleen and medLN. We measured the frequencies of T_{FH} cells (identified as CD4+CD19-Foxp3-CXCR5^{high}PD-1^{high}) and T_{FR} cells (identified as CD4+CD19-Foxp3+CXCR5^{high}PD-1^{high}) (**Fig. 17a**) by flow cytometry. As previously described, T_{FR} cells also co-express ICOS on their cell surface (**Fig. 17a**) and are of an antigen-experienced phenotype, as evidenced by the downregulation of CD62L (**Fig. 17b**). Germinal centre responses based on the expression of Gl7 and Fas (in some experiments Bcl6) on CD19+ B cells can be detected as early as day 5 post infection, and peaked at day 9 and 14 in the spleen and medLN respectively (**Fig. 17c**). We measured plasma cell (PC) responses using the expression of CD138 on CD19 intermediate cells and showed that the peak of the response occurred at day 9 in the spleen after infection (**Fig. 17d**). In the medLN, PCs were detected at day 9 and persisted to day 14 after the infection (**Fig. 17d**).

At day 9 after infection, T_{FH} cells constitute approximately 3 and 8% of the total conventional CD4 T cell pool in the spleen and medLN respectively (**Fig. 17e**), and their frequencies decreased by day 14. T_{FR} cells represent approximately 4% of Treg cells in the spleen and medLN at day 9, and further expand to approximately 5 - 7% at day 14 after infection (**Fig. 17f**). On the basis of these observations, we chose to analyse germinal centre cell types at day 9, the approximate peak of the adaptive immune response.

Further phenotyping of splenic T_{FR} cells demonstrated that a small proportion of T_{FR} cells express the co-inhibitory receptor, KLRG1 in comparison to non-T_{FR} and T_{FH} cells. Additionally, T_{FR} cells expressed high levels of ICOS when compared to non-T_{FR} cells but similar to that of T_{FH} cells (**Fig. 17g**). As Bcl6 has been shown to be essential for the development of both follicular T cell subsets (Nurieva, Chung et al. 2009, Linterman, Pierson et al. 2011), we assessed the expression of Bcl6 via flow cytometry in T_{FH} and T_{FR} cells after influenza infection. Staining of CXCR5-positive splenic and medLN conventional CD4 and Treg cells with Bcl6 revealed Bcl6 expression in a proportion of CXCR5-hi conventional CD4 T cells. The expression of Bcl6 in CXCR5-hi Treg cells however was negligible (**Fig. 17h**). Here, we concluded that the resolution of Bcl6 expression via flow cytometry was not informative and as such, Bcl6 staining was not included in the experiments to identify T_{FR} cells going forward.

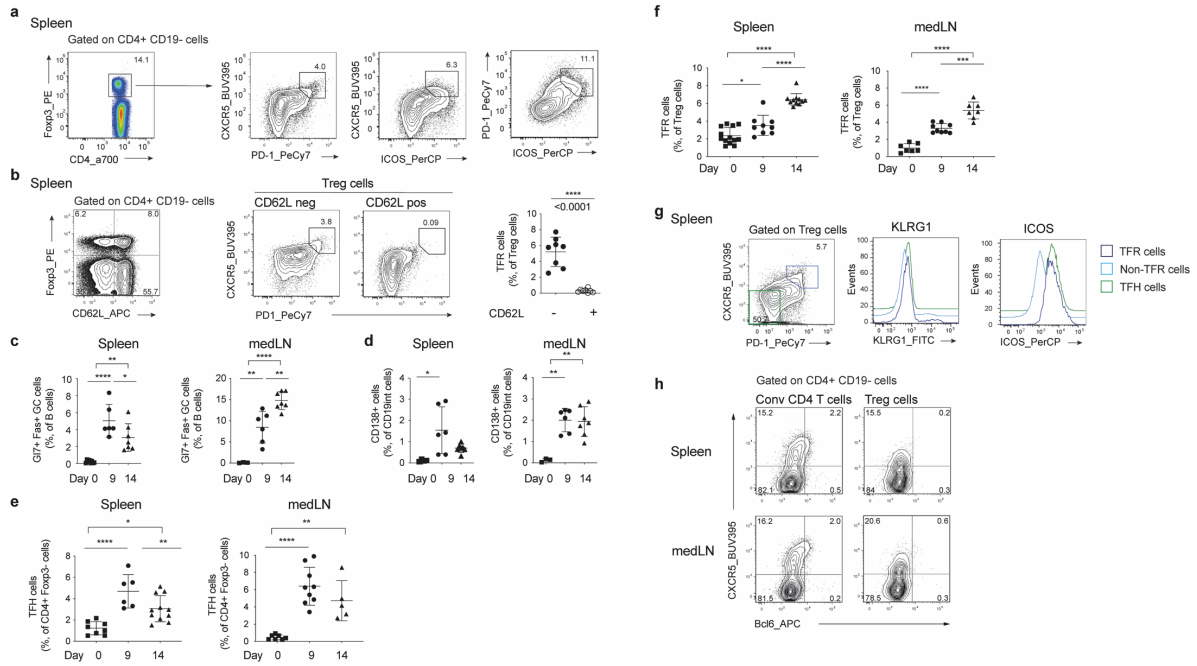


Figure 17. Kinetics of the germinal centre response after influenza infection.

(a) Flow cytometric plots showing the gating strategy of splenic Treg and T_{FR} cells day 9 after influenza infection. T_{FR} cells co-express CXCR5, ICOS and PD-1. (b) Flow cytometric plots showing that splenic T_{FR} cells are more effector-like and express low levels of CD62L. (c–f) Graphs showing frequencies of (c) G17⁺Fas⁺CD19⁺ GC B cells, (d) CD138⁺ plasma cells, (e) CD4⁺CD19⁺Foxp3⁺CXCR5^{high}PD-1^{high} T_{FH} cells and (f) CD4⁺CD19⁺Foxp3⁺CXCR5^{high}PD-1^{high} T_{FR} cells from the spleen and medLN of wild type mice at baseline, and days 9 and 14 after influenza infection. (g) Flow cytometric plot showing non-T_{FR} Treg cell gating strategy. Histogram on the right showing cell surface expression of KLRG1 and ICOS in T_{FR} (green), T_{FR} (blue) and non-T_{FR} Treg (light blue) cells. (h) Flow cytometric plots depicting Bcl6 expression of T_{FR} and T_{FR} cells from the spleen and medLN of infected wild type mice. (i) Intracellular expression of Bcl6 as determined by flow cytometry in splenic CD19⁺ B (purple), T_{FR} (green) and T_{FR} (blue) cells.

Numbers adjacent to gated areas indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). Analysed using (b–d) unpaired Student's t-test and (e–f) one-way ANOVA (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of two independent experiments with three mice per group. GC, germinal centre; medLN, mediastinal lymph node; T_{FR}, follicular T helper; T_{FR}, follicular Treg; WT, wild type.

4.2 T_{FR} cells express the transcription factor Blimp-1 and its target IL-10.

I) Blimp-1 is essential for the terminal differentiation of effector Treg cells.

To determine the expression pattern of Blimp-1 (encoded by *Prdm1*) on T_{FH} and T_{FR} cells, we analysed secondary lymphoid organs of *Foxp3^{RFP}Blimp1^{GFP/+}* dual reporter mice (described in the previous chapter) after influenza infection. Analysis of *Blimp1^{GFP}* fate mapping reporter mice identified that *Blimp1^{GFP}* reporter is stably expressed by all eTreg cells. We observed that Treg cells that expressed the maturation marker KLRG1, expressed the highest level of *Blimp1^{GFP}* reporter compared to CD4+Foxp3- T cells and naïve Treg cells in the spleen (**Fig. 18a**). Additionally, a proportion of KLRG1 negative Treg cells also expressed *Blimp1^{GFP}* reporter (**Fig. 18a**). At day 9 following infection, we observed that splenic T_{FH} cells do not express *Blimp1^{GFP}* reporter when compared to T_{FR} and CXCR5^{low} PD-1^{low} non-follicular Treg (non-T_{FR}) cells (**Fig. 18b**). *Blimp1^{GFP}* reporter positive T_{FR} cells expressed low levels of CD62L and CCR7 (**Fig. 18c**). Approximately 20-22% of T_{FR} and non-T_{FR} cells expressed *Blimp1^{GFP}* reporter (**Fig. 18d**). Similar to observations from the non-follicular CD4 T cell populations (Cretney, Xin et al. 2011), relative to T_{FR} cells that did not express the *Blimp1^{GFP}* reporter, *Blimp1^{GFP}* reporter positive cells expressed significantly higher levels of ICOS (**Fig. 18e-f**).

II) Blimp-1 expression is restricted to eTreg cells where it is required for Il10 expression.

To determine if T_{FR} cells express suppressive molecules such as IL-10, we analysed influenza infected *Il10^{GFP}* reporter mice (described previously (Kamanaka, Kim et al. 2006) that were crossed to *Foxp3^{RFP}* reporter mice (Wan and Flavell 2005) at day 9 after infection for the expression of IL-10 protein. Consistent with a recent publication (Laidlaw, Lu et al. 2017), we demonstrated that approximately 10% and 5% of Treg cells from the spleen and medLN respectively express *Il10^{GFP}* reporter (**Fig. 19a**). Comparing T_{FR} to non-T_{FR} Treg cells in both the spleen and medLN, we observed that T_{FR} cells contribute to majority of the *Il10^{GFP}* reporter expression (**Fig. 19b-c**). However, only 10-15% of T_{FR} cells are responsible for the production of *Il10^{GFP}* (**Fig. 19d**). We also observed T_{FH} cells to express *Il10^{GFP}* reporter at similar levels to T_{FR} cells when compared to non-T_{FR} Treg cells in both the spleen and medLN of these reporter mice 9 days after influenza infection (**Fig. 19e**). Splenic T_{FR} cell populations expressed the cell surface immune receptor, TIGIT, which correlates with

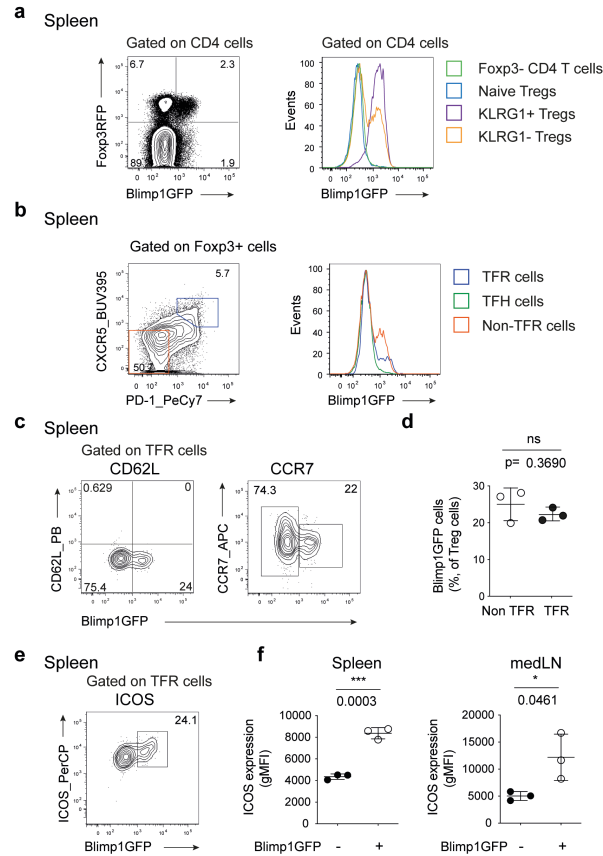


Figure 18. T_{FR} cells express the transcription factor Blimp-1 and its target IL-10.

(a) Flow cytometric plot showing the expression of *Blimp1*^{GFP} reporter by splenic Treg cells of *Foxp3*^{RFP}*Blimp1*^{GFP/+} reporter mice 9 days after influenza infection. Histogram comparing the expression of *Blimp1*^{GFP} reporter by naïve conventional CD4 (light green) and Treg (light blue), KLRG1⁺ (purple) and KLRG1⁻ (orange) Treg cells. (b) Flow cytometric plot (left) and histogram (right) of *Blimp1*^{GFP} reporter expression by T_{FH} (green), non T_{FR} (light blue) and T_{FR} (blue) cells. (c) Flow cytometric plots of the expression of *Blimp1*^{GFP} reporter and CD62L and CCR7 by splenic T_{FR} cells of mice as in (a). (d) Graph showing the frequency of *Blimp1*^{GFP} reporter T_{FR} and non-T_{FR} Treg cells in the spleen of mice as in (a). (e) Flow cytometric plot showing the expression of ICOS by T_{FR} cells. (f) Graph showing the gMFI of ICOS expression by *Blimp1*^{GFP} positive and negative T_{FR} cells from the spleen and medLN.

Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). *p=0.0461, ***p=0.0003 (unpaired Student's t-test). Flow cytometric plots and data are representative of two to three independent experiments with three mice per group. gMFI, geometric Mean Fluorescence Intensity; KLRG1, killer cell lectin-like receptor G1; medLN, mediastinal lymph node; ns, not significant; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

effector function (Joller, Lozano et al. 2014) and that TIGIT⁺ T_{FR} cells contribute significantly to the expression of *Il10*^{GFP} reporter (Fig. 19f).

Collectively, our data show that a proportion of the T_{FR} cell pool express *Blimp1*^{GFP} reporter and its target, IL-10 and have an effector phenotype, which fits our hypothesis that T_{FR} cells are the eTreg cell of the germinal centre.

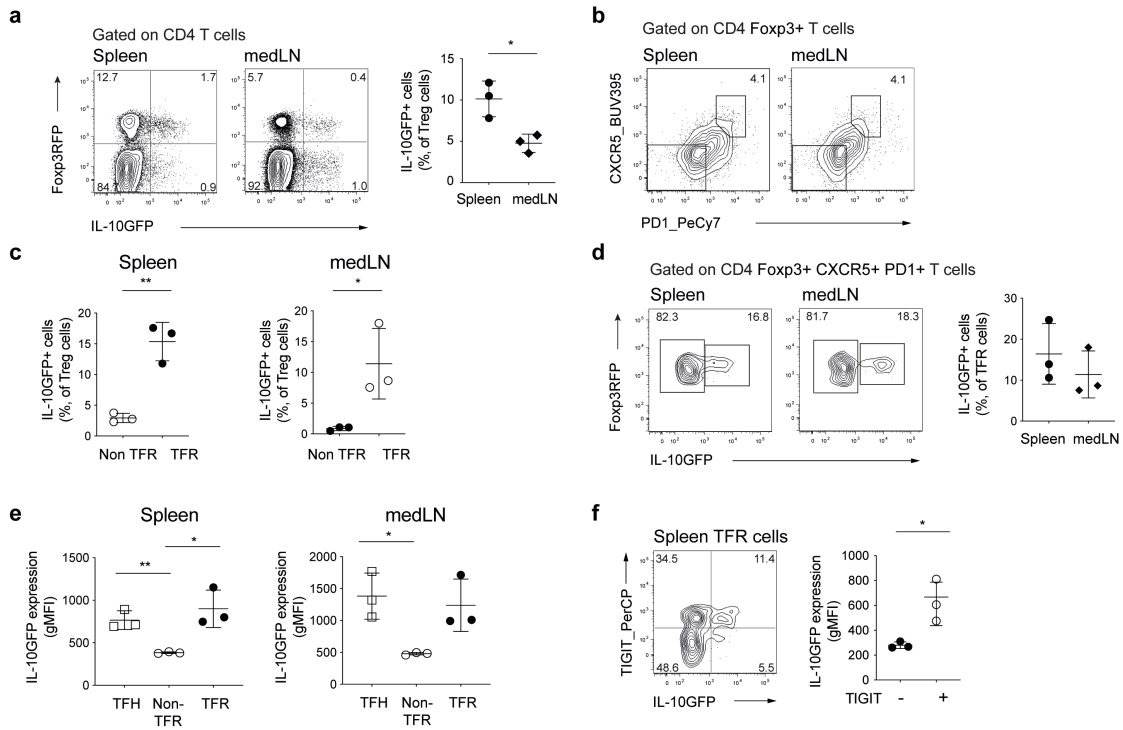


Figure 19. Blimp-1 expression is restricted to eTreg cells where it is required for *Il10* expression.

(a) Flow cytometric plots showing *Il10*^{GFP} reporter expression by splenic and medLN Treg cells from *Foxp3*^{RFP}*Il10*^{GFP} reporter mice 9 days after influenza infection. Right, quantification. (b) Flow cytometric plots of T_{FR} cells in the spleen and medLN from mice in (a). (c) Bar graphs showing the frequency of *Il10*^{GFP}-expressing T_{FR} and non-T_{FR} Treg cells from the spleen and medLN of mice in (a). (d) Flow cytometric plots (left) and quantification (right) of *Il10*^{GFP} reporter expression from splenic and medLN T_{FR} cells of mice as in (a). (e) Expression of *Il10*^{GFP} reporter by splenic and medLN T_{FH}, T_{FR} and non-T_{FR} Treg cells. (f) Flow cytometric plot (left) and quantification (right) showing expression of TIGIT and *Il10*^{GFP} reporter by splenic T_{FR} cells. Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using (a,c-d,f) unpaired Student's t-test and (e) one-way ANOVA (*p<0.05, **p<0.01, ***p<0.0001, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of two to three independent experiments with three mice per group. gMFI, geometric mean fluorescence index; medLN, mediastinal lymph node; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

III) T_{FR} cells continually experience T cell receptor signals.

T cell receptor (TCR) signal strength instructs CD4⁺ thymocyte fate and regulatory T cell differentiation (Fassett, Jiang et al. 2012). In addition, TCR signalling has been shown to drive IRF4 expression in CD8, CD4 and Treg cells (Lohoff, Mittrucker et al. 2002, Bollig, Brustle et al. 2012, Man, Miasari et al. 2013). To determine if T_{FR} cells show hallmarks of ongoing TCR signalling, we utilized transgenic mice that express the green fluorescence protein (GFP) reporter under the *Nur77* gene locus that specifically reports TCR signalling (Moran, Holzapfel et al. 2011). *Nur77*^{GFP} reporter mice were infected with influenza virus and splenic and medLN T cell populations were analysed at day 9 after infection. Similar to published data, Treg cells showed higher *Nur77*^{GFP} reporter expression compared to conventional CD4 T cells, supporting the notion that Treg cells perceive stronger TCR signalling (**Fig. 20a**). CXCR5^{high} PD-1^{high} T_{FR} cells expressed higher levels of *Nur77*^{GFP} compared to T_{FH} and non-T_{FR} Treg cells (**Fig. 20b-c**). This finding suggests that T_{FR} cells, like Treg cells (Levine, Arvey et al. 2014, Vahl, Drees et al. 2014), continue to rely on TCR signals to mediate T_{FR} cell suppressive function despite having undergone functional adaptation.

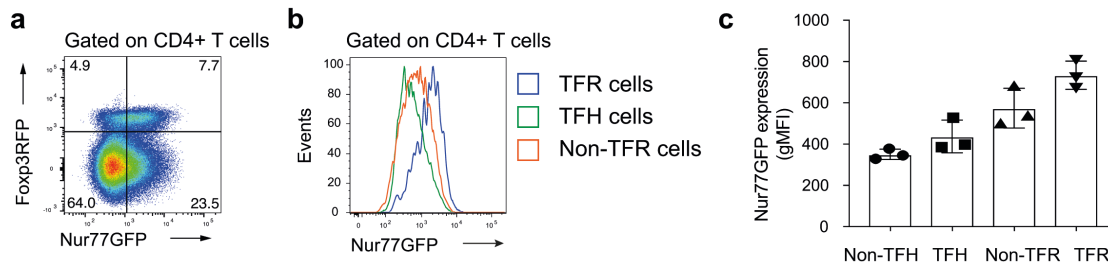


Figure 20. T_{FR} cells perceive TCR signals.

(a) Flow cytometric plot showing *Nur77*^{GFP} reporter expression by splenic Treg cells. (b) Representative histogram of *Nur77*^{GFP} reporter expression by T_{FH}, T_{FR} and non-T_{FR} Treg cells from *Foxp3*^{RFP}*Nur77*^{GFP} reporter mice day 9 following influenza infection. (c) Bar graph showing gMFI of *Nur77*^{GFP} reporter expression by non-T_{FR}, T_{FR}, non-T_{FR} and T_{FR} cell subsets. Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Flow cytometric plots and data are representative of two independent experiments with three mice per group. gMFI, geometric mean fluorescence index; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

4.3 IRF4 is highly expressed by T_{FR} cells.

T_{FH} cell differentiation has previously been shown to depend on IRF4 (Bollig, Brustle et al. 2012). In order to examine the expression of IRF4 in eTreg cell populations, we made use of our novel *Irf4^{tdTom/+}* reporter mouse. Consistent with earlier reports from ourselves and others (Zheng, Chaudhry et al. 2009, Cretney, Xin et al. 2011), we observed Treg cells from non-infected *Irf4^{tdTom/+}* reporter mice to express higher levels of *Irf4^{tdTom}* reporter than conventional CD4 T cell populations as assessed by flow cytometry (**Fig. 21a**).

However, *Irf4^{tdTom}* reporter expression was heterogeneous among Treg cells, with tdTomato reporter expression correlating with activation- and effector-associated molecules, such as ICOS, CD44 and TIGIT (not shown), and inversely correlated with quiescence-associated lymphoid homing receptors CD62L and CCR7 (**Fig. 21b**).

To determine the level of IRF4 expression in T_{FR} cells, we infected *Irf4^{tdTom/+}* reporter mice with influenza virus and assessed *Irf4^{tdTom}* reporter expression in the spleen by flow cytometry on day 10 post infection. As in resting animals, we observed Treg cells to express higher levels of *Irf4^{tdTom}* reporter than the conventional CD4 T cell pool (**Fig. 21c**). We further observed T_{FR} cells to express considerably higher *Irf4^{tdTom}* reporter levels compared to non-T_{FR} Treg cells (**Fig. 21d**). Interestingly, compared to T_{FH} cells, the level of *Irf4^{tdTom}* reporter expression was only mildly increased (**Fig. 21e**). *Irf4^{tdTom/+}* expressing Treg cells contributed to the majority of T_{FR} cells (**Fig. 21f**). We also observed higher expression of *Irf4^{tdTom}* reporter in CTLA-4 positive compared to CTLA-4 negative Treg cells (**Fig. 21g**).

Together, these observations indicate that IRF4 expression is induced following T cell activation and with the subsequent effector differentiation of Treg cells, consistent with previous reports (Zheng, Chaudhry et al. 2009, Cretney, Xin et al. 2011, Man, Miasari et al. 2013, Krishnamoorthy, Kannanganat et al. 2017).

4.4 Foxp3-specific deletion of IRF4 results in dysregulated germinal centre responses.

To determine the precise role of IRF4 in T_{FR} cell commitment, we utilized the *Irf4^{fl/fl}Foxp3^{Cre}* mouse strain where mice carrying floxed *Irf4* alleles (*Irf4^{fl/fl}*) were crossed to *Foxp3^{Cre}* mice that expressed a yellow fluorescent protein (YFP)-Cre recombinase fusion protein under the control of the *Foxp3* locus. Notably, *Irf4^{fl/fl}* mice carried an internal GFP cassette that reports gene inactivation (Klein, Casola et al. 2006). Therefore, these mice specifically delete *Irf4*

from all mature Treg cells (Zheng, Chaudhry et al. 2009). These mice, born in the expected Mendelian ratio appear healthy at birth but progressively suffer from weight loss,

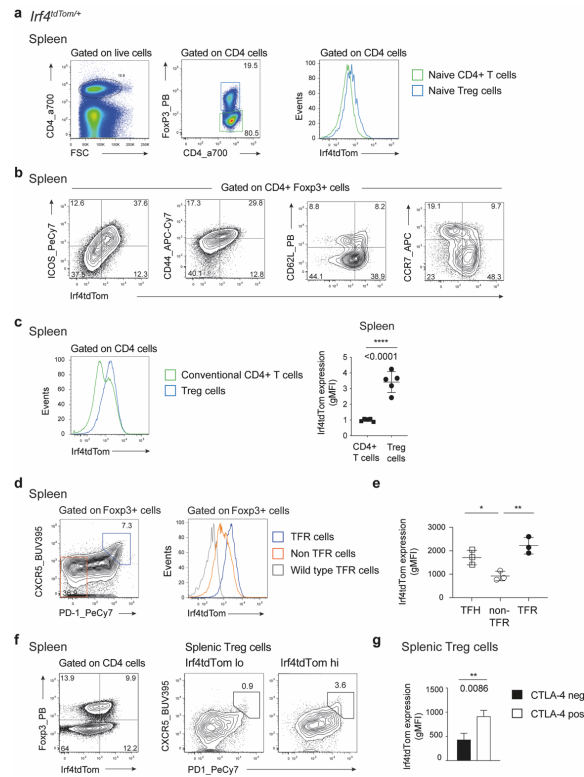


Figure 21. IRF4 is highly expressed by T_{FR} cells.

(a) Flow cytometric plots (left) and histogram (right) showing the gating strategy of naïve conventional CD4 and Treg cells from non-infected *Ir4tdTom*^{+/+} reporter mice. (b) Co-expression of *Ir4tdTom* reporter expression with indicated activation-associated molecules by Treg cells of *Ir4tdTom*^{+/+} reporter mice. (c) Representative histogram (left) and quantification (right) of *Ir4tdTom* reporter expression by naïve conventional CD4 and Treg cells from *Ir4tdTom*^{+/+} reporter mice 9 days after influenza infection. (d) Flow cytometric plot showing CXCR5 and PD-1 expression by Treg cells. Right, Representative histogram showing *Ir4tdTom* reporter expression by T_{FR} cells (blue) and non-T_{FR} cells (orange). Gray line indicates background fluorescence in non-reporter cells. (e) Graph showing gMFI of *Ir4tdTom* reporter expression from T_{FH}, non-T_{FR} and T_{FR} cells. (f) Flow cytometric plot showing *Ir4tdTom* reporter expression by CD4 T cells (left) and the co-expression of CXCR5 and PD-1 based on the expression of *Ir4tdTom* reporter. (g) Bar graph showing *Ir4tdTom* reporter expression by CTLA4 positive and negative splenic Treg cells. Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using (c,g) unpaired Student's t-test and (d) one-way ANOVA (*p<0.05, **p<0.01, ****p<0.0001, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of two to three independent experiments with three mice per group. Ctrl, control; gMFI, geometric mean fluorescence index; T_{FH} follicular T helper; T_{FR}, follicular Treg.

splenomegaly, and lymphadenopathy. Eventually, *Irf4^{fl/fl}Foxp3^{Cre}* mice succumb to fatal, multi-organ autoimmune disease within 6-8 weeks of life. Compared with *Foxp3^{Cre}* control animals, we observed spontaneous germinal centre formation and accumulation of T_{FH} cells (**Fig. 22a and c**). Flow cytometric analysis revealed significant increase in the frequency of Gl7 and Fas expressing germinal centre B cells and T_{FH} cells in the spleen and peripheral LN (pLN) of *Irf4^{fl/fl}Foxp3^{Cre}* animals (**Fig. 22b and d**). Next, we analysed the plasma cell compartment in both spleen and pLN. Plasma cells were identified by their high surface expression of CD138 (**Fig. 22e**). There was a trend to increased plasma cells in *Irf4^{fl/fl}Foxp3^{Cre}* mice, though this was not significant (**Fig. 22f**). There was a strongly reduced T_{FR} cell compartment within the lymphoid organs of *Irf4^{fl/fl}Foxp3^{Cre}* mice (**Fig. 22g**) despite increased frequencies of Foxp3-expressing Treg cells in the spleen compared to controls (**Fig. 22h**). The expression of Helios, a marker of thymic (t)Treg cell (Thornton, Korty et al. 2010), in *Irf4^{fl/fl}Foxp3^{Cre}* mice was unchanged compared with control mice (**Fig. 22i**).

Together, these data indicate that IRF4 was dispensable for tTreg cell development and maintenance. However, the loss of IRF4 results in defective T_{FR} cell differentiation and substantially dysregulated germinal centre responses, which culminates in systemic autoimmunity.

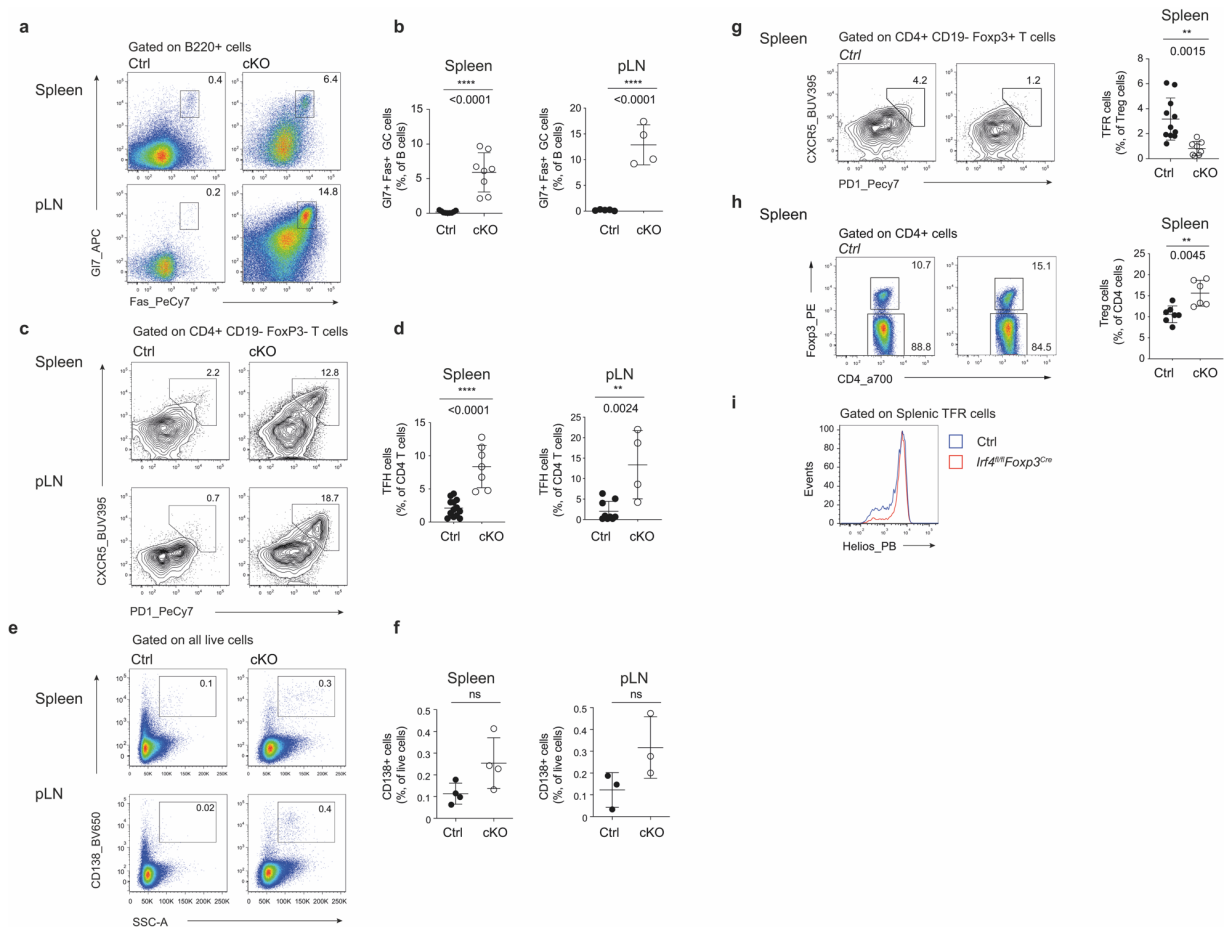


Figure 22. IRF4 regulates T_{FR} cell differentiation.

(a) Flow cytometric plots showing CD19+G17+Fas+ GC B cells from both spleen and pooled pLN of non-infected controls and *Irf4^{fl/fl}Foxp3^{Cre}* (cKO) mice. (b) Bar graphs showing the frequency of splenic and pLN GC B cells as in (a). (c) Flow cytometric plots showing the frequencies of T_{FH} cells in the spleen and pLN of non-infected controls and cKO mice. (d) Frequencies of T_{FH} cells from the spleen and pLN of cKO mice compared to controls. (e) Flow cytometric plots showing CD138+ plasma cells from spleen and pLN of non-infected controls and cKO mice. (f) Bar graph showing frequencies of plasma cells in both spleen and pLN of cKO and control mice. (g) Frequencies of T_{FR} cells from the spleen of non-infected controls and cKO mice. Right, quantification. (h) Flow cytometry plots (left) and quantification (right) showing the frequency of conventional CD4 T and Treg cells from mice in (a). (i) Histogram showing Helios expression by T_{FR} cells.

Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). ** p <0.01, *** p <0.001, **** p <0.0001 (unpaired Student's t-test). Flow cytometric plots and data in a, b, c, d and e are representative of 2-3 independent experiments with at least 3 mice per group. GC, germinal centre; ns, not significant; pLN, peripheral lymph nodes; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

4.5 The requirement for IRF4 in T_{FR} cell differentiation is cell intrinsic.

I) Global IRF4-deficient mice demonstrated an absent T_{FR} cell compartment.

As previously described, *Irf4^{fl/fl}Foxp3^{Cre}* mice that have a Treg specific deletion of IRF4 invariably succumb to fatal autoimmune disease with a heightened immune response before 6 weeks of age. Given that, we were unable to undertake provoked immune challenge in these mice. In addition, the observed systemic autoimmune disease in these mice could be a functional consequence of the loss of T_{FR} cells. As such, to assess whether the defect in T_{FR} cell differentiation was Treg cell intrinsic, we generated mixed bone marrow chimeric mice containing congenically marked wild type (CD45.1) and *Irf4*-sufficient or deficient (CD45.2) haematopoietic compartments (**Fig. 23a**). These chimeric mice showed normal development of wild type and *Irf4*^{-/-} Treg cells in the spleen after infection with influenza virus 6 weeks after reconstitution (not shown). Indeed, analysis of the *Irf4*^{-/-} T cell compartment (CD45.2) demonstrated a complete absence of T_{FR} cells in both the spleen and medLN when compared to the wild type (CD45.1) compartment in the same mouse (**Fig. 23b**). Compared to mice that received *Irf4*-sufficient donor bone marrow, the Treg cells of mice that were reconstituted with *Irf4*-deficient donor bone marrow expressed less KLRG1 (**Fig. 23c**). In the analysis of the lymphoid-homing molecules that are essential for Treg cell egress from lymphoid organs, CCR7 and CD62L, we observed that Treg cells from mice that received *Irf4*-deficient donor bone marrow were unable to downregulate CCR7 (**Fig. 23d**). In addition, Treg cells from *Irf4*-deficient chimeric mice were unable to upregulate activation-associated molecules such as CD44 and ICOS (**Fig. 23e**). Along with the activation defect, *Irf4*-deficient Treg cells also failed to upregulate PD-1, a negative regulator of TCR-induced activation (Sage, Francisco et al. 2013), though their ability to express CXCR5 was unaffected (**Fig. 23e**). Notably, the expression of the transcription factor essential for follicular T cell differentiation, c-Maf (Kroenke, Eto et al. 2012), was also reduced in the absence of IRF4 (**Fig. 23f**).

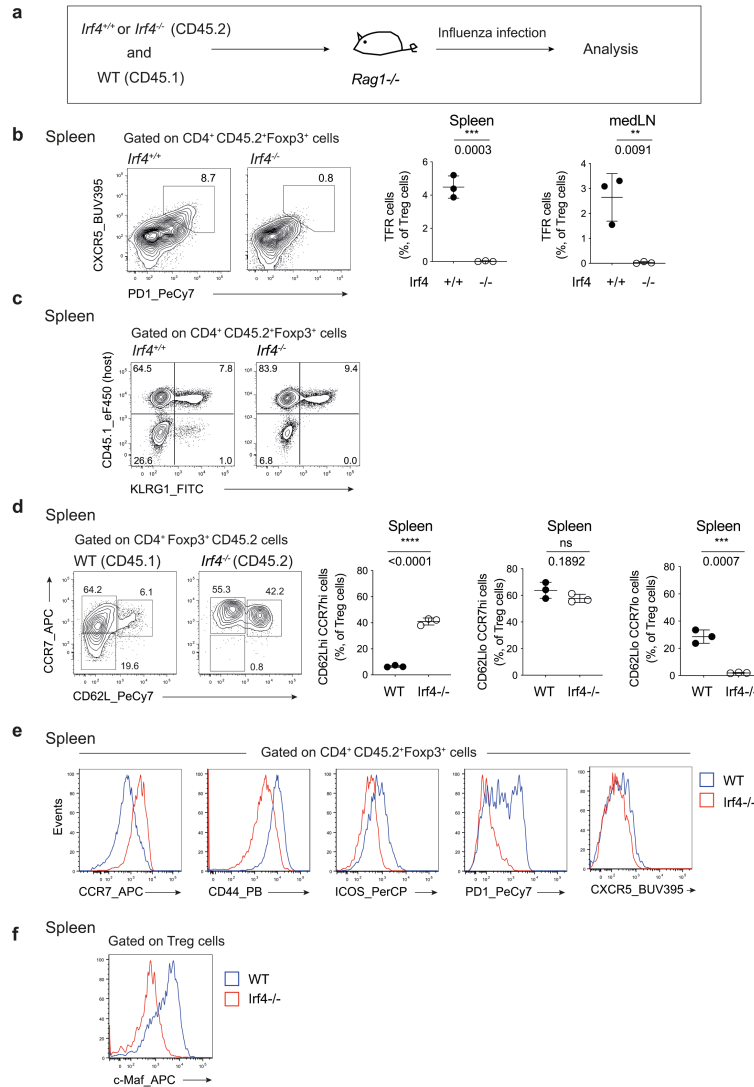


Figure 23. IRF4 is intrinsically required for T_{FR} cell differentiation.

(a) Model of mixed bone marrow chimeras generated by reconstitution of sublethally irradiated *Rag1*^{-/-} mice with *Irif4*^{+/+} or *Irif4*^{-/-} (each CD45.2⁺) and WT (CD45.1⁺) bone marrow, followed by infection with influenza virus strain HKx31. (b) Flow cytometric plot of splenic T_{FR} cells showing complete absence of T_{FR} cells in the *Irif4*^{-/-} (Ly5.2⁺) compartment. Bar graphs show the frequencies of splenic and medLN T_{FR} cells. (c) Co-expression of CD45.1 and KLRG1 in Treg cells from mice as in (a). (d) Flow cytometric plots (left) and quantification (right) of CD62L and CCR7 expression by Treg cells from the WT (Ly5.1⁺) and *Irif4*^{-/-} (Ly5.2⁺) compartment in the same mouse. (e) Histograms showing the expression of Treg-cell activation-associated molecules by WT and *Irif4*^{-/-} Treg cells from chimeric mice as in (a). (f) Representative histogram showing c-Maf expression by WT and *Irif4*^{-/-} Treg cells.

Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). ***p*<0.01, ****p*<0.001, *****p*<0.0001 (unpaired Student's *t*-test). Flow cytometric plots and data are representative of 2-3 independent experiments with at least 3 mice per group. medLN, mediastinal lymph node; ns, not significant; T_{FH} follicular T helper; T_{FR}, follicular Treg; WT, wild type.

II) Treg-specific deletion of IRF4 resulted in an absent T_{FR} cell compartment.

To exclude an activation or maturation defect which was seen in mice with a global deletion of IRF4, we generated competitive bone marrow chimeric mice reconstituted with *Irf4*^{+/+}*Foxp3*^{Cre} (CD45.1) and *Irf4*^{fl/fl}*Foxp3*^{Cre} (CD45.2) donor bone marrow into lethally irradiated C57BL/6 (CD45.1/2) recipients (**Fig. 24a**). As expected, analysis of the lymphoid organs demonstrated a near complete absence of T_{FR} cells among the *Irf4*^{fl/fl}*Foxp3*^{Cre} Treg compartment (**Fig. 24b**). Similarly, we observed an impaired ability of the *Irf4*-deficient Treg cell compartment to upregulate activation-associated and functional molecules such as ICOS and CTLA-4 (**Fig. 24c**). Overall, our results demonstrate that the requirement of *Irf4* for the generation of T_{FR} cells was cell-intrinsic, as influenza infected mixed bone marrow chimeric mice containing congenically marked wild-type and *Irf4*^{fl/fl}*Foxp3*^{Cre} hematopoietic cells showed a pronounced reduction in T_{FR} cells among the *Irf4*-deficient Treg cells.

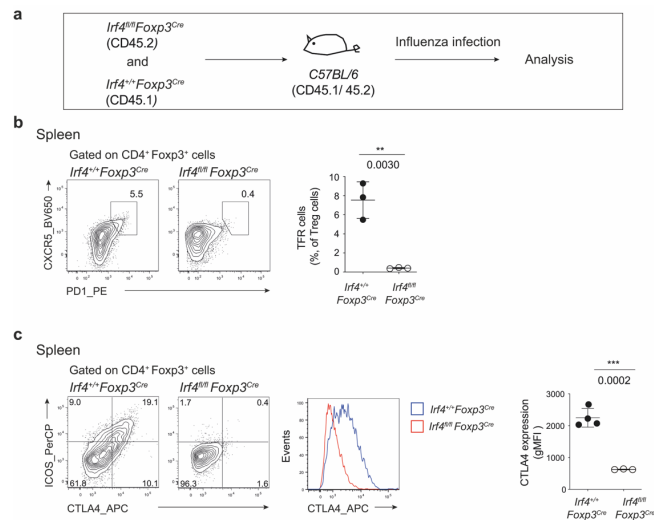


Figure 24. Treg cell specific deletion of *Irf4* results in a near complete absence of T_{FR} cells.

(a) Model of mixed bone marrow chimeras generated by reconstitution of lethally irradiated CD45.1/CD45.2 mice with *Irf4*^{fl/fl}*Foxp3* (CD45.2) and WT (CD45.1) bone marrow followed by infection of recipient mice with influenza virus strain HKx31. (b) Flow cytometric plot (left) and quantification (right) of splenic T_{FR} cells of mice in (a) day 9 after infection. (c) Flow cytometric plot showing co-expression of ICOS and CTLA4 in splenic Treg cells from bone marrow chimeras as in (a). Right, histogram showing CTLA4 expression. Far right, bar graph showing gMFI of CTLA4 expression of *Irf4*-deficient and -sufficient Treg cells from mice as in (a).

Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). **p=0.003, ***p=0.0002 (unpaired Student's t-test). Flow cytometric plots and data are representative of 2-3 independent experiments with at least 3 mice per group. gMFI, geometric mean fluorescence index; ns, not significant; T_{FR}, follicular Treg.

III) *Irf4*-deficient Treg cells from hemizygous *Irf4^{fl/fl}Foxp3^{Cre}* transgenic mice fail to generate T_{FR} cells.

Next, we made use of female *Irf4^{fl/fl}* mice hemizygous for the *Foxp3^{Cre}* transgene (*Irf4^{fl/fl}Foxp3^{Cre/+}*). As the *Foxp3* gene is located on the X chromosome and due to random X inactivation, female mice hemizygous for *Foxp3^{Cre}* express the Cre transgene in only half of their Treg cells. Thus, *Irf4^{fl/fl}Foxp3^{Cre/+}* mice will harbour *Irf4*-sufficient and -deficient Treg cells. *Irf4*-deficient Treg cells are marked by the YFP *Foxp3^{Cre}* reporter and by activation of GFP from the recombination of the *Irf4*-flox transgene (**Fig. 25a**). *Irf4^{fl/fl}Foxp3^{Cre/+}* mice are healthy like their *Foxp3^{+/+}* littermates, allowing us to investigate the behaviour and development of *Irf4*-deficient Treg cells within a healthy animal. To induce T_{FR} cell differentiation, we infected *Irf4^{fl/fl}Foxp3^{Cre/+}* mice with influenza and assessed the YFP/GFP⁻ and YFP/GFP⁺ Treg cell compartment on day 9 post infection by flow cytometry. While the YFP/GFP⁻ compartment contained CXCR5 and PD-1-expressing T_{FR} cells, the frequency of T_{FR} cells were reduced in the YFP/GFP⁺ compartment in both the spleen and medLN of *Irf4^{fl/fl}Foxp3^{Cre/+}* mice (**Fig. 25b**). In contrast, *Irf4*-deficient YFP/GFP⁺ Treg cell compartment was dominated by CCR7^{high} Treg cells, with significant reduction in the frequencies of CCR7^{low} CD62L^{low} cells compared to *Irf4*-sufficient YFP/GFP⁻ Treg cells (**Fig. 25c**). When we analysed the ICOS expression of these two compartments, we saw a significant reduction of ICOS expression by *Irf4*-deficient Treg cells (**Fig. 25d-e**).

Taken together, using various chimeric models, our data supports a cell-intrinsic role for IRF4 in the generation of T_{FR} cells. In addition, the T_{FR} signature molecules including ICOS, PD-1, and c-Maf were diminished in the absence of IRF4. It is important to note that in these chimeric settings, the functional effect of T_{FR} cell deficiency cannot be assessed. Therefore, we had to design a different chimeric model to analyse germinal centre responses in the absence of IRF4.

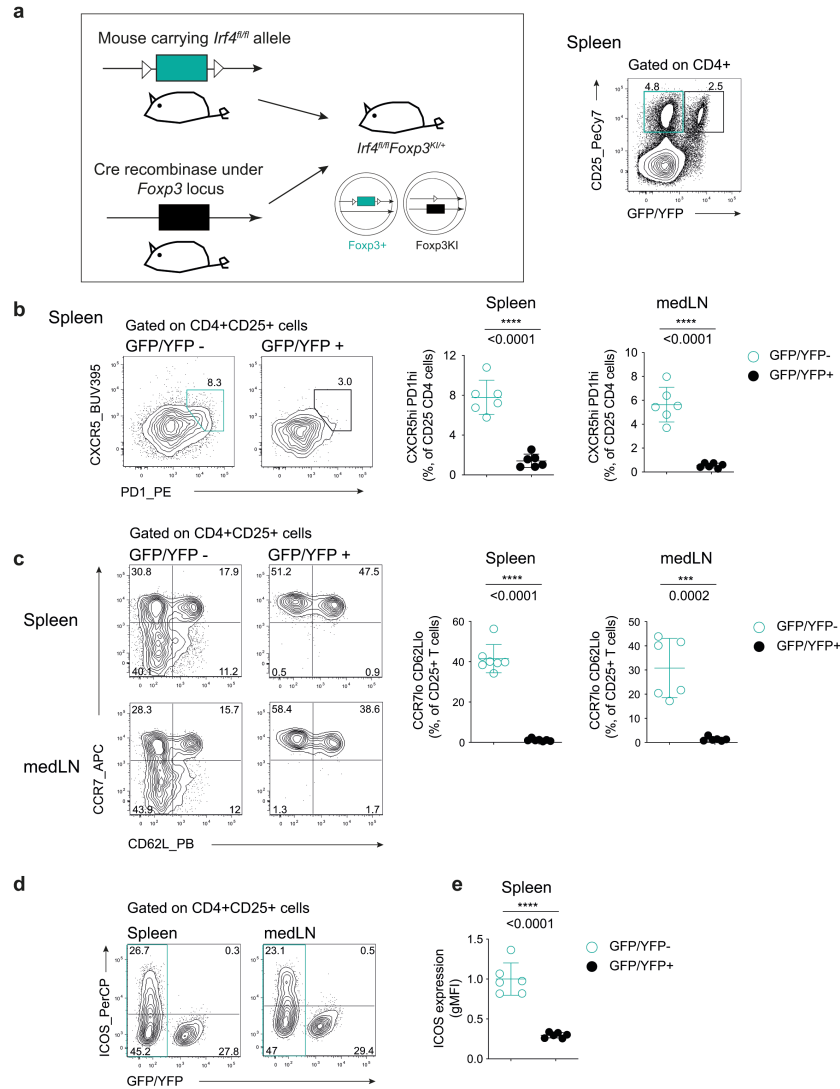


Figure 25. T_{FR} cell differentiation is dependent on IRF4 expression.

(a) Schematic illustration showing the female *Irf4^{fl/fl} Foxp3^{Cre/+}* mice hemizygous for the *Foxp3^{Cre}* transgene, which has a mix of both *Irf4*-sufficient (aqua) and -deficient Treg cells (black). Right, GFP/YFP expression identifies *Irf4*-deficient Treg cells in *Irf4^{fl/fl} Foxp3^{Cre/+}* female mice. (b) Flow cytometric plots (left) and quantification (right) showing frequencies of T_{FR} cells in *Irf4*-sufficient and -deficient Treg cells. (c) Flow cytometric plots showing CCR7 and CD62L expression of *Irf4*-sufficient and -deficient Treg cells. Right, quantification. (d) Reduced ICOS expression by *Irf4*-deficient splenic and medLN Treg cells of *Irf4^{fl/fl} Foxp3^{Cre/+}* female mice. (e) Bar graph showing the gMFI of ICOS expression by *Irf4*-sufficient and -deficient Treg cells. Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). *** $p=0.0002$, **** $p<0.0001$ (unpaired Student's t-test). Flow cytometric plots and data are representative of three independent experiments with three mice per group. gMFI, geometric mean fluorescence index; ns, not significant; T_{FH} follicular T helper; T_{FR}, follicular Treg.

4.6 T_{FR} cell function is dependent on the expression of IRF4.

I) Minor plasma cell dysregulation in the absence of T_{FR} cells.

Due to systemic autoimmune disease that occur in the *Irf4^{fl/fl}Foxp3^{Cre}* mice, we were unable to ascertain the direct functional consequences of the loss of T_{FR} cell development due to the deficiency of IRF4. Therefore, we generated a mixed bone marrow chimeric mouse model that allowed the inducible deletion of wild type T_{FR} cells from a germinal centre response in a healthy mouse while leaving behind either wild type or *Irf4*-deficient T_{FR} cells. To this end, lethally irradiated *Foxp3^{DTR}* (CD45.1) mice (Kim, Rasmussen et al. 2007) were reconstituted with donor bone marrow from *Foxp3^{DTR}* (CD45.1), and either *Irf4^{fl/fl}Foxp3^{Cre}* or control (*Foxp3^{Cre}*, CD45.2). In the *Foxp3^{DTR}* mice, the human diphtheria toxin (DT) receptor was knocked-in to the *Foxp3* locus, whereby administration of DT punctually and specifically depletes all *Foxp3* expressing cells (Hori, Nomura et al. 2003). Thus, in chimeric mice containing *Foxp3^{DTR}* (CD45.1) and *Irf4^{fl/fl}Foxp3^{Cre}* (CD45.2) Treg cells, autoimmunity will be controlled and normal development of germinal centre will be promoted by the presence of a fully functional *Foxp3^{DTR}* Treg cell compartment. Indeed, 6-8 weeks after reconstitution, chimeric *Irf4^{fl/fl}Foxp3^{Cre}Foxp3^{DTR}* mice appeared healthy with no signs of autoimmune disease. They were then infected with influenza virus and treated with diphtheria toxin (DT) at days 0, 1 and 6-post infection to deplete the *Foxp3^{DTR}* CD45.1 Treg cell compartment (**Fig. 26a**). Mice were subsequently sacrificed at day 9. The deletion of *Foxp3*-expressing cells from the *Foxp3^{DTR}* compartment was very efficient (**Fig. 26b**). As expected, *Irf4*-deficient Treg cells were unable to form T_{FR} cells (**Fig. 26c**). Endogenous germinal centre responses were identified by examining B cell expression of GL7 and Fas. There was a trend to reduced frequency of germinal centre B cells in the medLN but not the spleen compared to control mice, though this was not significant (**Fig. 26d**). We observed higher representation of CD19^{int}CD138⁺ plasma cells in the spleen but not medLN of mice that received *Irf4^{fl/fl}Foxp3^{Cre}* bone marrow (**Fig. 26e**). Unexpectedly, there was no difference in T_{FH} cell frequencies compared with controls (**Fig. 26f**). Similar to the bone marrow chimeric mouse models discussed above, we documented an impaired ability of *Irf4*-deficient Treg cells to downregulate CCR7 (**Fig. 26g**), which would account for the failure of Treg cells to migrate to the germinal centre. Here, we also saw reduced ICOS expression and complete absence of KLRG1 expression by Treg cells in mice that received *Irf4^{fl/fl}Foxp3^{Cre}* donor bone marrow

(Fig. 26h). Consistent with our hypothesis, the loss of IRF4 leads to a failure of T_{FR} cell generation following viral infection. Surprisingly, the direct functional consequence of an absent T_{FR} cell compartment was the promotion of splenic plasma cell formation.

II) An *in vitro* approach to assess the functional capacity of T_{FR} cell.

To confirm or refute our *in vivo* findings of an augmented plasma cell response in the absence of T_{FR} cells, we performed *in vitro* co-cultures of B, T_{FH}, and Treg cells as published (Sage, Ron-Harel et al. 2016) using Treg cell from either wild type or *Irf4*^{-/-} mice. Here, we co-cultured CTV labelled purified CD19⁺ B cells from spleens of wild type mice infected with influenza virus at day 9 after infection (see **Chapter 2: Methods and Materials**) with flow cytometry sorted CD4⁺Foxp3⁺CD62L^{low} T cells from the same influenza infected wild type mice, and flow cytometry sorted Treg cells from the *Foxp3*^{RFP} reporter mice that were either deficient or sufficient for IRF4. However, despite numerous attempts, even for wild type co-cultures we were unable to reproduce the results published by Sage et al, 2016. The cultures were dominated by excessive cell death, especially when cells were cultured for more than 4 days. We did not observe altered B cell proliferation or differentiation in the presence or absence of Treg cells.

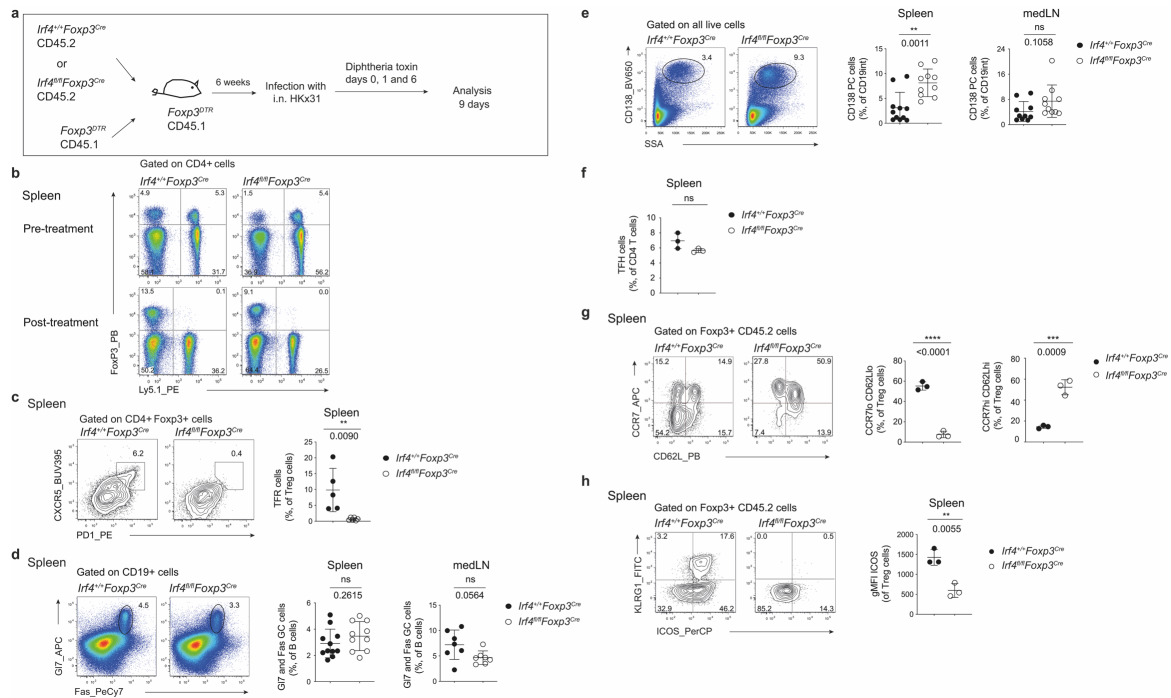


Figure 26. IRF4 is required for the expression of T_{FR} cell migration and functional molecules.

(a) Model of mixed bone marrow chimeric mice generated by reconstitution of lethally irradiated *Foxp3^{DTR}* (CD45.1) mice with *Irf4^{+/+}Foxp3* or *Irf4^{fl/fl}Foxp3* (each CD45.2) and *Foxp3^{DTR}* (CD45.1) bone marrow, followed by infection of recipient mice with influenza virus strain HKx31. Mice also received treated with DT at days 0, 1 and 6 of the experiment. (b) Flow cytometric plot of splenic Treg cells showing effective deletion of *Foxp3⁺* cells from the Ly5.1 compartment. (c) Flow cytometric plots (left) and quantification (right) showing the frequency of splenic T_{FR} cells 9 days after influenza infection. (d-e) Flow cytometric plots (left) and quantification (right) of the frequencies of (d) splenic Fas⁺GIT⁺ GC B cells and (e) splenic CD138⁺ plasma cells of mice in (a). (f) Graph showing frequencies of splenic T_{FH} cells from mice in (a). (g) Flow cytometry plots showing expression of CCR7 and CD62L by Treg cells from mice in (a). Right, graphs showing quantification of CCR7^{low}CD62L^{low} and CCR7^{high}CD62L^{high} Treg cells from mice in (a). (h) Flow cytometry plots showing KLRG1 and ICOS expression by Treg cells from mice in (a). Right, graph showing the gMFI of ICOS.

Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; horizontal lines indicate the mean ($\pm$ SD). **p<0.01, ***p=0.0009, ****p<0.0001 (unpaired Student's t-test). Flow cytometric plots and data are representative of two independent experiments with three mice per group. GC, germinal centre; medLN, mediastinal lymph node; ns, not significant; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

4.7 IRF4 is essential for T_{FR} cell maintenance.

Next, to determine if there was a requirement of IRF4 for the maintenance of the T_{FR} cell compartment after germinal centres have already formed, we made use of the Cre^{ERT2} system, in which a constitutively expressed Cre becomes active following provision of tamoxifen (Feil, Valtcheva et al. 2009). We utilized mice with an inducible deletion of *Irf4* via the Cre^{ERT2} system (*Irf4^{fl/fl}-Cre^{ERT2}*), where tamoxifen treatment results in Cre-mediated deletion of the loxp flanked *Irf4* allele. Notably, upon loxp deletion of the *Irf4* floxed allele, GFP expression reported gene inactivation, as previously published (Klein, Casola et al. 2006). To create a model in which IRF4 expression could be abrogated specifically in T_{FR} cells at specific time points during an infection, we made mixed bone marrow chimeras using bone marrow from *Irf4^{fl/fl}Foxp3^{Cre}* (which we have found unable to produce T_{FR} cells) and *Irf4^{fl/fl}-Cre^{ERT2}* donors (which may generate any hematopoietic compartment prior to tamoxifen treatment) (**Fig. 27a**). By making these chimeras within T cell-deficient *Rag1^{-/-}* hosts, this ensures that the only T_{FR} cells produced could have come from the *Irf4^{fl/fl}-Cre^{ERT2}* genotype marrow, and allows us to track a developed T_{FR} cell population following inducible loss of *Irf4* expression. As controls, we generated mixed bone marrow chimeras from *Irf4^{+/+}Cre^{ERT2}* and *Irf4^{fl/fl}Foxp3^{Cre}* donors, such that after tamoxifen treatment, mice will contain a mix of *Irf4*-sufficient and -deficient Treg cells. Six weeks following reconstitution, we infected bone marrow chimeric mice with influenza virus and treated mice with tamoxifen via oral gavage for 3 consecutive days from day 6 post infection to delete IRF4. Spleen and medLN were analysed at day 14 following infection. Compared with controls that received *Irf4^{+/+}Cre^{ERT2}* bone marrow, recipients of *Irf4^{fl/fl}-Cre^{ERT2}* bone marrow had reduced frequencies of T_{FR} cells both in the spleen and medLN (**Fig. 27b**) CD25⁺ YFP/GFP⁺ CD4 T cells from mice that received *Irf4^{fl/fl}-Cre^{ERT2}* bone marrow were unable to downregulate CCR7 (**Fig. 27c**). Furthermore, CD25⁺ YFP/GFP⁺ CD4 T cells from mice that received bone marrow from *Irf4^{fl/fl}-Cre^{ERT2}* donors showed significantly reduced expression of ICOS and PD-1 (**Fig. 27d**). Taken together, these data confirmed that IRF4 is essential to maintain the expression of key follicular T cell molecules and regulate the expression of lymphoid-homing molecules in an established germinal centre reaction.

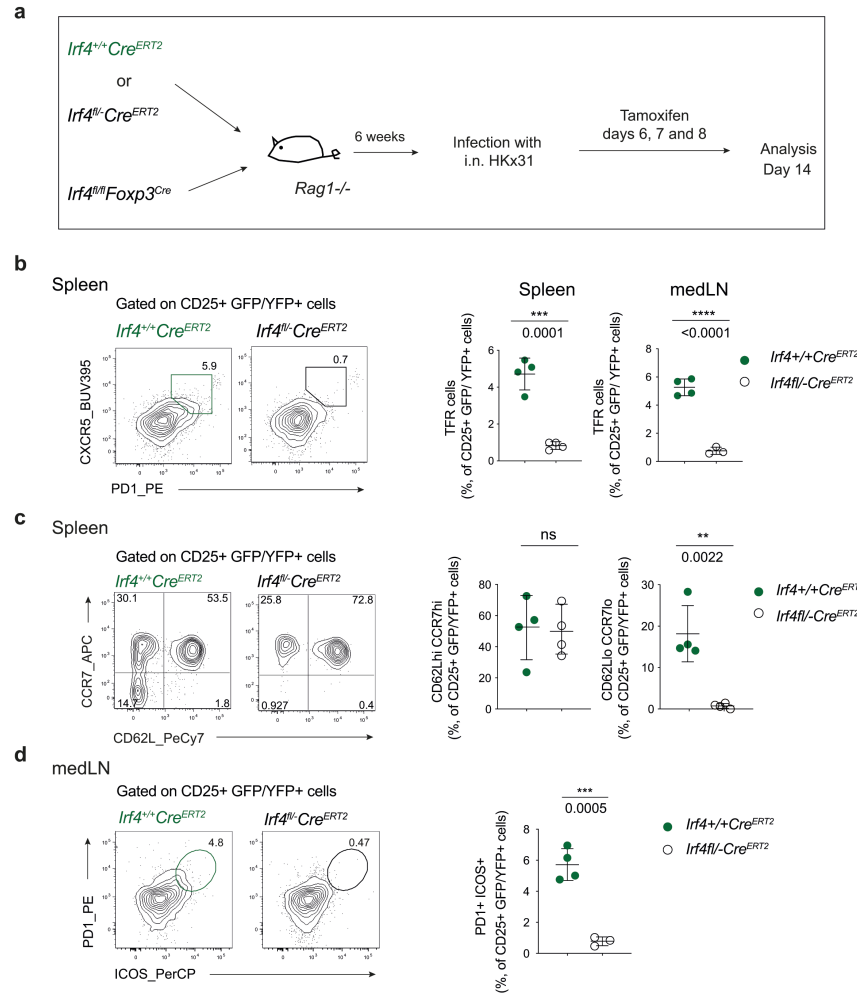


Figure 27. IRF4 is required for T_{FR} cell maintenance.

(a) Model of mixed bone marrow chimeric mice generated by reconstitution of sublethally irradiated *Rag1*^{-/-} hosts with *Irf4*^{fl/fl} *Foxp3*^{Cre} and *Irf4*^{fl/-} *Cre*^{ERT2} or *Irf4*^{+/+} *Cre*^{ERT2} donor bone marrow that were infected with influenza virus and subsequently treated with tamoxifen by oral gavage on days 6,7 and 8 after infection. (b) Flow cytometric plots (left) and quantification (right) of the expression of CXCR5 and PD-1 by CD4⁺CD25⁺ cells from the spleen of mice in (a) at day 14 after influenza infection. (c) Flow cytometric plots showing CCR7 and CD62L expression on CD4⁺CD25⁺ GFP/YFP⁺ cells from the spleen of from mice in (a). Right, quantification. (d) Co-expression of ICOS and PD-1 by medLN CD4⁺ CD25⁺ GFP/YFP⁺ cells of mice from (a). Right, graph showing the percentage of PD-1 and ICOS-expressing CD4⁺CD25⁺ GFP/YFP⁺ cells. Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). ***p*=0.0022, ****p*<0.001, *****p*<0.0001 (unpaired Student's t-test). Data is from one experiment with four mice per group. medLN, mediastinal lymph node; ns, not significant; T_{FH} follicular T helper; T_{FR}, follicular Treg.

4.8 IRF4 regulates molecules essential for T_{FR} cell migration and localization.

To confirm our previous findings on the effect of IRF4 on the regulation of genes that are functionally related to tissue homing and trafficking of Treg cells, we analysed the expression of CCR7 and CD62L in *Foxp3^{RFP}* reporter mice that were either sufficient or deficient for IRF4 (**Fig. 28a**). We observed that *Irf4*-deficient Treg cells had a defect in CCR7 downregulation (**Fig. 28b**), which indicates that IRF4 is critically required for the maintenance of immune homeostasis through the regulation of Treg cell trafficking.

Next, to determine the localization of T_{FR} cells within the germinal centre, we examined the location of Foxp3-expressing cells within the germinal centre of *Irf4*-deficient mice histologically. In these experiments, we generated mixed bone marrow chimeric mice from wild type and *Irf4^{-/-}Foxp3^{RFP}* bone marrow, as *Irf4*-deficient mice are unable to form germinal centres (Bollig, Brustle et al. 2012, Willis, Good-Jacobson et al. 2014). As controls, we also made mixed bone marrow chimeras using bone marrow from wild type and *Irf4^{+/+}Foxp3^{RFP}* donors (**Fig. 28c**). In the chimeric mice that received *Irf4*-deficient bone marrow, we relied on the wild type Treg cells for the initial germinal centre reaction. We looked by confocal microscopy to identify RFP expressing Treg cells at day 9 after influenza infection (**Fig. 28d**). *Irf4*-deficient *Foxp3^{RFP}* reporter positive cells were predominantly found within the T cell zone, though in reduced numbers when compared to *Irf4*-sufficient Treg cells. There was also a marked reduction in germinal centre *Foxp3^{RFP}* reporter positive cells in *Irf4*-deficient bone marrow chimeras compared to controls (**Fig. 28d-e**).

Taken together, IRF4 is not only essential for the generation of T_{FR} cells but appear to also be required for the downregulation of lymphoid homing receptors, which is necessary for T_{FR} cells to appropriately localize to, and within, the germinal centres.

(d) Immunofluorescence staining of spleen sections from *Irf4*-sufficient and deficient mice 9 days after influenza infection showing localization of T_{FR} cells in the germinal centre. The germinal centre is demarcated by blue lines. The T cell zone is demarcated by red lines. Dashed lines indicate the B cell follicles. (e) Quantification of Treg cells in the T cell zone and germinal centre of *Irf4*-sufficient and deficient mice. Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). **** $p < 0.0001$ (unpaired Student's t-test). GC, germinal centre; ns, not significant. Flow cytometric plots and data are representative of three independent experiments with three mice per group.

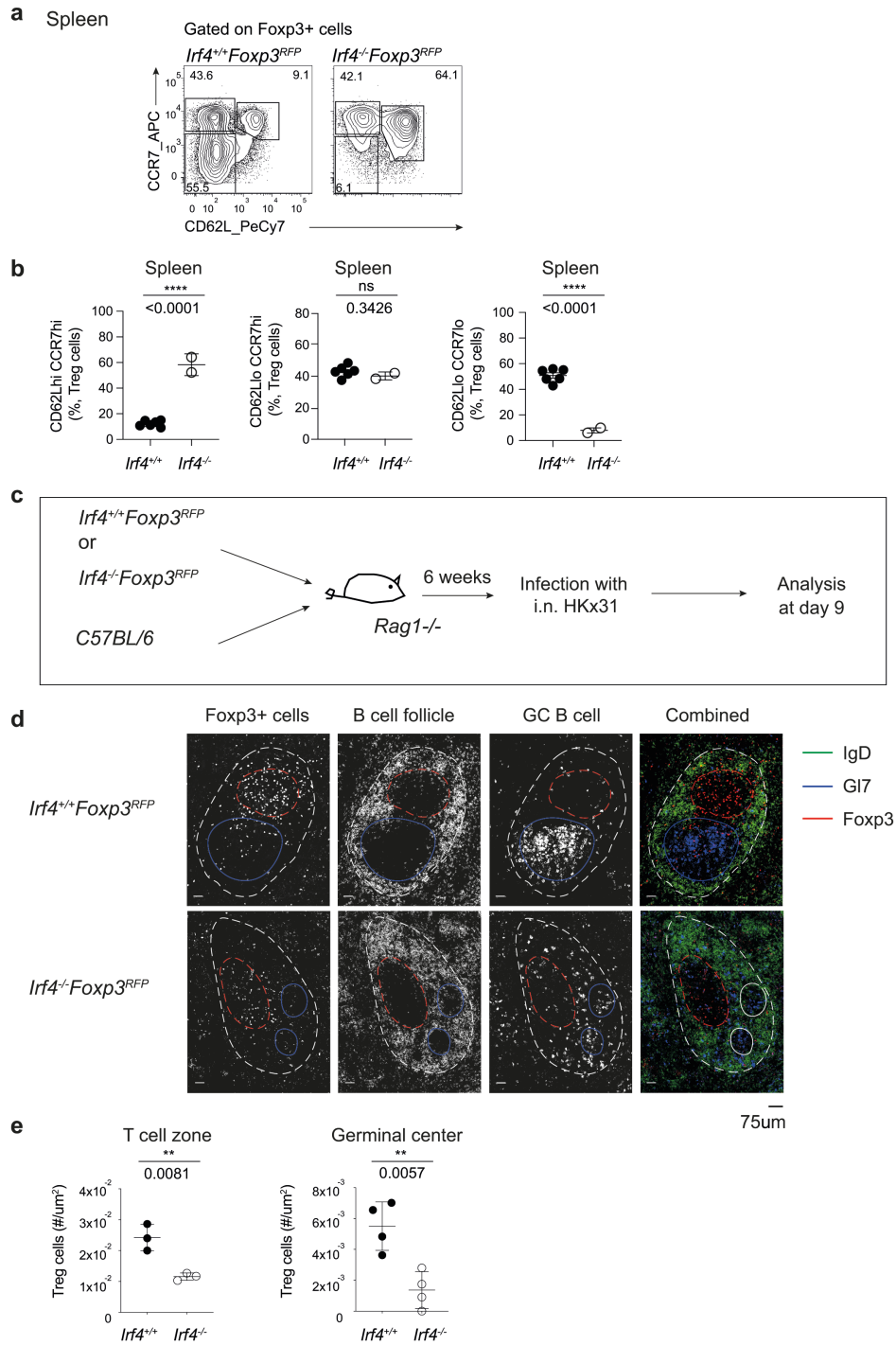


Figure 28. T_{FR} cell localization is dependent on chemokine receptor expression.

(a) Expression of CCR7 and CD62L in splenic Treg cells from *Ir4*^{+/+}*Foxp3*^{RFP} and *Ir4*^{-/-}*Foxp3*^{RFP} mice 9 days after influenza infection. (b) Graphs showing the frequency of CCR7^{hi}CD62L^{hi}, CCR7^{hi}CD62L^{lo}, and CCR7^{lo}CD62L^{lo} Treg cells from *Ir4*-sufficient and deficient mice. (c) Model of mixed bone marrow chimeric mice generated by reconstitution of sublethally irradiated *Rag1*^{-/-} hosts with *Ir4*^{+/+}*Foxp3*^{RFP} or *Ir4*^{-/-}*Foxp3*^{RFP} and C57BL/6 donor bone marrow that were infected with influenza virus.

4.9 Gene regulation of IRF4 in naïve CD4, regulatory T and follicular T cells.

Given our *in vivo* findings that IRF4 is required for T_{FR} cell commitment, we next sought to identify if IRF4 directly regulates genes that are required for T_{FR} cell differentiation. Our lab has previously shown by RNA sequencing (RNA-seq) that eTreg (CD62L^{low}*Blimp1*^{GFP} positive) cells have a distinct transcriptional profile compared to cTreg (CD62L^{high}*Blimp1*^{GFP} negative) cells (Cretney, Xin et al. 2011). Treg cells that express the transcription factor Blimp-1 upregulated functionally essential genes including *Ctla4*, *Icos*, *Klrg1* and *Tigit*, in keeping with the finding that eTreg cells are the most suppressive Treg cells (Vasanthakumar, Moro et al. 2015). In this following section, we utilized several molecular techniques including RNA-seq and chromatin immunoprecipitation (ChIP) followed by next generation sequencing (ChIP-seq) to unravel the transcriptional programs regulated by IRF4 in T_{FR} cells.

I) Identification of ‘follicular signature genes’ expressed in T cell associated with germinal centre B cells.

To identify the core T_{FR} cell gene expression signature, we performed RNA-seq analysis in collaboration with Dr Julie Tellier (Nutt lab, WEHI). To this end, we used flow cytometry to sort naïve (CD62L^{hi}) conventional CD4 and Treg cells, IL-21-negative (T_{FH}), and IL-21-positive T_{FH} (T_{FH}-21), and T_{FR} cells from influenza infected *Foxp3*^{RFP}*Il21*^{GFP} reporter mice (Luthje, Kallies et al. 2012) at day 9 after infection (**Fig. 29a**). We observed the follicular T cells to cluster closely and away from naïve T cells. This also revealed that T_{FR} cells closely resembled both Treg and follicular T cells (**Fig. 29b**). Next, to exclude all genes that are common to CD4 T cells, we compared T_{FH} cells to naïve CD4 cells and T_{FH}-21 cells with naïve CD4 cells and found 2,232 and 1,835 differentially expressed (DE) genes respectively. Similarly, we compared T_{FR} cells to naïve Treg cells to exclude all genes that are common to Treg cells, such as *Foxp3*, and found 548 DE genes. Genes common to two or all of the three follicular CD4 T cells were considered the “*follicular signature genes*”. There were 509 genes identified in this overlap (**Fig. 29c**). This list was enriched for genes essential for T_{FH} cell differentiation and function, such as *Cebpa*, *Ctla4*, *Cxcr5*, *Icos*, *Maf*, *Pdcd1* and *Sh2d1a* (SAP) that were shared with T_{FR} cells. Due to the stringent application of statistics, *Bcl6* was not included in the *follicular signature genes*, though it is clear and has been shown by numerous groups to be essential for the development of T_{FR} cells (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011, Leavenworth, Verbinnen et al. 2015, Wu, Chen et al. 2016).

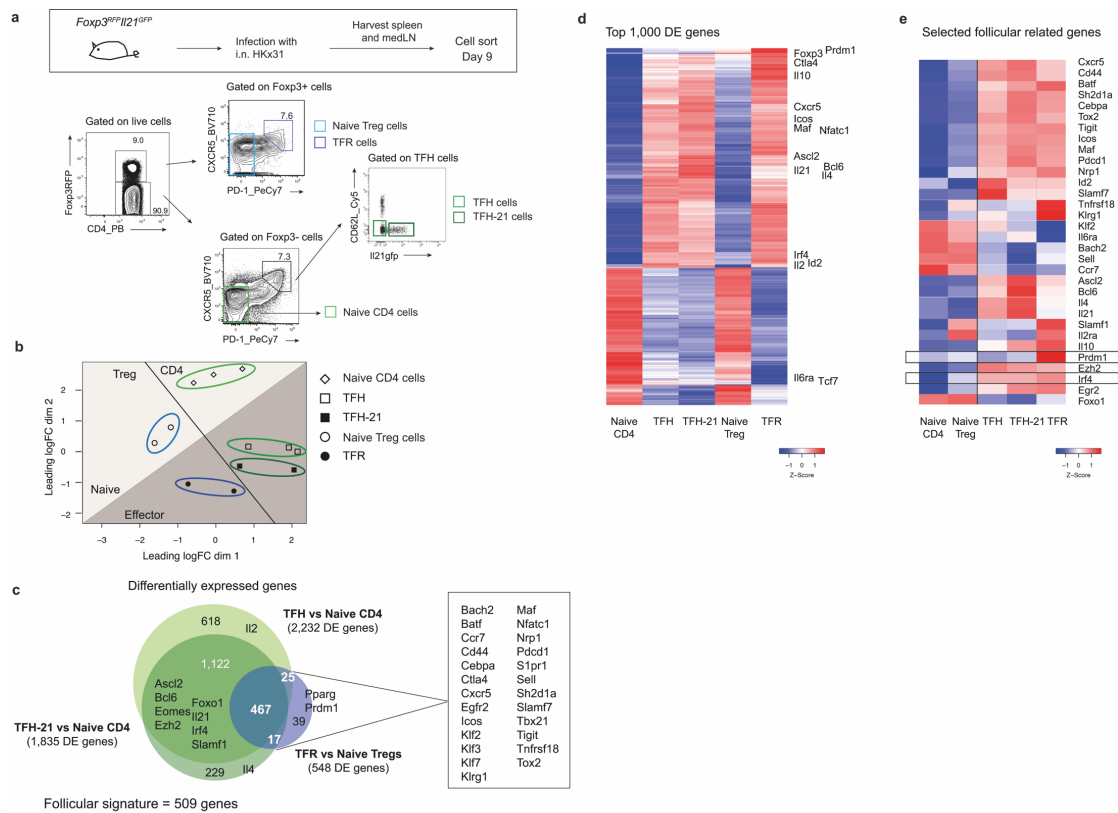


Figure 29. T_{FR} cell gene signature.

Gene regulation in naïve and follicular CD4 T cells. (a) Flow cytometry plots illustrating the gating strategy of flow cytometry-sorted naïve Treg, T_{FR}, naïve (CD62L^{hi}) conventional CD4 T, IL-21-negative (T_{FH}), and IL-21-positive T_{FH} (T_{FH}-21) cells from influenza infected *Foxp3^{RFP}Il21^{GFP}* dual reporter mice at day 9 that were subjected to transcriptional profiling by RNA sequencing (RNA-seq). (b) Multidimensional scaling plot showing the relationship between transcriptional profiles of sorted cells from (a). Foxp3-expressing Treg cells are highlighted with blue circles whilst CD4 T cells that do not express Foxp3 are in green. (c) Numbers of genes significantly differentially expressed (DE) between T_{FR} and naïve Treg cells, T_{FH} and naïve CD4 T cells, and T_{FH}-21 and naïve CD4 T cells that constitute the *follicular signature* (p<0.05). (d) Heatmap showing relative expression (Z-score) of the top 1,000 DE genes for naïve CD4 T, T_{FH}, T_{FH}-21, naïve Treg and T_{FR} cells from mice 9 days after influenza infection (FDR <0.1). (e) Heatmap showing relative expression (Z-score) of common follicular T cell genes DE in naïve CD4 T, T_{FH}, T_{FH}-21, naïve Treg and T_{FR} cells from mice 9 days after influenza infection (FDR <0.1).

Data are representative of 2 independent experiments. Treg, regulatory T; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

T_{FH} and T_{FH}-21 cells had a similar enrichment for follicular helper specific genes such as *Ascl2*, which is required for the expression of CXCR5 in T_{FH} cells (Liu, Chen et al. 2014), *Il21* (Luthje, Kallies et al. 2012) and *Slamf1*, a surface receptor specifically required for IL-4 production (Yusuf, Kageyama et al. 2010).

In the list of 39 DE genes that were identified between the T_{FR} and naïve Treg cell comparison, we found *Prdm1* (encoding Blimp-1) supporting that T_{FR} cells are indeed a subset of eTreg cells. The discovery of *Ppary* in this short list would suggest that the effector program is switched on in all eTreg cells, however each tissue-specific eTreg cell relies on external cues, such as the cytokine milieu, for its functional specialization. To visualise the transcriptional profile of the naïve and follicular CD4 T cells, we generated a heatmap displaying the top 1,000 DE genes in these five CD4 T cells subtypes with a fold change cut-off of 2.0 (**Fig. 29d**). We narrowed this list to known follicular T and Treg cell-related genes and found similarities in the gene expression profile of follicular CD4 T cells, irrespective of *Foxp3* expression (Fig. 30e). Consistent with our *in vivo* experiments, we observed *Irf4* to be upregulated in follicular T cells. Additionally, we observed T_{FR} cells to express the highest levels of *Prdm1* compared with all other cell types examined.

II) Regulation of the T_{FR} cell transcriptome by IRF4.

To determine the transcriptional target of IRF4 in Treg cells, we performed RNA-seq on Treg cells isolated from pooled spleen and pLN from wild type and *Irf4*^{-/-} mice (with A. Vasanthakumar, Kallies lab). It is important to note that we performed RNA-seq on total Treg cell populations as loss of IRF4 severely impairs activation and differentiation of Treg cells, and *Irf4*^{-/-} mice lack eTreg cells (Cretney, Xin et al. 2011). Interestingly, a number of genes encoding Treg cell activation-associated and migration molecules were downregulated in the absence of IRF4, including *Icos*, *Ctla4*, *Egr2*, *Tigit*, *Klrg1*, *Ccr7* and *Sell* (**Fig. 30a**). Approximately 50% of the eTreg cell signature was deregulated in the absence of IRF4, which included *Bach2*, *Ccr7*, *Icos*, *Il10*, *Il1rl1* and *Prdm1*. In line with the finding of an absent T_{FR} cell compartment in *Irf4*-deficient mice, 40% of the *follicular signature genes* were poorly expressed by *Irf4*-deficient splenic Treg cells (**Fig. 30b**). A vast majority of these genes encoded factors that regulate the differentiation and function of T_{FR} cells and were significantly downregulated in *Irf4*-deficient Treg cells. These included *Cebpa*, *Ctla4*, *Cxcr5*, *Icos*, *Maf*, *Nfatc1*, *Nrp1* and *Pdcd1*.

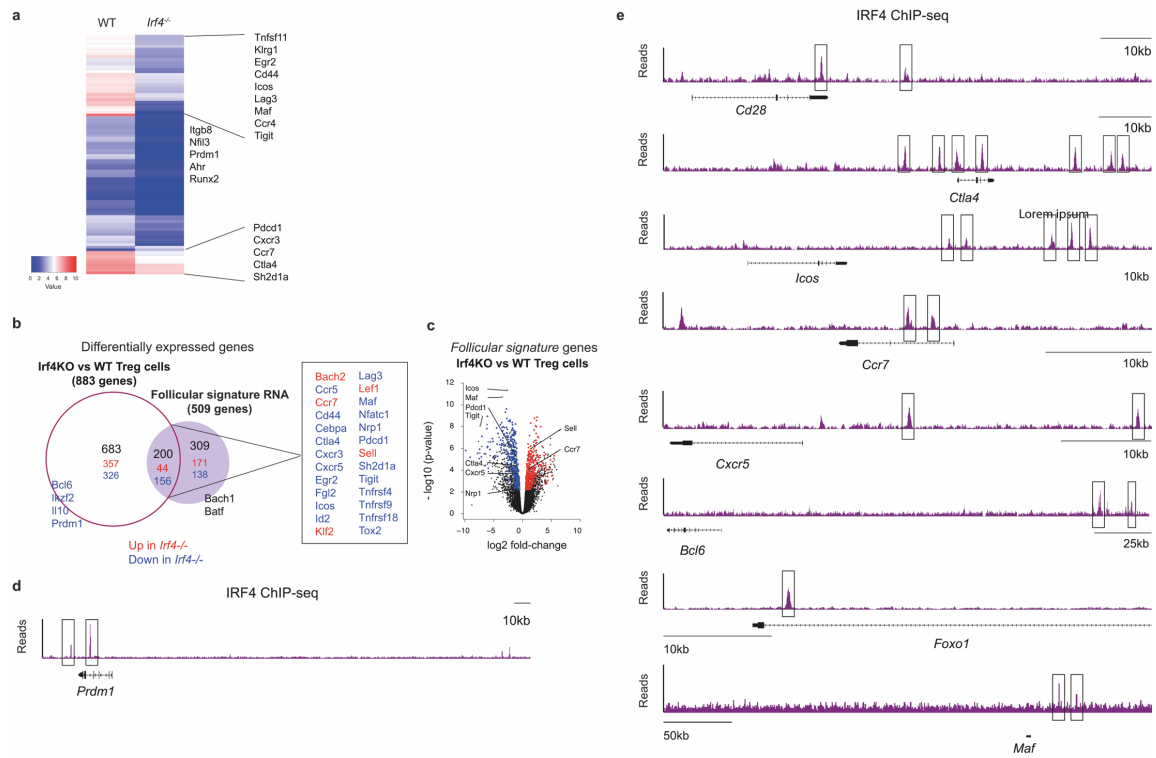


Figure 30. Identification of direct IRF4 target gene candidates in Treg cells.

(a) Heatmap showing top 100 DE in Treg cells from wild type and *Irf4*^{-/-} (p<0.05). (b) Venn diagram showing the DE genes in the absence of *Irf4* and our *follicular signature* genes. Genes highlighted in red are upregulated in *Irf4*^{-/-} Treg cells and blue for those that are downregulated. (c) MA plot showing the *follicular signature* genes from *Irf4*-sufficient and -deficient Treg cells. (d-e) IRF4 ChIP-seq tracks of WT Treg cells. Read density showing IRF4 binding at loci of key eTreg cell activation genes such as *Prdm1* (d), *Icos*, *Ctla4* and *Ccr7*, and known T_{FR} cell signature genes (e). Both *Foxo1* and *c-Maf* are targets of IRF4. Boxes highlight IRF4 binding sites that are statistically significant.

Data are representative of 2 independent experiments. KO, knock out; Treg, regulatory T; WT, wild type.

Consistent with our *in vivo* data, we observed that the transcription of lymphoid-homing molecules such as *Ccr7* and *Sell* were upregulated in the absence of *Irf4* (**Fig. 30c**).

To identify possible direct transcriptional effects of IRF4 on T_{FR} cell differentiation, we utilized our published IRF4 ChIP-seq dataset (Vasanthakumar, Liao et al. 2017). Here, ChIP-seq analysis of purified wild type Treg cells revealed 3,817 accessible IRF4 binding sites, targeting 2,575 genes. Putative IRF4 binding sites were found to be enriched in genes that are important for eTreg cell differentiation and function (**Fig. 30d-e**). This included two previously identified binding sites in the *Prdm1* locus in CNS (conserved non-coding

sequences) 9 and in the 3' region of the gene (Cretney, Xin et al. 2011, Ochiai, Maienschein-Cline et al. 2013) (**Fig. 30d**). Notably, several follicular signature genes such as *Bcl6*, *Cxcr5* and *Icos* were also IRF4 targets. IRF4 binding was also observed in the loci of genes that promote lymphoid organ homing including *Ccr7*, *Klf2* and *Slpr1* (genomic tracks for the latter 2 genes not shown). In addition, genes that were essential for the development of both tTreg and peripheral-induced Treg cells such as *Foxo1* was also identified to be an IRF4 target. We observed an IRF4 binding site proximal to the promoter region of *Maf*, suggesting that IRF4 regulates *Maf* expression in Treg cells (**Fig. 30**).

Together, our data confirm that T_{FR} cells are a subset of eTreg cells, found within germinal centres, and its transcriptional profile is dysregulated in the absence of the TCR-induced transcription factor, IRF4. In addition, IRF4 bound to genes essential for T_{FR} cell generation. Given these findings, we sought to determine if the two transcription factors, IRF4 and c-Maf, collaborate to orchestrate the generation of T_{FR} cells.

4.11 c-Maf in T_{FR} cell differentiation.

I) c-Maf is required for T_{FR} cell differentiation.

The transcription factor c-Maf is a member of the activator protein (AP)-1 family and has been long implicated in the differentiation and function of T_{FH} cells (Bauquet, Jin et al. 2009, Kroenke, Eto et al. 2012), suggesting that it may also play an important role in T_{FR} cells. Our RNA-seq analysis identified *Maf* as part of the *follicular signature* genes common to T_{FH}, T_{FH}-21 and T_{FR} cells (**Fig. 29c**). As such, we first analysed the B and T cell compartment of wild type mice by flow cytometry to determine the level of c-Maf expression *in vivo* and found that Treg cells express high levels of c-Maf when compared to CD19⁺ B and naïve CD4⁺ T cells (**Fig. 31a**). Next, to assess the role of c-Maf in T_{FR} cell differentiation, we generated c-Maf conditional knock out mice, where *Maf^{fl/fl}* mice (Wende, Lechner et al. 2012) were crossed to *Foxp3^{Cre}* mice. *Maf^{fl/fl}Foxp3^{Cre}* (c-MafKO) mice lack expression of c-Maf in all mature Treg cells. Similar to the *Irf4^{fl/fl}Foxp3^{Cre}* mice, in non-infected c-MafKO, we observed a diminished splenic T_{FR} cell compartment compared to *Foxp3^{Cre}* controls despite the presence of Foxp3-expressing Treg cells (**Fig. 31b**). A similar finding was observed within the Peyer's patches of c-MafKO mice compared to controls (**Fig. 31c**). In contrast to the *Irf4^{fl/fl}Foxp3^{Cre}* mice, there were no observable difference in the T_{FH} cell compartment of c-MafKO mice (**Fig. 31d**). Intriguingly, there appeared to be a trend of reduced T_{FH} cell

frequencies compared to controls though this was not significant. c-MafKO mice showed normal frequencies of conventional CD4 T cells in the spleen compared to controls (**Fig. 31e**). Given the known requirement of c-Maf for the regulation of ROR γ t Treg cells within the colon (Xu, Pokrovskii et al. 2018), we also analysed the frequencies of conventional CD4 T and Treg cells within the lamina propria of the colon, and found no difference compared to controls (**Fig. 31f**). Key activation-associated molecules typically expressed by eTreg cells such as Nrp-1, KLRG1 and TIGIT were generally unaltered in c-MafKO mice indicating normal activation status of c-MafKO mice (**Fig. 31g**). Analysis of the germinal centre response as measured by the expression of Gl7 and Bcl6 in B cells, and CD138+ plasma cells revealed that there were no differences found in the B and plasma cell compartment in both the spleen and Peyer's patches of cMafKO mice compared to controls (**Fig. 31h**).

Next, to determine the functional outcomes due to the absence of T_{FR} cells, we subjected c-MafKO mice to influenza infection. At day 9 after infection, c-MafKO mice showed no overt phenotype. Examining the spleen and draining LN of c-MafKO and *Foxp3*^{Cre} control mice showed that whilst T_{FR} cells successfully developed in control animals, the population was absent in c-MafKO mice (**Fig. 31i**). Similarly, there were no observable differences in the frequencies of T_{FH}, germinal centre B and plasma cells seen in c-MafKO mice compared to controls (**Fig. 31j**). Combined, our data revealed that whilst c-Maf loss specifically in Treg cells resulted in an absent T_{FR} cell compartment, there were no significant functional consequences.

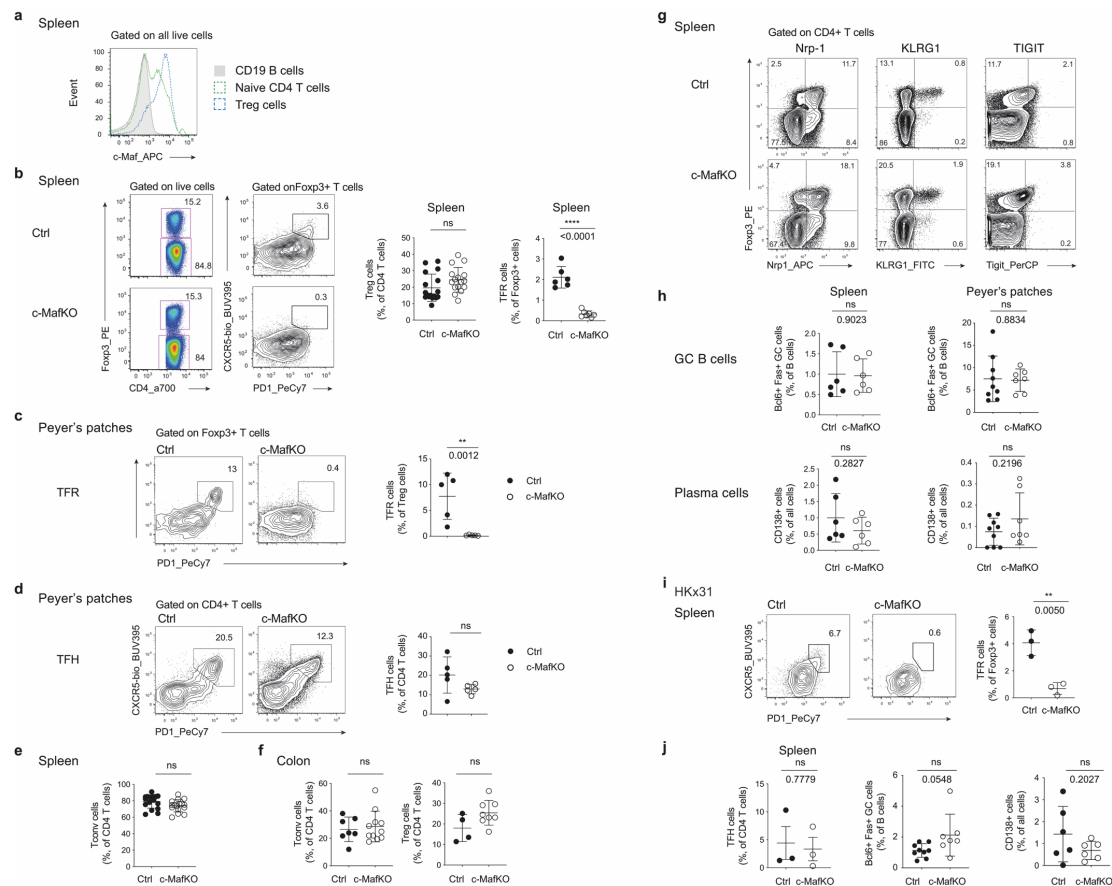


Figure 31. c-Maf is required for T_{FR} cell commitment.

(a) Representative histogram showing the expression of c-Maf by CD19⁺ B, naïve conventional CD4 T and Treg cells from non-infected wild type mice. (b) Flow cytometric plots (left) and quantification (right) of the frequency of Treg cells, and T_{FR} cells from *c-Maf^{fl/fl}Foxp3^{Cre}* (c-MafKO) and control mice. (c-d) Flow cytometric plots (left) and quantification (right) of (c) T_{FR} and (d) T_{FH} cells from the PP of c-MafKO and control mice. (e-f) Graph showing the frequencies of (e) conventional CD4 T cells in the spleen and (f) conventional CD4 T and Treg cells in the colonic lamina propria of c-MafKO and control mice. (g) Flow cytometric plots showing the co-expression of Foxp3 and key Treg cell activation-associated molecules by splenic Treg cells from c-MafKO and control mice. (h) Frequencies of GC B cells (Fas⁺Bcl6⁺) and CD138⁺ plasma cells from the spleen and PP of c-MafKO and control mice. (i) Flow cytometric plots (left) and quantification (right) showing the absence of T_{FR} cells in the spleen of c-MafKO and control mice 9 days after influenza T infection. (j) Frequencies of T_{FH}, GC B and plasma cells from c-MafKO and control mice as in (i).

Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). ** $p < 0.01$, *** $p < 0.0001$ (unpaired Student's t-test). Flow cytometric plots and data are representative of three independent experiments with three mice per group. GC, germinal centre; ns, not significant; PP, Peyer's patches; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

II) Treg cell intrinsic requirement for c-Maf in T_{FR} cells.

To assess the cell-intrinsic requirement of c-Maf in T_{FR} cell differentiation, we generated mixed bone marrow chimeras using donor bone marrow from congenically marked wild type (CD45.1) and c-MafKO (CD45.1/2) to reconstitute sub-lethally irradiated *Rag1*-deficient hosts. Mice were left to reconstitute for 6 weeks and subsequently subjected to influenza virus infection. Spleens and medLN were harvested at day 9 after infection for flow cytometric analysis (**Fig. 32a**). In line with findings from the non-chimeric c-MafKO mice, there a striking absence of T_{FR} cells in the spleen and medLN of mice that received c-MafKO bone marrow compared to controls (**Fig. 32b**). Interestingly, in contrast to *Irf4^{fl/fl}Foxp3^{Cre}* mice, there was no defect in the capacity of Treg cells from the c-MafKO compartment to downregulate CCR7 (**Fig 32c**).

Together, these results indicate that c-Maf is required in a cell-intrinsic manner for the commitment of activated Treg cells to T_{FR} cells, but is not essential for the expression of CCR7.

III) T_{FR} cells jointly express IRF4 and c-Maf.

To understand the expression pattern of IRF4 and c-Maf and their relationship in T_{FR} cell differentiation, we first analysed the Treg cell compartment in the spleens of uninfected *Irf4^{-/-}Foxp3^{RFP}* and wild-type mice and found that *Irf4^{-/-}* Treg cells did not upregulate the expression of c-Maf (**Fig. 33a – top**). Analysis of c-Maf expression based on Treg cell activation status in the spleen of uninfected mice revealed no difference in c-Maf expression by naïve (CD62L^{high}) Treg cells in *Irf4*-deficient vs -sufficient mice but a lack of c-Maf expression by activated (CD62L^{low}) Treg cells in the absence of *Irf4* (**Fig. 33a – bottom**). The defect became more apparent 9 days after an infective challenge with influenza virus (**Fig. 33b**). Here, we observed that both naïve and activated Treg cell populations from *Irf4*-deficient mice showed reduced c-Maf expression (**Fig. 33c**) confirming a possible role for IRF4 in the regulation of c-Maf.

Additionally, we had observed that the expression pattern of c-Maf closely resembled that of IRF4, with Treg cells expressing higher levels of both c-Maf and IRF4 compared to conventional CD4 T cells suggesting that both are dependent on TCR signalling (**Fig. 31a**). To determine if c-Maf was a downstream target of IRF4 in eTreg cells, we used flow cytometry to assess the expression of c-Maf by Treg cells from the spleen, mesenteric lymph

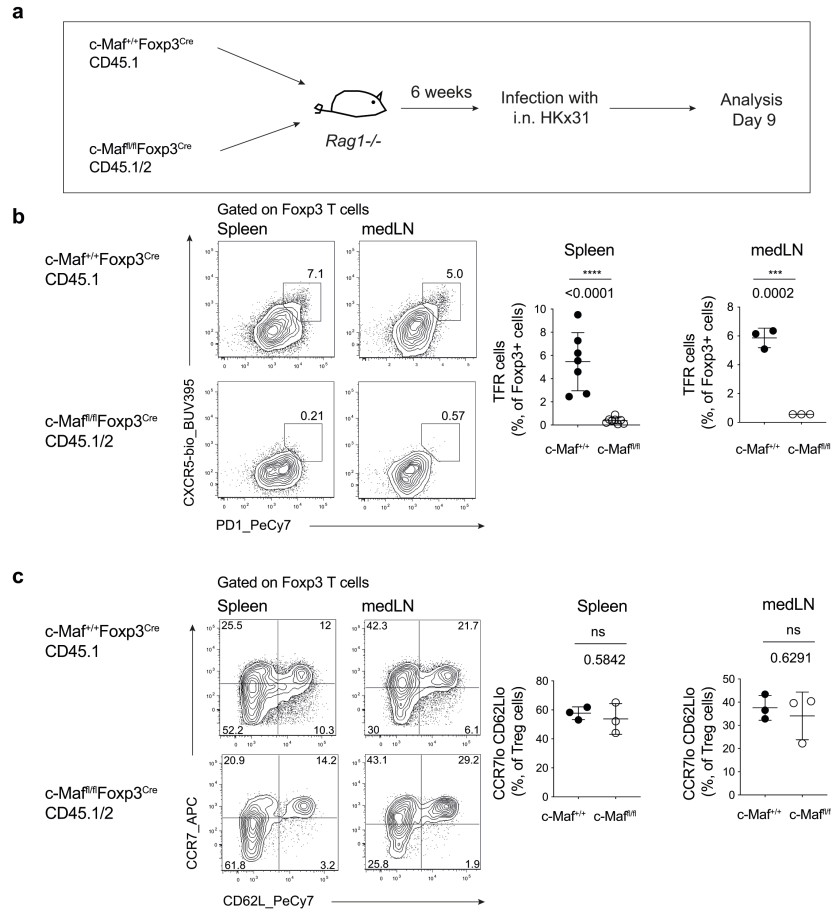


Figure 32. c-Maf is intrinsically required for T_{FR} cell generation.

(a) Mixed bone marrow chimeric mice generated by sublethally irradiating *Rag1*^{-/-} mice and reconstitution with bone marrow cells from wild type controls (CD45.1) and *c-Maf*^{fl/fl}*Foxp3*^{Cre} (CD45.1/2) that were subsequently infection with influenza virus. (b) T_{FR} cells were absent in the CD45.1/2 compartment of spleen and medLN of infected mixed bone marrow chimeric mice as in (a). Right, graphs depict the frequencies of T_{FR} cells in the spleen and medLN. (c) Flow cytometric plots showing the expression of CCR7 and CD62L in splenic and medLN Treg cells of c-MafKO and control bone marrow chimeric mice as in (a). Right, quantification. Numbers adjacent to gated areas or in quadrants indicate percent cells in each throughout. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). ***p=0.0002, ****p<0.0001 (unpaired Student's t-test). Flow cytometric plots and data are representative of four independent experiments with three mice per group. gMFI, geometric mean fluorescence index; medLN, mediastinal lymph node; ns, not significant; T_{FH} follicular T helper; T_{FR}, follicular Treg.

node, Peyer's patches and lamina propria of the SI and colon from *Irf4*^{-/-} and control mice (**Fig. 33d**). We observed a global reduction in c-Maf expression by Treg cells found in non-lymphoid organs of *Irf4*-deficient mice (**Fig. 33d**) confirming that IRF4 is required for the expression of c-Maf.

Next, using our novel *Irf4*^{tdTom} reporter mice, we sought to identify if IRF4 and c-Maf were co-expressed by T_{FR} cells. We first assessed the splenic Treg cell compartment by flow cytometry and found a strong correlation between *Irf4*^{tdTom} reporter and c-Maf expression (**Fig. 33e**). A similar finding was also seen in Peyer's patches and SI Treg cells highlighting that these two TCR-induced transcription factors likely cooperate to regulate the effector differentiation of Treg cells (**Fig. 33e**). Nine days after influenza infection, majority of the T_{FR} cells expressed both c-Maf and *Irf4*^{tdTom} reporter compared to non-T_{FR} Treg cells (**Fig. 33f**) suggesting a common requirement of both transcription factors for T_{FR} cell differentiation.

Combined, our data showed that T_{FR} cells jointly express IRF4 and c-Maf and both are required for T_{FR} cell differentiation. Of note, since we started this project, there have been three publications showing the c-Maf-dependent development of colonic Treg and T_{FR} cells, including our own (Wheaton, Yeh et al. 2017, Xu, Pokrovskii et al. 2018, Neumann, Blume et al. 2019).

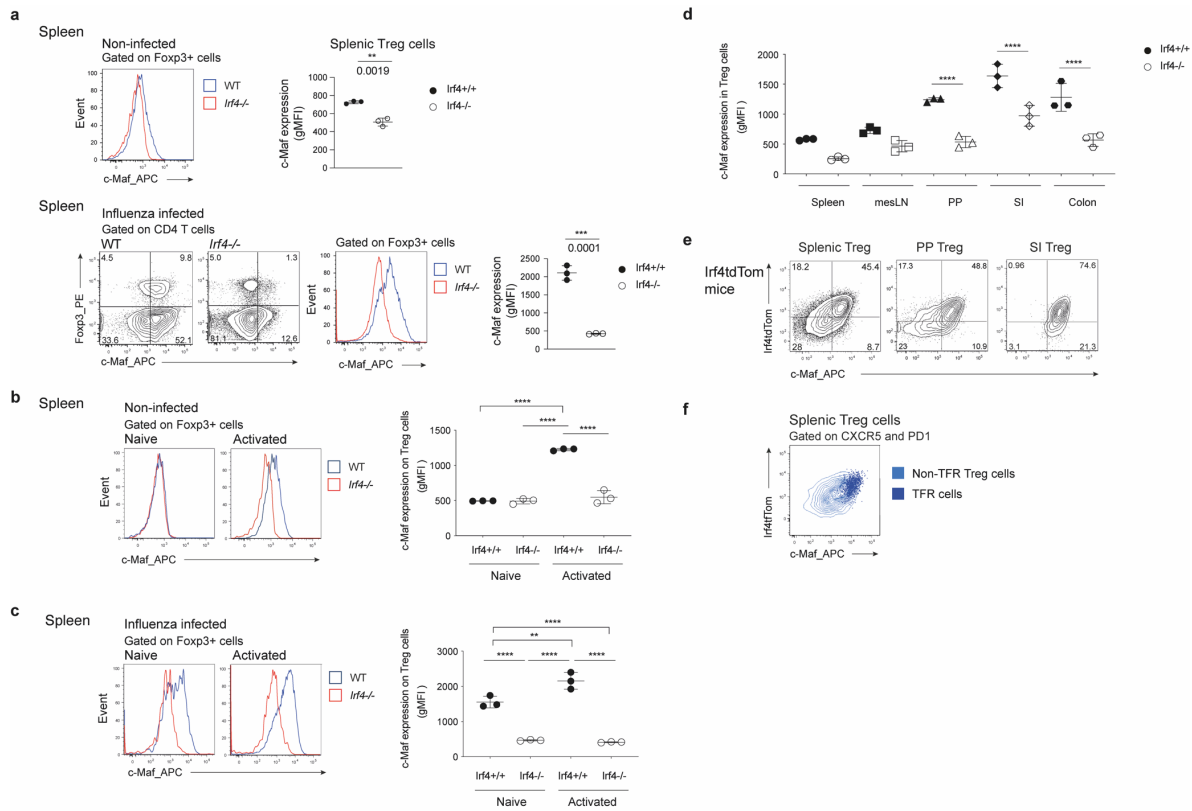


Figure 33. Effector Treg and T_{FR} cells jointly express IRF4 and c-Maf.

(a) Top – Representative histogram (left) and quantification (right) of c-Maf expression by Treg cells from non-infected WT and *Irf4*^{-/-}*Foxp3*^{RFP} mice. Bottom – Flow cytometric plots (left) and histogram (middle) of c-Maf expression by Treg cells from influenza infected WT and *Irf4*^{-/-}*Foxp3*^{RFP} mice at day 9 after infection. Right, quantification of c-Maf expression by Treg cells from the same mice. (b) Representative histograms (left) and quantification (right) of c-Maf expression by naïve and activated Treg cells from the spleen of mice as in (a-top). (c) Histogram (left) and quantification (right) of c-Maf expression by naïve and activated Treg cells from the spleen of mice as in (a-bottom). (d) Intracellular expression of c-Maf by activated Treg cells from splenic, mesLN, PP, lamina propria of SI and colon Treg cells of mice as in (a-top). (e) Flow cytometric plots showing the co-expression of *Irf4*^{tdTom} reporter and c-Maf by Treg cells from the spleen, PP and SI of *Irf4*^{tdTom/+} reporter mouse. (f) Representative mixed contour and dot plot showing the co-expression of *Irf4*^{tdTom} reporter and c-Maf by splenic T_{FR} and non-T_{FR} Treg cells from the *Irf4*^{tdTom/+} reporter mouse 9 days after influenza infection.

Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). Analysed using (a) unpaired Student's t-test and (b-d) ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, non-significant comparisons are unlabelled). Flow cytometric plots and data are representative of four independent experiments with three mice per group.

gMFI, geometric mean fluorescence index; medLN, mediastinal LN; mesLN, mesenteric lymph node; PP, Peyer's patches; SI, small intestine; T_{FH} follicular T helper; T_{FR}, follicular Treg; WT, wild type.

4.12 The transcription factor Foxo1 in T_{FR} cell differentiation.

The Foxo family of transcription factors have been implicated in regulating Treg cell homeostasis through a variety of mechanisms (Ouyang, Liao et al. 2012). By ChIP-seq, we observed that Foxo1 was a direct target gene bound by IRF4 in Treg cells (**Fig. 30**). As such, we examined if Foxo1 acts downstream of IRF4 in the differentiation of T_{FR} cells. Here, we made use of *Foxo1^{fl/fl}pLck^{Cre}* mice, where all positively selected T cells were deficient for Foxo1. In *Foxo1^{fl/fl}pLck^{Cre}* mice, thymic T cell development was previously shown to be relatively normal, though T cell response to TCR activation was dysregulated (Gubbels Bupp, Edwards et al. 2009). We analysed lymphoid organs from *Foxo1^{fl/fl}pLck^{Cre}* mice 9 days after influenza infection. Similar to published findings (Gubbels Bupp, Edwards et al. 2009), frequencies of activated splenic T cells as gated on CD62L^{low}CCR7^{low} Foxp3-negative CD4 T cells were increased in *Foxo1^{fl/fl}pLck^{Cre}* mice, suggestive of a requirement for Foxo1 to regulate T cell activation (**Fig. 34a**). We observed that peripheral deletion of Foxo1 driven by the distal-Lck promoter led to increased T_{FH} and T_{FR} cell frequencies, compared to controls (**Fig. 34b-c**). Analysing the germinal centre compartment, plasma cells were increased in both the spleen and medLN of *Foxo1^{fl/fl}pLck^{Cre}* mice compared to controls (**Fig. 34d**). There were reduced frequencies of germinal centre B cells in the medLN of *Foxo1^{fl/fl}pLck^{Cre}* mice compared to controls but this was not observed within the spleen (**Fig. 34e**). There were increased frequencies of activated splenic Treg cells in mice that had a specific deletion of Foxo1 in positively selected T cells (**Fig. 34f**). Additionally, Treg cells in mice that had a specific deletion of Foxo1 expressed functional Treg cell molecules, such as ICOS and CTLA-4, though the expression of ICOS was increased compared to that of Foxo1 sufficient mice (**Fig. 34g-h**), in keeping with increased Treg cell activation. Of note, I performed four independent experiments using the *Foxo1^{fl/fl}pLck^{Cre}* mouse strain. However, the results from the only experiment with data showing increased frequencies of activated splenic Treg cells that is in line with previously published data (Gubbels Bupp, Edwards et al. 2009) are displayed in the figures.

Given the inconsistencies found in the *in vivo* model, we turned to an *in vitro* stimulation assay to determine if IRF4 regulated the expression of Foxo1. Here, purified naïve conventional CD4 T cells from the spleens and pooled peripheral lymph nodes from *Irf4*-sufficient and -deficient mice were stimulated with plate bound α CD3, IL-2 and α CD28 for 3 days. Cells were then harvested and processed for immunoblot analysis of Foxo1

expression. The results of this experiment captured the inefficient deletion of Foxo1 at the protein level in CD4 T cells, as the Foxo1 band was present in one experiment and not the second (**Fig. 34i**). It appears that in the absence of Foxo1, IRF4 protein levels were not increased. Conversely, in the absence of IRF4, there were also no overt differences in the expression of Foxo1 protein suggesting that IRF4 does not regulate Foxo1.

Based on the Western blot findings (and unrelated experiments using the same mouse strain), we came to the conclusion that deletion of target genes using *pLck^{Cre}* is incomplete. This is particularly problematic for genes critical for naïve T cell survival and quiescence such as Foxo1. Indeed, *Foxo1^{fl/fl}pLck^{Cre}* mice showed variable deletion of Foxo1 in their T cell compartment (data not shown). Thus, although our *in vivo* results suggest a role for Foxo1 in T_{FR} cell biology, more definitive mouse models need to be developed to examine this in more detail.

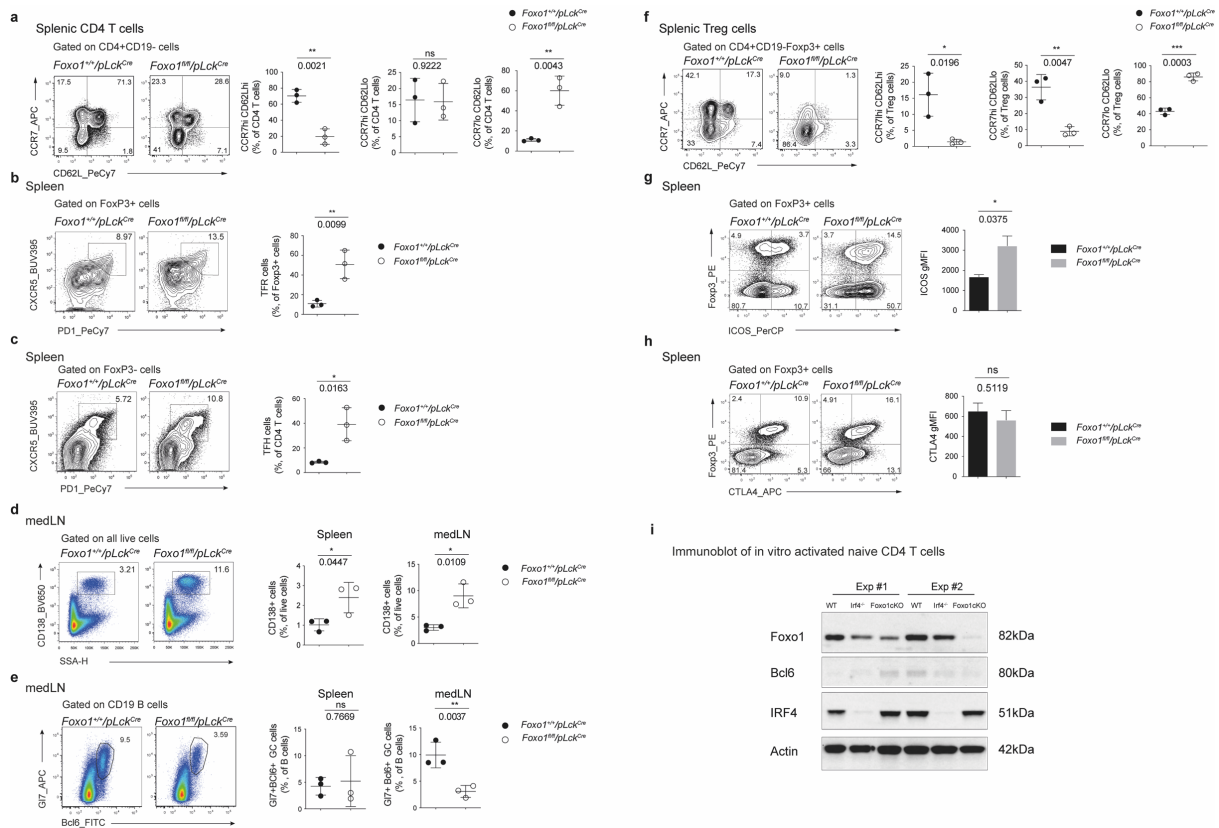


Figure 34. T_{FR} cell generation in the absence of Foxo1.

(a) Flow cytometric plots (left) and quantification (right) of the expression of CCR7 and CD62L by splenic CD4 T cells from mice that have a specific deletion of *Foxo1* and controls 9 days after influenza infection. (b–c) Flow cytometric plots showing T_{FR} (b) and T_{FH} (c) cells in the spleen of mice as in (a). Right, quantification. (d–e) Flow cytometric plots showing the frequency of plasma cells and GC B cells from the medLN of mice as in (a). Right, quantification. (f) Flow cytometric plots (left) and quantification (right) of the expression of CCR7 and CD62L on splenic Treg cells from mice as in (a). (g–h) Flow cytometric plots (left) and quantification (right) of ICOS expression and intracellular CTLA4 expression by splenic Treg cells from mice in (a). (i) Western blot analysis of Foxo1, Bcl6, IRF4 and actin (loading control) using whole lysates of naïve CD4 T cells from WT, *Irf4*^{-/-} and *Foxo1*^{Δ/Δ}*pLck*^{Cre} mice stimulated for 3 days with αCD3, αCD28 and IL-2.

Numbers adjacent to gated areas or in quadrants indicate percent cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±SD). *p<0.05, **p<0.01, ***p=0.0003 (unpaired Student's t-test). Flow cytometric plots and data are representative of one out of four independent experiments with three mice per group. Assay in (i) was performed in duplicates. gMFI, geometric mean fluorescence index; medLN, mediastinal LN; ns, not significant; T_{FH} follicular T helper; T_{FR}, follicular Treg.

inefficient deletion of Foxo1 at the protein level in CD4 T cells, as the Foxo1 band was present in one experiment and not the second (**Fig. 34i**). It appears that in the absence of Foxo1, IRF4 protein levels were not increased. Conversely, in the absence of IRF4, there were also no overt differences in the expression of Foxo1 protein suggesting that IRF4 does not regulate Foxo1.

Based on the Western blot findings (and unrelated experiments using the same mouse strain), we came to the conclusion that deletion of target genes using *pLck^{Cre}* is incomplete. This is particularly problematic for genes critical for naïve T cell survival and quiescence such as Foxo1. Indeed, *Foxo1^{fl/fl}pLck^{Cre}* mice showed variable deletion of Foxo1 in their T cell compartment (data not shown). Thus, although our *in vivo* results suggest a role for Foxo1 in T_{FR} cell biology, more definitive mouse models need to be developed to examine this in more detail.

4.13 TGF-β signalling does not alter the T_{FR} cell generation.

As TGF-β played a role in the early specification of T_{FH} cell precursors and T_{FH} cell differentiation (Marshall, Ray et al. 2015), we wanted to investigate if it was required for T_{FR} cell differentiation. TGF-β transcription is induced in influenza infection with the neuraminidase enzyme cleaving latent TGF-β complex into its bioactive form (Schultz-Cherry 1996, Carlson 2010). To determine whether TGF-β signalling deficiency impaired T_{FR} cell differentiation after viral infection, we examined the germinal centre responses in mice that had a selective deficiency for TGF-β in differentiated T cells after thymic positive selection using the *Tgfb²^{fl/fl}Lck^{Cre}* (hereafter referred to as *Tgfb^{-/-}*) mouse 9 days after influenza infection. *Tgfb^{-/-}* mice showed normal frequencies of splenic Treg cells compared to controls (**Fig. 35a**). Interestingly, both splenic and medLN Treg cells from *Tgfb^{-/-}* mice expressed less Helios (**Fig. 35b-c**), suggesting impaired tTreg cell development or reduced Treg cell activation. However, we observed similar frequencies of CCR7 and CD62L-expressing Treg cells in *Tgfb^{-/-}* mice compared to controls suggesting that TGF-β signalling did not affect Treg cell activation (**Fig. 35d-e**). Deletion of TGF-β in the mature CD4 compartment did not result in an aberrant T_{FR} cell population after influenza infection compared to controls (**Fig. 35f**). Contrary to published data using adoptive transfer

experiments (Marshall, Ray et al. 2015), we observed no difference in the T_{FH} cell response in both the spleen and medLN of *Tgfbr*^{-/-} mice compared to controls (**Fig. 35g**).

Whilst there was a reduction in germinal centre B cell response within the spleen of *Tgfbr*^{-/-} mice, this was not seen in the medLN (**Fig. 35h**). CD138⁺ CD98⁺ expressing plasma cells in both the spleen and medLN of *Tgfbr*^{-/-} mice were unperturbed compared to controls (**Fig. 35i**).

To assess if TGF- β was required for the expression of Treg cell activation-associated molecules, we analysed the expression of KLRG1 in Treg cells and observed a significant increase within the medLN of *Tgfbr*^{-/-} mice compared to controls, though this was not seen in the splenic Treg cell compartment (**Fig. 35j**). Whilst there was a trend to reduced Treg cell expression of ICOS and PD-1 in *Tgfbr*^{-/-} mice, these were not significant when compared to controls (**Fig. 35k**).

Overall, we found that TGF- β signalling deficiency did not impair T_{FR} cell differentiation though it may have some effects on thymic-derived Treg cells in the periphery. Based on the findings of this experiment, a repeat experiment was not performed. These findings could be attributed to the pleiomorphic nature of TGF- β as a cytokine for T cell proliferation, and activation. Additionally, the transgenic mouse system utilizing a distal Lck-Cre could be masking the true effects of TGF- β deficiency as only T cells with lower TCR affinity have been selected. Further, these mice showed no signs of autoimmune disease.

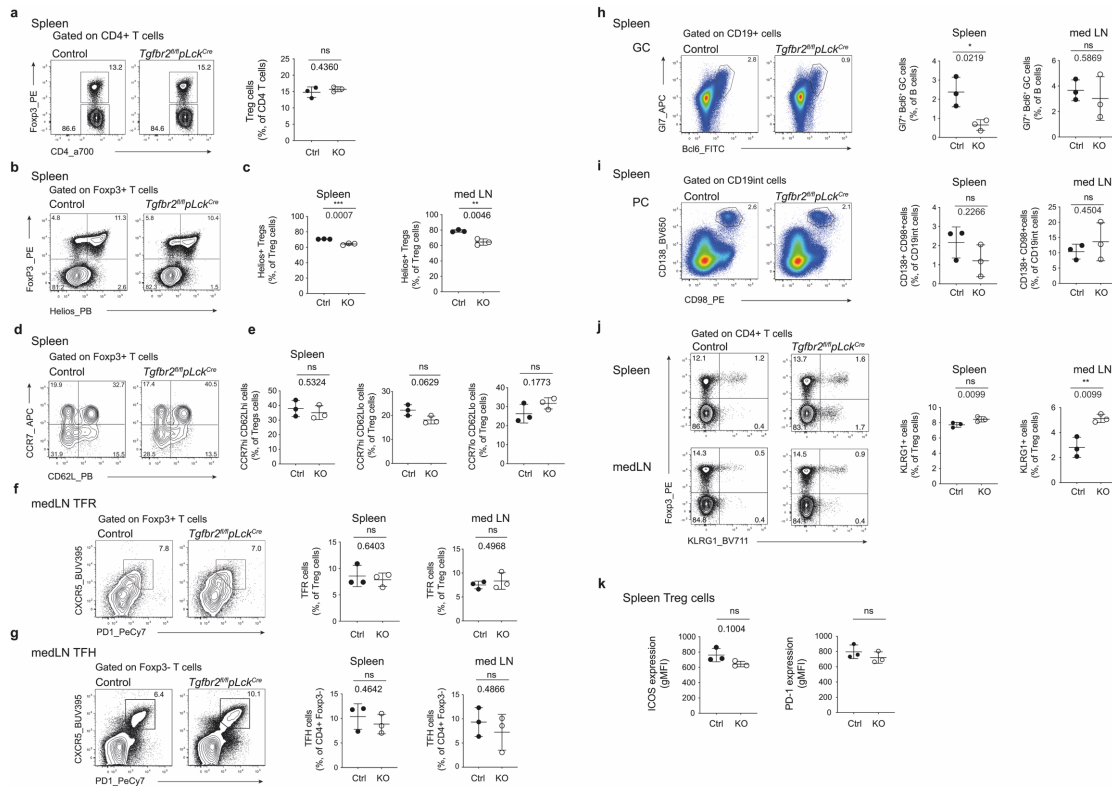


Figure 35. TGF- β deletion does not restrict follicular T cell differentiation.

(a) *Tgfb2^{fl}/pLck^{Cre}* (KO) mice that lack TGF- β in their mature CD4 compartment express similar frequencies of Treg cells compared to controls day 9 after influenza infection. Right, quantification. (b) Flow cytometric analysis of splenic Treg cells from KO mice showing Helios expression compared to controls. (c) Graphs reflect the frequency of Helios⁺ Treg cells in both the spleen and medLN. (d–e) Flow cytometric plots (left) and quantification (right) showing CCR7 and CD62L expression by Treg cells from the spleen of mice as in (a). (f–g) Both KO and control mice had similar follicular T cell responses shown by the expression of CXCR5^{high}PD-1^{high} (f) T_{FR} and (g) T_{FH} cells. Right, quantification. (h) CD19⁺G17⁺Bcl6⁺ GC B cells from the spleen of mice as in (a). Right, quantification. (i) Flow cytometric plot (left) and quantification (right) of plasma cell populations from the spleen of mice as in (a). (j) Cell surface expression of KLRG1 by Treg cells from the spleen and medLN. Right, quantification. (k) ICOS and PD-1 expression by Treg cells in KO mice compared to controls.

Flow cytometric plots and data are representative of one experiment with 3 mice in each group. Numbers adjacent to gated areas or in quadrants indicate percent cells in each. Each symbol represents an individual mouse; small horizontal lines indicate the mean ($\pm$ SD). * $p=0.0219$, ** $p<0.01$, *** $p=0.0007$ (unpaired Student's t-test). Ctrl, control; GC, germinal centre; medLN, mediastinal LN; ns, not significant; T_{FH} follicular T helper; T_{FR}, follicular Treg.

Discussion

For almost a decade now, the developmental pathway and functionality of T_{FR} cells both in mice and humans have been intensively researched (Sage, Francisco et al. 2013, Vaeth, Muller et al. 2014, Maceiras, Fonseca et al. 2017, Sayin, Radtke et al. 2018, Xie and Dent 2018). Excessive T_{FH} cell expansion and perturbed germinal centre responses in the context of diminished T_{FR} cell presence culminates in deleterious outcomes as evidenced by various autoimmune diseases in mice and humans (Linterman, Pierson et al. 2011, Wu, Chen et al. 2016, Fonseca, Romao et al. 2018, Yang, Wang et al. 2019). There has also been extensive research towards investigating the role of T and B cell interactions within the Peyer's patches in the regulation of intestinal IgA production (Cerutti, Chen et al. 2011). Similar to the findings within the germinal centre of secondary lymphoid organs, the lack of T_{FR} cells results in dysregulated IgA production in the colon (Wheaton, Yeh et al. 2017, Xu, Pokrovskii et al. 2018, Neumann, Blume et al. 2019).

In this chapter, we utilized influenza infection in various transgenic and chimeric mice models to study T_{FR} cell differentiation and to uncover the role of IRF4 in their generation. As previously mentioned, eTreg cells are a specialised subset of Treg cells that are predominantly found within non-lymphoid organs. Whilst T_{FR} cells are found in a specialized compartment of secondary lymphoid organs, its differentiation pathway mirrored that of other well documented eTreg cell populations (Cipolletta, Feuerer et al. 2012, Vasanthakumar, Moro et al. 2015, Panduro, Benoist et al. 2016). In particular, T_{FR} cells adopt a transcriptional program and respond to cytokines that are specific for the germinal centre environment. To participate in local immune homeostasis, we know that T_{FR} cells co-opt the same transcriptional machinery to Th cells, in this case T_{FH} cells, but also largely conserved the Treg cell phenotype, maintained by the expression of Foxp3 and various effector and functional molecules such as CTLA-4, ICOS, and KLRG1 (Sage and Sharpe 2016). Interestingly, we showed that only 25% of T_{FR} cells expressed the transcription factor specific for eTreg cells, Blimp-1 (Cretney, Xin et al. 2011). We posit that the likely explanation for this is the gating strategy to identify T_{FR} cells by flow cytometry, which is based on placing a gate on CD4⁺ Foxp3⁺ T cells with the highest expression of CXCR5 and PD-1. As such, we postulate that only the Blimp-1 expressing Treg cells are bona fide T_{FR} cells. In line with this, our results demonstrated that compared to T_{FR} cells that did not express *Blimp1*^{GFP}

reporter, *Blimp1*^{GFP} reporter-expressing T_{FR} cells downregulated CD62L, CCR7 and expressed high levels of ICOS. In the absence of a double reporter mouse for *Blimp-1* and *Il10* expression, we were unable to determine if the *Blimp1*^{GFP} reporter positive T_{FR} cells were indeed the IL-10 producers. Further studies will be required to resolve this.

As tools for documenting the expression of IRF4 were limited, in particular the ability to distinguish the levels of *Irf4* transcript and protein (Man, Miasari et al. 2013), we generated a novel reporter mouse to enable the study of IRF4 protein expression at a single cell level. Analysis of the IRF4 protein expression from the *Irf4*^{tdTom} reporter transgenic mouse at different stages of Treg cell activation and T_{FR} cell generation showed that T_{FR} cells express high levels of IRF4. Similar to other tissue-Treg cells for example, the visceral adipose tissue Treg cells (Vasanthakumar, Moro et al. 2015), we showed that the requirement of IRF4 for T_{FR} cell differentiation is also cell-intrinsic. T_{FR} cells preferentially locate within the germinal centre after influenza infection and its homing to the lymphoid follicle is not only in response to CXCL13 (Linterman, Pierson et al. 2011), but also requires the downregulation of CCR7. Our data showed that mice with a Treg cell specific deletion of IRF4 had an impaired ability to downregulate CCR7, which resulted in markedly reduced Treg cells within the germinal centre. Combined, our data confirms that IRF4 is critically required for the maintenance of peripheral immune homeostasis through the regulation of Treg cell trafficking and homing receptors.

Consistent with published data (reviewed in Sage and Sharpe 2016, Fazilleau and Aloulou 2018), our research confirmed that T_{FR} cells are phenotypically similar to their germinal centre T cell counterparts, the T_{FH} cells. The transcriptional profile of T_{FR} cells however has yet to be fully described. Despite the expression of canonical Treg cell markers by T_{FR} cells, our RNA-seq analysis revealed similarities in the transcriptional profile of T_{FR} cells to IL-21 secreting and non-secreting T_{FH} cells. We uncovered a *follicular signature* gene set that comprised of 509 genes, which included known key follicular T cell genes. This finding strongly supports the notion that T_{FR} cells follow the same multistep differentiation process and share a common transcriptional program to T_{FH} cells. We observed that many of these *follicular signature* genes are targets of the TCR-induced transcriptional regulator, IRF4. We showed that the core transcriptional program for T_{FR} cell commitment and function was

deregulated in the absence of IRF4. In line with the known critical function of IRF4 to regulate the expression of key eTreg cell related genes (Vasanthakumar, Moro et al. 2015), our findings confirm that IRF4 is also critically required for the regulation of follicular T cell related genes.

One of the key roles of T_{FR} cells is to regulate T_{FH} cell responses in the germinal centre, which is essential for the production of high quality antibody generating B cells (Wollenberg, Agua-Doce et al. 2011, Wing, Ise et al. 2014). We observed spontaneous autoimmune disease in the absence of a T_{FR} cell compartment and the expansion of T_{FH} cells seen in the *Irf4^{fl/fl}Foxp3^{Cre}* mice. In our transcriptomic analysis of IRF4, we found that c-Maf, another key transcriptional regulator of follicular T cell differentiation (Kroenke, Eto et al. 2012), was also a target of IRF4. Intriguingly, we did not see a similar fate in mice that had a Treg cell-specific deletion of c-Maf. Whilst these mice had an absent T_{FR} cell compartment, they were healthy and had almost normal frequencies of T_{FH}, germinal centre B and plasma cells. It is unclear why there is such a dichotomy in response to the deficiency of an important eTreg cell subset, and as such, much work has to be done to understand the complexity of the T_{FH}-T_{FR} cell relationship in the regulation of germinal centre responses.

One of the many reported functions of T_{FR} cells is the regulation of the magnitude and size of germinal centre reactions (reviewed in Sage and Sharpe 2015). However, in both the *Irf4^{fl/fl}Foxp3^{Cre}* and *c-Maf^{fl/fl}Foxp3^{Cre}* mice, despite the striking absence of T_{FR} cells, we saw inconsistent results on germinal centre B and plasma cell consequences. There was spontaneous germinal centre formation with a trend to increased plasma cells in mice that had a specific deletion of IRF4 in the Treg cell compartment, whilst there were no differences seen in mice that had a Treg cell-specific deletion of c-Maf. This suggests that the loss of T_{FR} cells does not result in a global defect of germinal centre nor plasma cell differentiation. Further experiments are required to determine the effects of the loss of either transcription factor on antigen-specific antibody production, as it has been shown that T_{FR} cells can directly regulate B cells (Sage, Paterson et al. 2014, Wu, Chen et al. 2016, Laidlaw, Lu et al. 2017, Clement, Daccache et al. 2019). In support of this, mice deficient for c-Maf in the Treg cell compartment showed increased IgA production (Neumann, Blume et al. 2019).

In sum, the generation of a *follicular signature* gene expression profile has allowed us to have a near complete view of the transcriptional signature of T_{FR} cells, showing that IRF4 is indeed an integral player to the regulation of key T_{FR} cell-related molecules. Our findings support the conclusion that cell-intrinsic expression of both IRF4 and c-Maf by Treg cells are required for the generation of T_{FR} cells. In the following chapter, we sought to further unravel the relationship between IRF4 and c-Maf in the molecular control of T_{FR} cells. Using various molecular techniques, we documented the chromatin landscape of this essential subset of eTreg cells and find the link between transcription and epigenetic modifications.

Chapter Five: Molecular regulation of T follicular regulatory cells

Introduction

The regulation of gene expression is a complex process that is critical for Treg cells to respond and adapt to the environment. It is well understood that Treg cell diversity is governed by distinct, tightly regulated molecular mechanisms. Upon T cell receptor (TCR) stimulation, Treg cells are activated and undergo fundamental changes in gene expression whereby genes can be turned on or off depending on the signals that they receive. Changes to the chromatin structure result in epigenetic changes that underpin the specific transcriptional programs. The recruitment of a specific combination of transcription factors regulates the differentiation and function of these Treg cells to maintain heterogeneity.

As previously discussed, mature Treg cells exit the thymus and undergo further differentiation based on various cues in the periphery. TCR stimulation results in the generation of effector (e)Treg cells. Using the lymphoid follicle as an inducible model of eTreg cell differentiation after influenza infection, we examined the transcriptional and epigenetic signature of follicular T regulatory (T_{FR}) cells. In the previous chapter, we described that the TCR signalling-induced transcription factor IRF4 is critical for driving the differentiation and fully suppressive function of T_{FR} cells, exemplified by perturbed germinal centre compartment after influenza infection, and dysregulated eTreg cell transcription profile seen in *Irf4*-deficient mice. We also showed that c-Maf is critical for the differentiation of Treg cells into T_{FR} cells and subsequently for the acquisition of a *follicular signature* gene expression profile. Whilst these two TCR-induced transcription factors are essential for T_{FR} cell development, the way in which either controls T_{FR} cell biology remains unclear. In this chapter, we used various molecular techniques to assess the transcriptional program of T_{FR} cells.

Results

5.1 Chromatin accessibility profiling.

I) The chromatin architecture of naïve and follicular CD4 T cells.

To determine the genome-wide accessibility of chromatin of follicular and non-follicular T cells, we used flow cytometry to isolate naïve (CD62Lhi) conventional CD4 T cells, IL-21-negative (T_{FH}) and IL-21-positive follicular helper T cells (T_{FH-21}) as well as naïve (CD62Lhi) Treg and T_{FR} cells from pooled spleen and mediastinal lymph nodes of influenza infected *Foxp3^{RFP}Il21^{GFP}* reporter mice at day 9 of the infection (**Fig. 36a** shows the gating strategy). We chose to segregate IL-21 expressing T_{FH} cells from those that did not, as we hypothesized that the chromatin landscape of T_{FH} and T_{FH-21} cells would be different because cytokine production may signal a broader differentiation state.

Next, we performed assay for transposase accessible chromatin followed by sequencing (ATAC-seq) (Buenrostro, Giresi et al. 2013) to assess the accessibility of chromatin in these T cell populations. Following alignment of reads to the mouse genome, visual assessment of the relationships among samples was performed as quality control. Accessible regions were assigned to the nearest gene and later annotated to the nearest transcription start site. In total, we identified 38,918 high confidence open chromatin regions across all cell types analysed (ATAC-seq peaks). Multidimensional scaling (MDS) of the normalized signal intensity of these regions revealed that follicular CD4 T cells clustered together independent of *Foxp3* expression (**Fig. 36b**). These data indicate that the chromatin architecture of CD4 T cells show greater similarity based on the cell's activation state. Next, we performed a series of pairwise comparisons between the various T cell subtypes to quantify the extent of chromatin changes. The numbers of open chromatin regions identified from each T cell subtype are shown in **table 3**.

Cell type	Number of open chromatin regions
Naïve CD4 T	25,778
T _{FH}	28,125
T _{FH} -21	18,543
Naïve Treg	28,533
T _{FR}	35,074

Table 3. Number of open chromatin regions identified in ATAC-seq samples.

Examination of raw ATAC-seq read distribution verified regions assigned to genes associated with T cell activation, such as *Bach2* and *Cd44* (**Fig. 36c-d**). We observed that certain chromatin regions lose chromatin accessibility (grey shade) with activation, whilst others gain accessibility (orange shade) in late effector T cell differentiation. Some areas of chromatin remain open regardless of the activation status of the cells (purple shade). Importantly, serving as an internal control, we identified a site of open chromatin in the Treg specific demethylated region of the *Foxp3* gene in Treg and T_{FR} cells, which was absent in CD4 and T_{FH} cells, consistent with its lack of accessibility in non-Fopx3 expressing CD4 T cells (**Fig. 36e**). Our data indicate that cellular differentiation or activation is paralleled by changes in chromatin accessibility.

II) Differences in the chromatin landscape of follicular T cells.

First, to determine the chromatin landscape of T_{FH} cells, we compared naïve CD4 T and T_{FH} cells and found 2,814 differentially accessible (DA) peaks associated with 2,256 nearest neighbour genes. We also compared naïve CD4 T and T_{FH}-21 cells and found 2,512 DA sites, associated with 2,041 genes (**Fig. 37a**). The overlap of these two comparisons encompasses high confidence peaks, which we termed the *T_{FH} cell chromatin signature*. This signature comprised of open chromatin regions in key follicular T cell genes, such as *Ascl2*, *Cebpa*, *Ctla4*, *Il10*, *Maf* and *Slpr1* (**Fig. 37a**). Notably, the areas of open chromatin were seen in the promoter regions of *Cebpa*, *Maf* and *Slpr1* (**Fig. 37b**), consistent with their active transcription. We also observed multiple open chromatin regions identified in follicular genes (black arrows) from both T_{FH} and T_{FH}-21 cells (**Fig. 37b**).

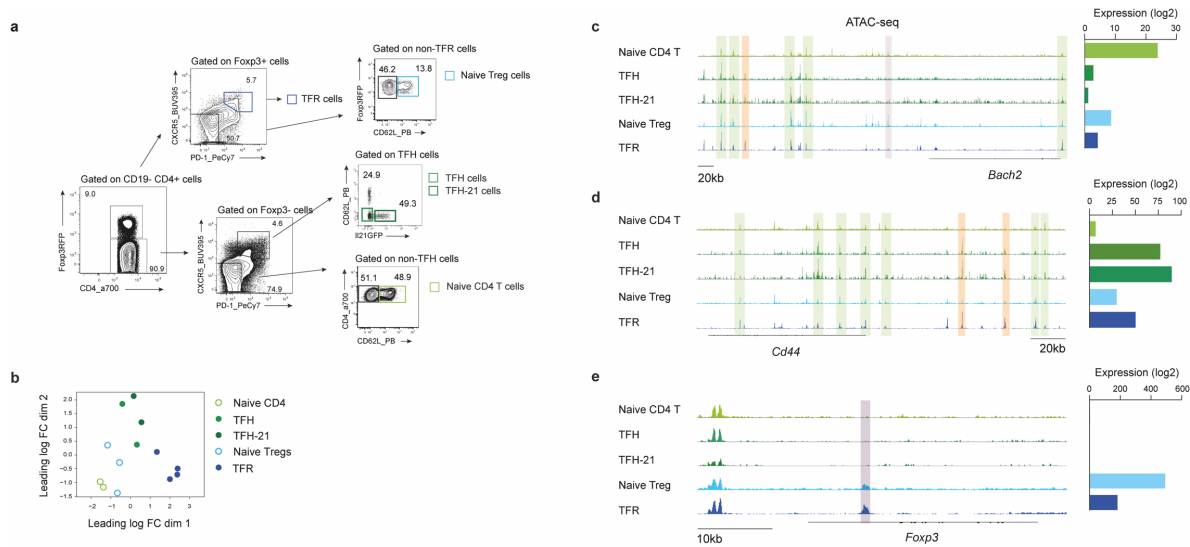


Figure 36. The chromatin architecture of naïve and follicular CD4 T cells.

(a) Gating strategy of sorted CD4 T cells. Naïve (CD62Lhi) CD4, IL-21-negative (T_{FH}), and IL-21-positive T_{FH} (T_{FH}-21), naïve (CD62Lhi) Treg and T_{FR} cells from pooled spleen and mediastinal lymph nodes were sorted at the peak of influenza infection from *Foxp3*^{RFP}*Il21*^{GFP} reporter mice and subjected to ATAC-seq. (b-c) Multidimensional scaling plot of the ATAC-seq data from the CD4 T cell subsets from (a) showing clustering of (b) naïve versus follicular samples after normalization. (c-d) Normalized ATAC-seq genome tracks (left) and mRNA expression patterns (right) of the indicated T cell subtypes of selected CD4 T cell activation genes, including *Bach2* (c), and *Cd44* (d) showing dynamic regulation of chromatin architecture across naïve CD4, T_{FH}, T_{FH}-21, naïve Treg and T_{FR} cells. Loss of chromatin accessibility (grey shade) with activation and gain of accessibility (orange shade) in follicular T cells. Areas of open chromatin in both naïve and follicular CD4 T cells are highlighted in green. (e) Genome tracks of the indicated T cell subtypes showing minimal accessibility at *Foxp3* in non-Treg CD4 T cell populations. Highlighted region shows cell type-specific ATAC-seq peak.

Data are pooled from two independent experiments. ATAC-seq, assay for transposase-accessible chromatin followed by next-generation sequencing; T_{FH}, follicular T helper; T_{FR}, follicular Treg; Treg, regulatory T.

Interestingly, the mRNA expression of the aforementioned genes was different with T_{FH}-21 cells expressing the highest levels of *Cebpa*, *Il10*, *Maf* and *Il21* compared to T_{FH} and naïve CD4 T cells. Furthermore, although the mRNA expression of *Il21* gene increased from naïve CD4 to T_{FH} cells, visually, we found no DA peaks unique to T_{FH}-21 cells suggesting that IL-21 production by T_{FH} cells is not limited by chromatin accessibility (**Fig. 37c**).

As we were interested to identify differences in the chromatin landscape of T_{FH} cells that secreted IL-21 compared to those that do not, we performed a pairwise comparison between

T_{FH} and T_{FH}-21 cells. This comparison identified 668 DA peaks associated with 599 genes. Whilst the majority of these genes were associated with cell development and adhesion, we observed an area of open chromatin in the *Cxcr4* locus in T_{FH} cells but not T_{FH}-21 cells (**Fig. 37d**). The chemokine receptor, CXCR4 mediates the migration of T_{FH} cells to the dark zone of the germinal centre where B cells undergo somatic hypermutation and class-switch recombination (Oropallo and Cerutti 2014). Interestingly, despite the difference in chromatin accessibility in the T_{FH} locus, it did not directly translate to an effect on the transcription of *Cxcr4*. We observed increased *Cxcr4* gene expression in T_{FH}-21 cells suggesting that IL-21 secreting T_{FH} cells may preferentially locate within the dark zone.

As we saw similarities between follicular T cells independent of Treg or non-Treg lineage identity in the MDS plot (**Fig. 36b**), we next wanted to identify a T_{FR} cell-specific chromatin accessibility pattern. To this end, we expanded our analysis to include the comparison of naïve Treg and T_{FR} cells. This yielded 6,965 DA sites associated with 4,573 genes, including open chromatin regions around the hallmark follicular T cell transcription factor *Bcl6*, and the germinal centre light zone homing receptor, *Cxcr5* (**Fig. 37e**).

Follicular T cell chromatin signature (f) Venn diagram showing the overlap of DA peaks in naïve Treg versus T_{FR} cells, naïve CD4 T versus T_{FH} cells, and naïve CD4 T versus T_{FH}-21 cells. The nearest neighbour genes of interest from the intersection of the three gene sets are shown in the box on the right. Numbers in the circles represent genes. Peaks were called using the reference set of 38,918 ATAC-seq peaks identified. (g) Genomic tracks showing chromatin accessibility (left) and mRNA expression (right) of the indicated T cell subtypes at (g) *Maf*, (h) *Cebpa* and (i) *Il10*. Regions in orange show follicular cell type-specific ATAC-seq peaks. Purple shade indicates a region of accessible chromatin in naïve Treg and follicular CD4 T cells. Arrow shows areas of open chromatin at promoter regions. (j) Unsupervised heat map of ATAC-seq peaks showing *follicular signature genes* identified by RNA-seq across naïve conventional CD4 and Treg, T_{FH}, T_{FH} 21, and T_{FR} cells from influenza infected *Foxp3^{RFP}Il21^{GFP}* reporter mice. Legend indicates Z-score.

Data are pooled from two independent experiments. ATAC-seq, assay for transposase-accessible chromatin followed by next-generation sequencing; DA, differential accessible; FDR, false discovery rate; Treg, regulatory T; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

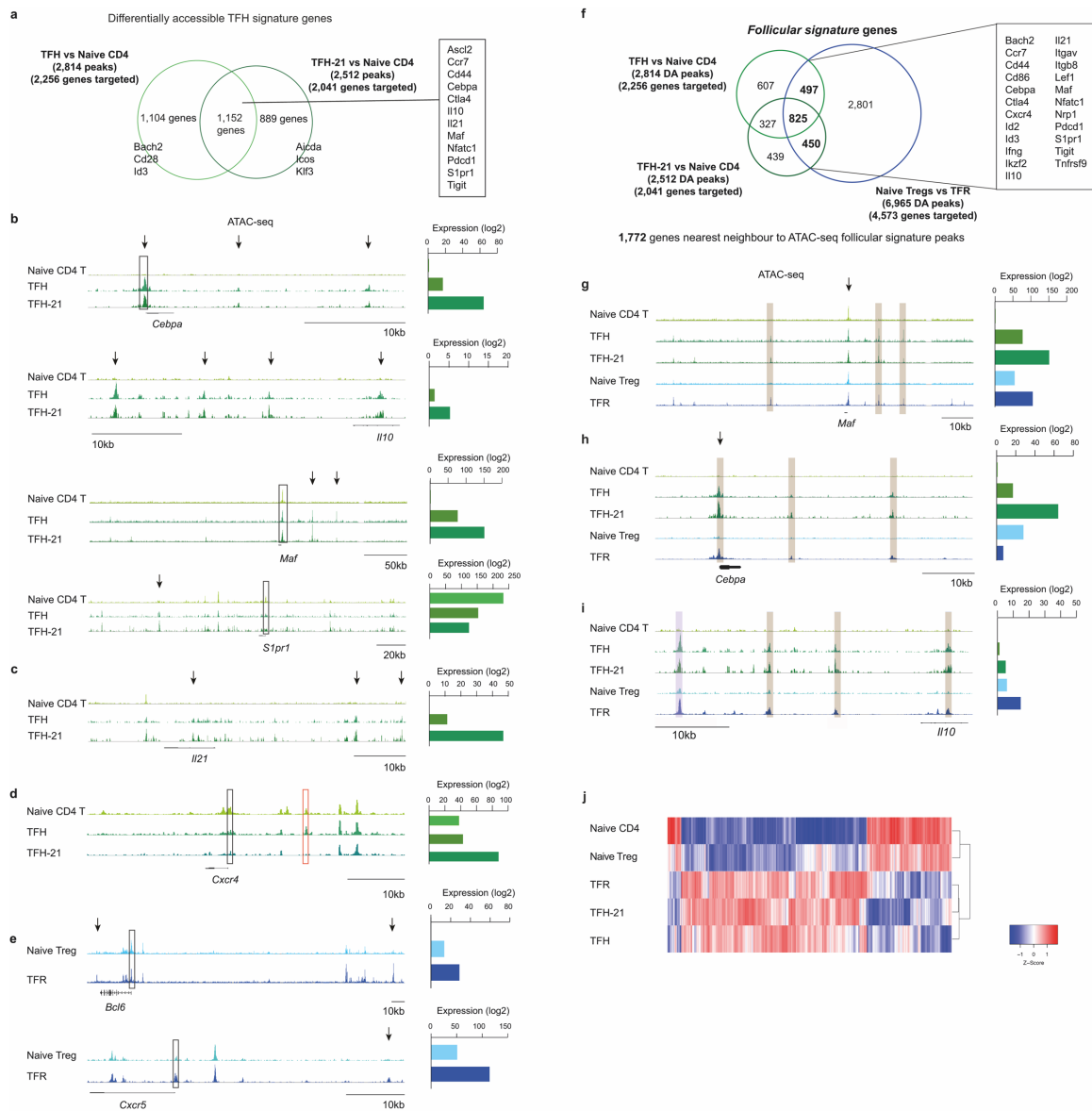


Figure 37. Identification of the chromatin landscape of follicular CD4 T cells.

T_{FH} cell chromatin signature (a) Venn diagram showing the overlap of DA peaks in naïve CD4 T versus T_{FH} cells, and naïve CD4 T versus T_{FH}-21 cells. (b) Normalized genome tracks of naïve CD4, T_{FH} and T_{FH} 21 showing dynamic changes of chromatin architecture in key follicular T cell related genes such as *Cebpa*, *Il10*, *Maf*, and *S1pr1*. Black arrows highlight DA peaks between naïve and follicular CD4 T cells. (c) ATAC-seq read density at the *Il21* locus from indicated cells. Black arrows indicate regions of significantly increased chromatin accessibility in T_{FH} and T_{FH} 21 cells. (d) ATAC-seq read density at the *Cxcr4* locus from indicated cells. Red box highlights the DA peak between T_{FH} and T_{FH} 21. (e) Genomic tracks showing DA peaks (black arrow) between naïve Treg and TFR cells in *Bcl6* and *Cxcr5*. Black boxes indicate the promoter region.

Next, we compared this set of T_{FR} cell-specific sites with the T_{FH} and T_{FH}-21 cell-specific sites (**Fig. 37a**), as the sites overlapping in this comparison would be specific to CD4 T cells that are found within the germinal centre. This identified 1,772 nearest neighbour genes at which chromatin was uniquely regulated in follicular T cells, regardless of Foxp3 expression (**Fig. 37f**). We considered this to be the *follicular T cell chromatin signature* and observed that this overlap included many genes known to be important for the follicular T cell phenotype, differentiation and function, such as *Il10*, *Il21*, *Lef1*, *Maf* and *Pdcd1* (encoding PD-1) (Ma, Deenick et al. 2012). Visual inspection of normalized ATAC-seq tracks of two genes that belong to the *follicular gene signature* identified by RNA-seq analysis (shown in **Chapter 4.9 – Fig. 29c**), *Maf* and *Cebpa*, demonstrated unique chromatin changes seen in all follicular T cell subsets (**Fig. 37g-h**, highlighted by areas shaded in orange). We observed an area of open chromatin in all CD4 T cell subsets analysed at the *Maf* promoter (**Fig. 37g**, black arrow). In contrast, only follicular CD4 T cells had an area of open chromatin in the *Cebpa* promoter, suggesting that the chromatin is poised to allow factors that promote gene transcription specific for follicular T cells (**Fig. 37h**, black arrow). Additionally, analysis of the *Il10* locus revealed open chromatin regions specific to follicular T cells (blue shade) with one area of chromatin upstream of the *Il10* promoter (purple shade) that showed a gain in accessibility in follicular T cells that was also accessible in naïve Treg cells (**Fig. 37i**).

We next generated a heatmap using unsupervised clustering of the ATAC-seq peaks based on the *follicular signature gene set* identified by RNA-seq. This showed that naïve CD4 and naïve Treg cells cluster together, while all follicular samples clustered together independent of Foxp3 expression (**Fig. 37j**).

Our data confirmed that as T cells differentiate from a naïve conventional or regulatory CD4 T cell into an effector state, dynamic changes occur in the chromatin accessibility and unique chromatin changes can be seen in follicular T cell subsets. Both naïve and follicular chromatin modules appear to be very divergent, highlighting the plasticity of epigenetic programming.

5.2 IRF4 and c-Maf regulate common and distinct genes in follicular T cell compartment.

In Chapter 4, we found that both IRF4 and c-Maf were critical for T_{FR} cell differentiation. To identify genes regulated by IRF4 and c-Maf in Treg cells, we analysed published RNA-seq

datasets comparing wild-type (WT) and *c-Maf*-deficient Treg cells isolated from the Peyer's patches, enriched for T_{FR} cells (Wheaton, Yeh et al. 2017) together with the *Irf4*^{-/-} RNA-seq dataset (A. Vasanthakumar, Kallies lab). In total, we identified 883 genes deregulated in the absence of IRF4 and 321 genes as differentially expressed (DE) in the absence of c-Maf (FC of 1.1, 0.05 FDR). We observed 106 DE genes that were shared between Treg cells from *c-Maf*- and *Irf4*-deficient mice in comparison to WT Treg cells (**Fig. 38a**). Consistent with the absence of T_{FR} cells in *c-Maf*- and *Irf4*-deficient mice, the overlap was enriched for genes that were essential for follicular T cell phenotype and function, including *Bcl6*, *Cebpa*, *Cxcr5*, *Il10*, *Maf*, *Pdcd1* and *Sh2d1a* (SAP), all of which were downregulated in the absence of both transcription factors. The core set of 106 genes that were common in the absence of both transcription factors also included some eTreg cell associated molecules such as *Egfr2*, *Nrp1* and *Tigit*.

Of the 777 differentially regulated genes that were exclusively found in *Irf4*^{-/-} Treg cells compared to controls (**Fig. 38a**), the vast majority of transcripts were reduced. This group was enriched for genes essential for Treg cell maintenance, activation and differentiation such as *Ahr*, *Cd44*, *Ikzf2* (Helios), *Klrg1*, *Tnfrsf4* (OX-40), *Tnfrsf9* (4-1BB) and *Tnfrsf18* (GITR) (Apetoh, Quintana et al. 2010, Cheng, Yuan et al. 2012, Vasanthakumar, Liao et al. 2017, Ye, Qiu et al. 2017, Thornton, Korty et al. 2018); and those critical for eTreg cell function, for example *Areg*, *Ctla4* and *Icos* (Cretney, Xin et al. 2011, Wing, Ise et al. 2014, Arpaia, Green et al. 2015). Kruppel-like factor (*Klf2*), which controls T cell trafficking through the regulation of sphingosine-1-phosphate receptor 1 (S1PR1) expression (Weinreich, Takada et al. 2009), was increased in *Irf4*^{-/-} Treg cells. Conversely, the downregulation of *Ccr7*, functionally required for tissue homing and trafficking was impaired in *Irf4*^{-/-} Treg cells (Campbell 2015). In addition, the transcription factor, *Bach2*, shown to inhibit eTreg cell differentiation was upregulated in *Irf4*^{-/-} Treg cells (Roychoudhuri, Hirahara et al. 2013, Sidwell, Liao et al. 2020).

In the c-Maf dataset, we found 215 genes that were exclusively DE in the absence of c-Maf, the majority of which were downregulated (**Fig. 38a**). Among these were key molecules associated with regulatory function in colonic Treg cells, including *Il23r* and *Rorc* (Sefik, Geva-Zatorsky et al. 2015), and *Myb*, essential for eTreg cell survival and proliferation (Dias, D'Amico et al. 2017). In addition, expression of some follicular CD4 T cell associated molecules were also downregulated, including *Il21* and *Slamf1* in the absence of c-Maf (**Fig.**

38a). Interestingly, the transcription factor, *Ascl2*, known to be important for the expression of CXCR5 in T_{FH} cells (Liu, Chen et al. 2014) was upregulated in *c-Maf*-deficient Treg cells. Next, to assess dysregulation of the T_{FR} cell transcription program in the absence of c-Maf, we integrated the *c-Maf*^{-/-} RNA-seq dataset with the *follicular signature gene* list identified in Chapter 4 (**Fig. 29c**). Of these, 59 genes (11%) were regulated by c-Maf (**Fig. 38b**), compared to the 39% of *follicular signature* genes regulated by IRF4 (**Fig. 30b**). Similar to the findings in *Irf4*^{-/-} Treg cells, the majority of the genes found in the overlap were significantly downregulated in the absence of c-Maf (**Fig. 38c**).

Interestingly, 22% of the Maf-regulated genes were also IRF4-regulated genes, suggesting a common role for both transcription factors in follicular T cell gene regulation. Taken together, our data demonstrate that both transcription factors play roles in follicular T cell generation and migration into the germinal centre by orchestrating shared and distinct changes in a network of transcription factors, cell surface molecules and homing receptors.

5.3 A minority of the follicular gene expression signature is affected by changes in the chromatin landscape.

Next, to determine the relationship between the gene expression and chromatin landscape of follicular CD4 T cells, we integrated the *follicular signature gene* set identified from our RNA-seq analysis (Chapter 4 – **Fig. 29c**) with the 1,772 *follicular T cell chromatin signature* genes identified by ATAC-seq. Unexpectedly, only 113 genes were shared, suggesting that the vast majority was not regulated by chromatin accessibility (**Fig. 39a**). Despite the small overlap, this comparison contained important follicular T cell genes such as *Lef1*, *Cebpa* and *Maf* (Choi, Gullicksrud et al. 2015, Neumann, Blume et al. 2019, Yang, Wang et al. 2019). Moreover, genes essential for follicular T cell migration *Ccr7*, *Slpr1*, *Sell* and *Nfatc1* (Haynes, Allen et al. 2007, Vaeth, Muller et al. 2014) and those that are required for T_{FR} cell function such as *Ctla4*, *Pdcd1* and *Tigit* (Sage, Francisco et al. 2013, Joller, Lozano et al. 2014, Sage, Paterson et al. 2014) were also found in this overlap (**Fig. 39b**). The expression of other key follicular T cell related genes such as *Bcl6*, *Cxcr5*, *Icos*, *Il21* and Slam associated proteins (SAP) were not found in the intersection of these two datasets suggesting that other factors regulate their expression. Here, our data revealed that only some of the *follicular signature* genes were regulated by epigenetic modifications.

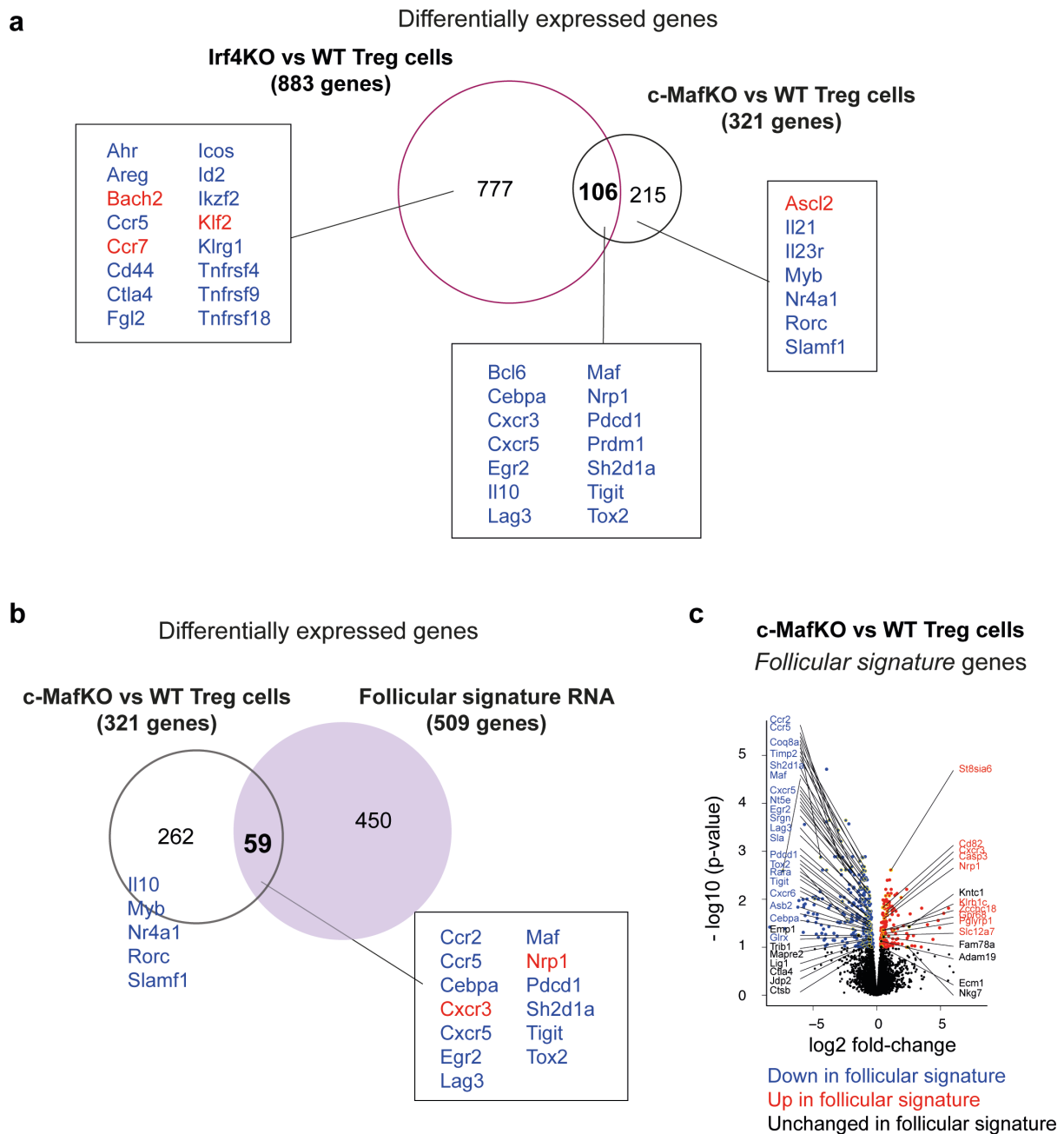


Figure 38. IRF4 and c-Maf regulate common and distinct genes in the follicular T cell compartment.

(a) Venn diagram illustrating the overlap of genes differentially expressed in *Irf4*-deficient versus control Treg cells compared with genes differentially expressed in *c-Maf*-deficient Treg cells compared with controls. (b) *c-Maf*-deficiency affects a small proportion of the follicular T cell transcriptome. (c) Key follicular related genes are downregulated in the absence of *c-Maf*.

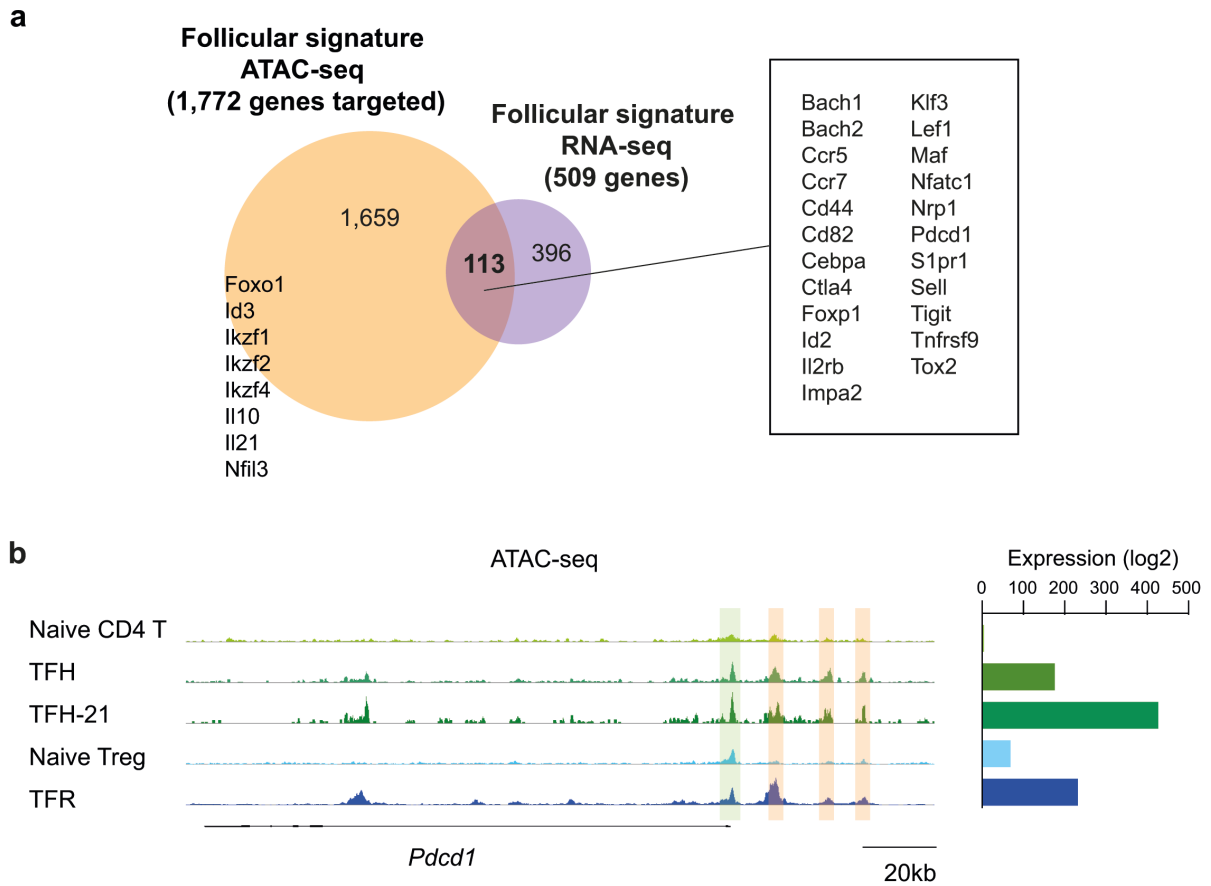


Figure 39. Some of the gene expression profile of follicular T cells is regulated by epigenetic modification.

(a) Venn diagram showing overlap of the *follicular signature* genes identified by ATAC-seq and RNA-seq. Genes of interest are shown in the box on the right. (b) Ensemble ATAC-seq data genome tracks for the indicated T cell subtypes in the *Pdcd1* locus. Regions highlighted in orange show follicular cell-specific peaks, and areas of open chromatin in all cells are shaded in green. Data are representative of 2 independent experiments. ATAC-seq, assay for transposase-accessible chromatin followed by next-generation sequencing; DA, differentially accessible; Treg, regulatory T; T_{FH}, follicular T helper; T_{FR}, follicular Treg.

5.4 Genomic binding of c-Maf.

Given the lack of T_{FR} cells in mice with a Treg cell-specific deficiency of *c-Maf*, we wanted to determine whether key follicular T cell genes are direct targets of c-Maf. To this end, we used published c-Maf chromatin immunoprecipitation (ChIP)-sequencing data (Ciofani, Madar et al. 2012) to assess genomic binding of c-Maf. We observed c-Maf binding sites in genes important for both CD4 T cell activation and follicular phenotype and function (**Fig. 40a**). We identified multiple Maf binding sites in key Treg cell genes such as *Prdm1* (encoding Blimp-1), *Cd28*, *Ctla4*, and *Icos*, and also saw similar increases in chromatin accessibility at discrete regions of genes essential for follicular CD4 T cell function such as *Il21*. Interestingly, Maf bound to its own gene, suggesting that this transcription factor also regulates its own expression (**Fig. 40b**).

Both IRF4 and c-Maf are essential for T_{FR} cell generation (discussed in Chapter 4). To understand the relationship of c-Maf and IRF4 in the regulation of genes required for T_{FR} cell generation, we interrogated c-Maf and IRF4 DNA binding using ChIP-seq data published by us and others (Ciofani, Madar et al. 2012, Vasanthakumar, Liao et al. 2017). This analysis identified 7,647 c-Maf binding sites and 3,817 IRF4 binding sites, assigned to 3,908 and 2,575 genes, respectively (FDR of 10^{-25} and 10^{-5}) (**Fig. 40c**). We observed 1,793 overlapping peaks associated with 1,271 genes. Multiple discrete regions were enriched for the binding of both transcription factors in genes that are essential for eTreg cell differentiation and function (Dias, D'Amico et al. 2017), including *Foxo1*, *Cd28*, *Ctla4*, *Icos*, *Prdm1* and *Il10* (**Fig. 40d**). We also identified several follicular associated genes such as *Bcl6*, *Il21* and *Maf* to have overlapping IRF4 and c-Maf binding sites, suggesting direct roles for both transcription factors in the regulation of T_{FR} cell generation (**Fig. 40e**). We also found co-binding of c-Maf and IRF4 to *Klf2*. Although we observed *Sh2d1a* (SAP) to also be a direct target of c-Maf (**Fig. 40f**), there was no evidence of direct binding of IRF4 to this locus, indicating that it is not an IRF4 target. Overall, our data suggests that IRF4 and c-Maf cooperate to establish some of the genes essential for follicular T cell phenotype.

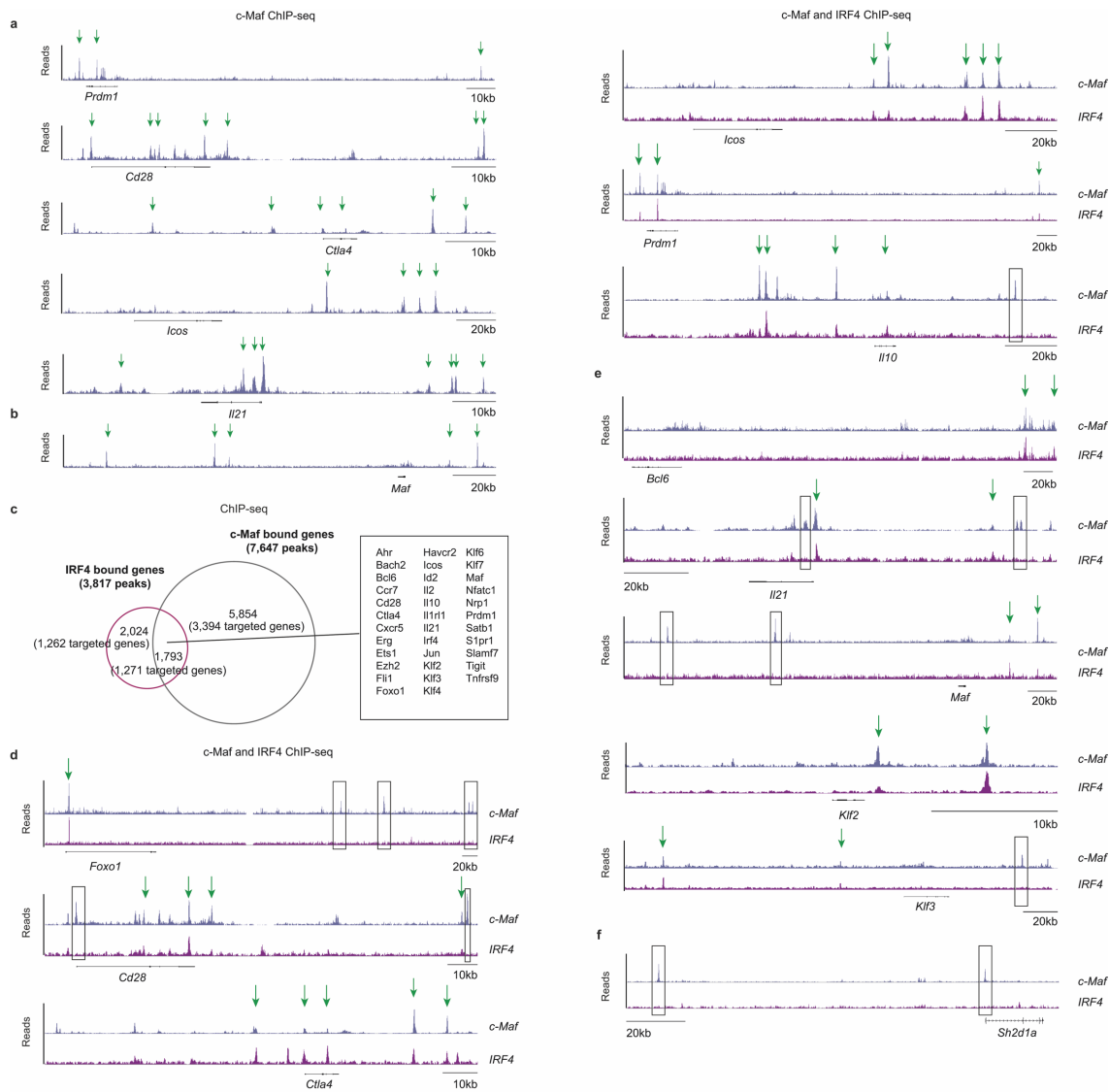


Figure 40. Genomic binding of c-Maf and IRF4.

(a-b) c-Maf ChIP-seq read density at selected loci: *Prdm1*, *Cd28*, *Ctla4*, *Icos*, *Il21* (a) and *Maf* (b). Arrows indicate peaks called as statistically significant. (c) Venn diagram of overlapping peaks identified by ChIP-seq dataset of c-Maf from Ciofani et al. (Ciofani, Madar et al. 2012) and IRF4 by Vasanthakumar et al. (Vasanthakumar, Moro et al. 2015) (FDR of 10^{-25} and 10^{-5} respectively). A list of key genes that were targeted by both transcription factors is shown in the box on the right. (d-f) IRF4 and Maf binding in key Treg cell (d) activation-associated, and (e) follicular T cell genes. Boxes indicate c-Maf binding alone. (f) No IRF4 binding seen in *Sh2d1a* locus. Arrows indicate overlapping peaks called as statistically significant. FDR, false discovery rate.

5.5 IRF4 and c-Maf establish the gene expression profile of T_{FR} cells.

To assess if IRF4 and c-Maf regulate the *follicular signature*, we integrated the RNA-seq dataset (**Fig. 29c**) with that of IRF4 and c-Maf ChIP-seq. We found 239 genes (46%) of the *follicular signature* to be direct targets of either IRF4 or Maf or both. Only a small fraction (114 genes) of the *follicular signature* genes were regulated by both IRF4 and c-Maf, which included genes such as *Bach2*, *Ccr7*, *Cd44*, *Ctla4*, *Cxcr5*, *Icos*, *Lef1*, *Maf*, *Nfatc1*, *Satb1* and *Slpr1* (**Fig. 41a**). As we had documented in Chapter 4, the essential follicular T cell transcription factor, *Bcl6*, was not included in this list due to stringent cut-offs in the RNA-seq analysis. In addition, genes for functional molecules such as *Il10* and *Il21*, known to be regulated by both c-Maf and IRF4 (**Fig. 40c**), were not found in the overlap also as a result of stringent cut-offs (**Fig. 41a**). Interestingly, the activator protein (AP)-1 family member basic leucine zipper transcription factor ATF-like (BATF), which is known to recruit IRF4 to AP-1-IRF composite element (AICE) sites (Li, Spolski et al. 2012) was a target of c-Maf (**Fig. 41a**). BATF directly regulates *Bcl6* and c-Maf, both of which are essential for T_{FR} cell development (Ise, Kohyama et al. 2011). In CD8 T cells, IRF4 cooperates with *Batf* and NFATc1/NFAT2 to promote exhaustion and *Pdcd1* expression (Oestreich, Yoon et al. 2008, Martinez, Hu et al. 2016, Man, Gabriel et al. 2017). In T_{FR} cells, both IRF4 and c-Maf partner with NFAT to regulate the expression of IL-4 (Ho, Hodge et al. 1996, Rengarajan, Mowen et al. 2002). Additionally, T_{FR} cells require NFATc1/NFAT2 to upregulate the expression of CXCR5, a key chemokine receptor that enables the positioning of T_{FR} cells within the light zone of the germinal centre (Vaeth, Muller et al. 2014). In our analysis, we identified IRF4 and c-Maf binding to *Nfatc1* indicating cooperation of these transcriptional regulators not only for IL-4 expression but for appropriate T_{FR} cell localization (**Fig. 41c**). Interestingly, we detected a c-Maf binding site in a region approximately 17 kb from the transcriptional start site of the inhibitory receptor, PD-1 (encoded by *Pdcd1*), but not IRF4, suggesting that the expression of PD-1 is related to the transcriptional activity of c-Maf but not IRF4 (**Fig. 41d**). Collectively, our data show that whilst half of the *follicular signature* is regulated by either IRF4 and/or c-Maf, only 20% (114 genes) are co-regulated by IRF4 and c-Maf.

T_{FR} cell generation, including *Maf* and *Il21*. Within these loci, we observed co-bound peaks aligned with regions of open chromatin specifically in the follicular CD4 T cells suggesting that both IRF4 and c-Maf cooperate to regulate the transcriptome of follicular T cells (**Fig. 42c**).

Next, we integrated ChIP-seq data and all DA peaks identified by ATAC-seq to define how much of the chromatin landscape could be regulated by IRF4 and c-Maf. Here, we identified c-Maf binding to 1,537 regions that contained areas of accessible chromatin identified by ATAC-seq and 486 DA peaks that had both IRF4 and c-Maf binding regions (**Fig. 42d**). Specifically, the combined evidence of open chromatin coinciding with c-Maf and IRF4 binding to the *Ctla4* locus confirmed both transcription factors to be positive regulators of CTLA-4 (**Fig. 42e**). Overall, we found evidence for IRF4 and c-Maf, alone and in combination, to bind to 5% of the open chromatin of follicular genes.

Finally, we were interested to determine whether DA peaks across all cell types examined represented any functional modules that could be regulated by either IRF4 or c-Maf, or both. Using the *Ctla4* locus as an example, we identified distinct patterns of chromatin accessibility for a naïve and follicular module. We found areas of open chromatin specific to naïve CD4 T cells highlighted in grey, and follicular T cells highlighted in orange. There were also areas of open chromatin common to all CD4 T cells (green shade) (**Fig. 42e**).

Combined, our data demonstrates that the chromatin landscape of naïve and follicular T cells is dynamic and reflects control by a transcriptional network. Furthermore, we show that the open chromatin regions can be distinguished into naïve, and follicular modules, providing a valuable resource for the interpretation of molecular control of follicular T cell differentiation.

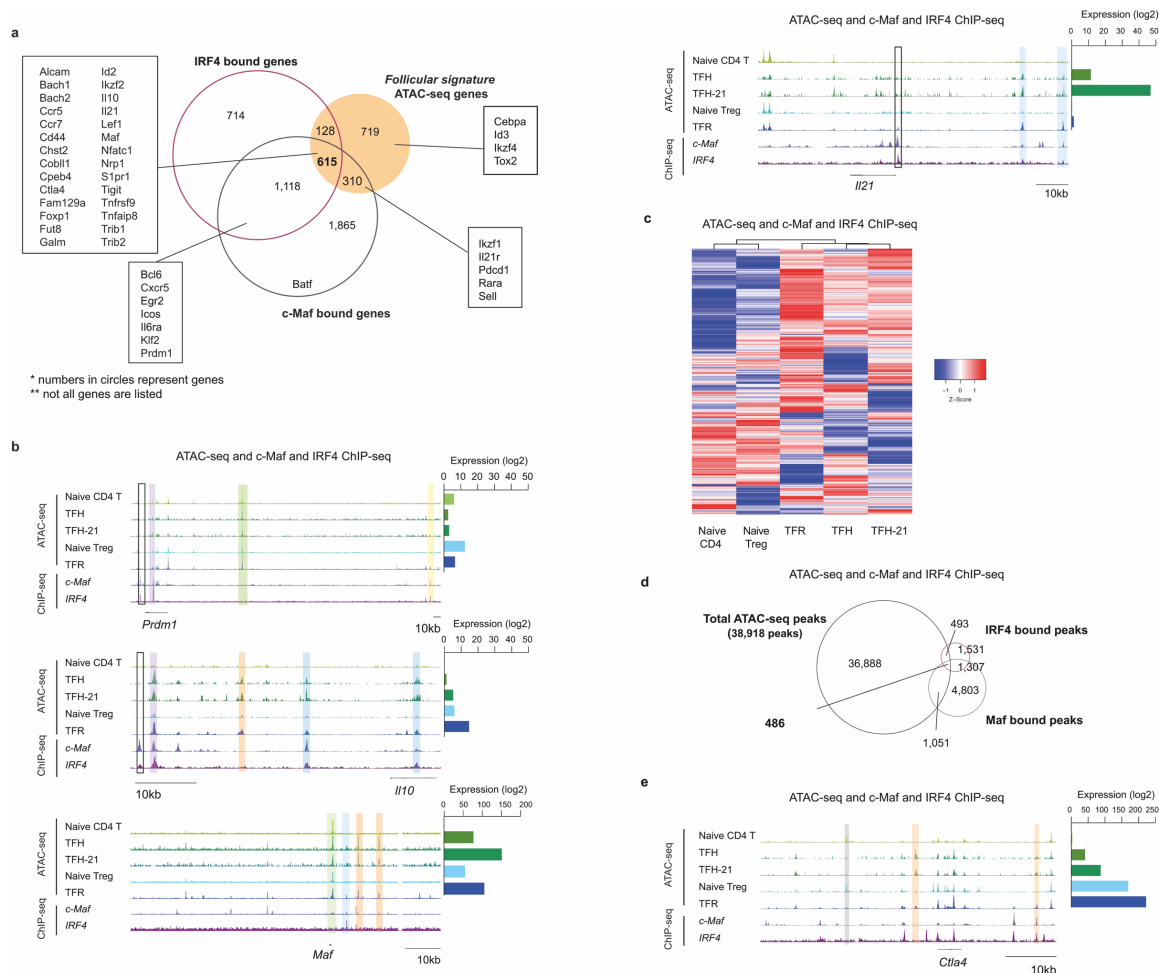


Figure 42. Epigenetic modification of T_{FR} cells is partially regulated by IRF4 and c-Maf.

(a) Venn diagram showing the overlap of genes bound by IRF4 and c-Maf as identified by ChIP-seq and the *follicular chromatin signature* genes (b) Read density at selected loci indicating chromatin accessibility. Areas of open chromatin found in all CD4 T cells (green shade) and are bound by IRF4 and c-Maf (purple shade), an area of open chromatin specific to T_{FR} cells (yellow), and areas of open chromatin in follicular CD4 T cells that are (blue) and are not (orange) associated with IRF4 and c-Maf binding. Boxes highlight overlapping IRF4 and c-Maf binding sites with no associated areas of open chromatin. ATAC and ChIP track heights are normalized to respective total number of mapped reads per sample. (c) Unsupervised heatmap of the indicated cell types showing relative expression (Z-score) of *follicular chromatin signature* genes that are bound by IRF4 and c-Maf. (d) Overlap of IRF4 and c-Maf bound peaks with total ATAC-seq peaks. (e) Genomic tracks of the indicated T cell subtypes at *Ctla4*. ATAC-seq peaks that fit a naïve module (grey) and a module that is unique to follicular T cells (orange) are shown. ChIP-seq, chromatin immunoprecipitation followed by next generation sequencing.

Discussion

The differentiation of Treg cells into T_{FR} cells is coordinated by a complex network of transcription factors. Using bioinformatic analyses of chromatin accessibility and transcription, we sought to create a comprehensive perspective on the T_{FR} cell transcriptional signature with molecular data generated by ourselves and those already published. We also analysed the data in parallel with the comparisons between naïve conventional CD4 T and T_{FH} cells to better understand the similarities and differences of the chromatin landscape when T cells become activated. As we had highlighted in the previous chapter, the two TCR-induced transcription factors c-Maf and IRF4 are critical for the regulation of T_{FR} cell differentiation. Here, we established their genomic binding in relation to the chromatin landscape of these CD4 T cell subsets to shed light on how they orchestrate this important transition.

Changes in chromatin landscape during CD4 T cell activation

In response to TCR stimulation, naïve conventional and Treg cells undergo activation and proliferation in a context specific manner (Ohkura and Sakaguchi 2010, Zhu, Yamane et al. 2010). Follicular CD4 T (T_{FH}) cells are a distinct subset of CD4 T cells that express Bcl6, and provide essential help to B cells to regulate the germinal centre reaction and differentiation in response to infection or immunization (Crotty 2019). Whilst much is known of their phenotype, differentiation and function (Vinuesa, Linterman et al. 2016), the chromatin landscape of T_{FH} cells has not been assessed. By performing ATAC-seq, we found that CD4 T cells with a follicular phenotype have distinct patterns of chromatin accessibility compared to naïve CD4 T cells, in line with the understanding that epigenetic changes shape T cell differentiation (Russ, Prier et al. 2013). We observed this pattern of chromatin accessibility to be conserved in T_{FR} cells unrelated to the expression of Foxp3, in keeping with evidence that Treg cells co-opt common transcriptional pathways of the cells in which they suppress (Cretney, Kallies et al. 2013).

IL-21, the prototypic cytokine provided by T_{FH} cells, is required not only for germinal centre B cell survival, proliferation and somatic hypermutation, but for the maintenance of T_{FH} cells themselves (King, Tangye et al. 2008, Crotty 2019). Mechanistically, IL-21 promotes the

expression of Blimp-1 in plasma cells and also induces Bcl6 (Ozaki, Spolski et al. 2004). We observed that only a proportion of T_{FH} cells secrete IL-21 and posited that they are likely to have a different chromatin landscape compared to T_{FH} cells that do not secrete IL-21. Not much is known about the differences in the transcriptome of these two cell types. We observed surprisingly few differences in the chromatin accessibility landscape of these two T_{FH} cell subsets, including at the *Il21* locus, suggesting that IL-21 secretion is predominantly governed by post-translational modification. However, there was a difference seen within the *Cxcr4* locus, with an area of open chromatin in naïve conventional and T_{FH} cells, but not T_{FH}-21 cells. This finding highlights that epigenetic changes can determine the localization of T_{FH}-21 cells within the light and dark zones of the germinal centre, and is likely important for the positioning of the cells based on their function.

The transcriptional program of follicular T cells is controlled by a core transcription network that includes Bcl6, Maf and STAT3 (Crotty 2014). Here, we generated a *follicular chromatin signature*, which contained many genes that are known to be essential for follicular T cell fate such as *Cebpa*, *Ctla4*, *Cxcr4*, *Il21*, *Maf*, *Nfatc1* and *Pdcd1* (Crotty 2019). Whilst we observed distinct differences in the chromatin landscape between naïve T and follicular T cells, the *follicular chromatin signature* did not include *Bcl6*, *Cxcr5* nor *Stat3*, suggesting that the transcription of some of the follicular signature is not accompanied by epigenetic changes alone. Unexpectedly, we also found that the overlap between the *follicular chromatin* and the *follicular gene signatures* was small, suggesting that much of the follicular T cell gene expression program is governed through other mechanisms such as post-translational modification and repression of alternative cell fate programs (Johnston, Poholek et al. 2009). This result was unexpected and suggested a less linear (direct) relationship between the chromatin landscape and gene expression in follicular T cells.

Specifically, examining the chromatin landscape of T_{FR} and T_{FH} (which included T_{FH}-21) cells showed that despite the expression of Foxp3 which defines the Treg cell lineage identity (Gavin, Rasmussen et al. 2007), it did not impact the expression of *follicular signature genes*. This suggests that the follicular T cell program is independent of Foxp3, and regulated by other transcription factors, such as Bcl6. We observed that the expression of certain molecules such as, *Lef1* and *Nfatc1*, that are essential for follicular T cell function (Choi,

Gullicksrud et al. 2015, Martinez, Hu et al. 2016) and tissue localization, in the *follicular chromatin signature*. These data suggest that the upstream regulators of T_{FR} cells are the same as that for T_{FH} cells.

Regulation of the T_{FR} cell transcriptional program by IRF4 and c-Maf

Treg cells are critical to the maintenance of self-tolerance in the periphery. Naïve Treg cells differentiate into Treg cells with effector function (eTreg cells) and migrate to sites of inflammation to suppress the magnitude of the inflammatory response. Additionally, eTreg cells are found in various non-lymphoid organs and play essential roles in maintaining immune homeostasis within the tissues (Teh, Vasanthakumar et al. 2015). We hypothesized that eTreg cells undergo a common Treg cell differentiation pathway and subsequently acquire functional specialisation induced by local tissue factors to adapt to the local tissue microenvironment. In a similar manner, Foxp3-expressing follicular T cells (or T_{FR} cells) that are found within the germinal centre utilise components of the follicular T cell transcriptional machinery and thus, share common cues with T_{FH} cells. Similar to a well-studied eTreg cell population in the visceral adipose tissue, (VAT) Treg cells (Burzyn, Benoist et al. 2013, Kolodin, van Panhuys et al. 2015, Vasanthakumar, Moro et al. 2015), T_{FR} cell development is also dependent on a complex network of transcription factors. These include Bcl6, BATF, and NFAT (Vaeth, Muller et al. 2014, Xie and Dent 2018). Like VAT Treg cells, T_{FR} cell activation through the TCR initiates transcriptional programs that drives their differentiation. We show that the TCR-induced transcription factor, IRF4 regulates the follicular T cell transcriptional profile. Consistent with the findings of an absent T_{FR} cell compartment in mice with the Treg cell specific deficiency of IRF4, we demonstrated that more than 35% of the *follicular signature* genes were deregulated in the absence of *Irf4*, with the vast majority of transcripts being reduced. Recent work by others and ourselves (Wheaton, Yeh et al. 2017, Neumann, Blume et al. 2019) have shown that Maf is also part of the core network of transcription factors that govern T_{FR} cell generation. c-Maf deficiency resulted in deregulation of 11% of the *follicular signature* genes. Among those were key T_{FR} cell genes consistent with the requirement of c-Maf for T_{FR} cell function and their maintenance. The core set of 106 genes that were common in the absence of both transcription factors also included some eTreg cell associated molecules such as *Prdm1*, *Il10*,

Nrpl and *Tigit*, suggesting that both transcription factors regulate a common, core eTreg cell differentiation pathway. Consistent with the severe fatal autoimmune disease seen in mice with a Treg cell deficiency of IRF4, compared to the milder phenotype observed in c-Maf deficient mice, our data showed that a larger frequency of the *follicular signature genes* appeared to be regulated by *Irf4* compared to *c-Maf*. Specifically, IRF4 critically regulated genes required for the Treg cell migratory capacity. This observation supports our hypothesis that IRF4 plays a more central role in the layered transcriptional requirement for the generation of T_{FR} cells, and as such, is critical required for the maintenance of peripheral immune homeostasis.

Finally, we observed genomic binding of both IRF4 and c-Maf to key Treg and T_{FR} genes. Specifically, the evidence of c-Maf and IRF4 binding to the *Maf* locus indicates that both transcription factors are positive regulators of Maf. In T cells, IRF4 is recruited by AP-1 transcription factors to AICE sites in the genome (Li, Spolski et al. 2012), while c-Maf binds the Maf recognition element (MARE) (Hiramatsu, Suto et al. 2010). As the MARE motif contains an AP-1 binding site, it is possible that the generation of T_{FR} cells requires IRF4 binding first and then subsequent recruitment of c-Maf. Our data suggests that both IRF4 and c-Maf together with AP-1 factors cooperatively regulate the transcription of critical genes required for T_{FR} cell differentiation.

Taken together, our data suggests that eTreg differentiation, function and maintenance, including T_{FR} cell generation, is critically dependent on IRF4, whilst c-Maf is essential for T_{FR} cell and colonic Treg cell differentiation only. It is likely that both transcription factors control follicular T cell generation and migration into the germinal centre by orchestrating a network of transcription factors, cell surface molecules and homing receptors. Further work using motif analysis would establish whether such cooperative binding does indeed occur. Additionally, ChIP-seq experiments to examine the genomic binding partners of c-Maf and IRF4 in the absence of each factor will further inform this important regulatory model.

Regulation of the T_{FR} cell chromatin landscape by c-Maf and IRF4

A recent study revealed that the chromatin landscape of eTreg-fated cells is primed in the lymphoid organs and subsequently, undergo further changes to adapt to the local tissue

environment (DiSpirito, Zemmour et al. 2018). Our bioinformatic analyses identified numerous areas of open chromatin in *follicular signature genes* from T_{FR} cells compared to naïve Treg cells, which paralleled regions of open chromatin in follicular CD4 T cells compared to naïve conventional CD4 T cells. We observed clear patterns of chromatin accessibility that are specific to follicular T cells. We found evidence for IRF4 and c-Maf, alone and in combination, to bind to 5% of the open chromatin of follicular genes. In particular, the combined evidence of open chromatin in the follicular T cell subsets coinciding with IRF4 and c-Maf binding in the *Maf* locus supports our hypothesis that both transcription factors are critical for T_{FR} cell differentiation and function.

It is well understood that the spatial and functional diversity of Treg cells is governed by distinct, tightly regulated molecular mechanisms. Based on our data, it is plausible to conclude that IRF4, together with tissue specific factors, establishes the transcriptional program of eTreg cells and cooperates with c-Maf to enforce the chromatin accessibility landscape, and the resultant transcriptional landscape of T_{FR} cells. Our findings bring us one step closer to understanding the intriguing and complex mechanisms of transcriptional regulation of T_{FR} cells by IRF4 and c-Maf.

Chapter Six: Final discussions

I) Blimp-1 expression in neonatal eTreg cell ontogeny.

Effector (e)Treg cells play fundamental roles to limit autoimmune pathology within non-lymphoid and lymphoid organs, and are involved in the regulation of tissue metabolism and repair (Arpaia, Green et al. 2015, Neumann, Blume et al. 2019, Vasanthakumar, Chisanga et al. 2020, Wang, Yaghi et al. 2020). Compared to naïve-like central (c)Treg and activated (a)Treg cells, eTreg cells express higher levels of effector molecules that are required for their function, such as CTLA4 and ICOS (reviewed in Teh, Vasanthakumar et al. 2015). Notably, the transcriptional regulator, Blimp-1 is expressed by all eTreg cells, which is required for the secretion of the immunosuppressive cytokine, IL-10 (Cretney, Xin et al. 2011). Whilst there is considerable knowledge regarding eTreg cell differentiation and function in the literature, there still remains a lot to be learned. For instance, do eTreg cells leave the thymus pre-programmed with a tissue-specific fate, destined to be a particular tissue Treg cell? Our data demonstrates the presence of *Blimp1*^{GFP} reporter-expressing Treg cells within the thymus of at 2-week-old neonates, which suggests that a proportion of tissue-specific Treg cells are likely to be programmed within the thymus prior to their export. It is essential however, to note that our experiments were not able to distinguish recirculating Treg cells from the periphery, though based on the ages of the mice analysed, perhaps recirculating *Blimp1*^{GFP} reporter-expressing Treg cells would contribute to only a small fraction. Our data also indicates that Treg cells with an effector phenotype that are destined to establish tissue residency exit the thymus around week 3 after birth. From our experiments, we were unable to further delineate if the eTreg cell migration from the thymus to the non-lymphoid organs occurred in a sequential manner reminiscent of the study by Dunon in 1997 (Dunon, Courtois et al. 1997).

The gastrointestinal tract is constantly exposed to an enormous diversity of benign commensal and pathological microbiota, and food-derived antigens that prime and modulate the immune system (reviewed in Belkaid and Harrison 2017). It is now evident that the complex interactions between the microbiota, the intestinal epithelium and the host immune system is important to maintain intestinal homeostasis (Hooper, Littman et al. 2012, Sefik, Geva-Zatorsky et al. 2015, Neumann, Blume et al. 2019). Breakdowns in the regulation of intestinal homeostasis can lead to localized chronic inflammatory pathology both in mice and

humans (Maloy and Powrie 2011, Schirmer, Franzosa et al. 2018). Further evidence suggests that effector CD4 T cells that drive intestinal inflammation can be controlled by host Treg cells (Pandiyani, Zheng et al. 2007, Lathrop, Bloom et al. 2011, Cebula, Seweryn et al. 2013). As such, the gastrointestinal tract usually has a constitutive Treg cell presence (Schiering, Krausgruber et al. 2014, Wheaton, Yeh et al. 2017, Xu, Pokrovskii et al. 2018, Neumann, Blume et al. 2019). Additionally, defects in the host-commensal relationship in the perinatal period have been associated with numerous diseases such as inflammatory bowel disease and asthma (Renz, Brandtzaeg et al. 2011, Gensollen, Iyer et al. 2016). Our experiments have demonstrated low frequencies of eTreg cells in the SI compartment of 2-week-old mice that rapidly expand to close to adult frequencies after weaning as their diet changes from maternal milk to fibre-containing foods. Further studies are needed to determine whether these SI eTreg cells are thymic-derived or peripherally-induced.

Collectively, our data showed that the pool of eTreg cells detected within non-lymphoid organs consisted of a combination of cTreg, aTreg and eTreg cells. As such, it seems reasonable to postulate from these insights that at least some of the eTreg cells within non-lymphoid organs undergo thymic programming prior to their migration to populate the various tissues. These findings would suggest that the eTreg cell program is not entirely dependent on stochastically encountered signals in the periphery.

II) IRF4 is central to eTreg cell generation.

T cell receptor (TCR) signalling mediates the activation and subsequent effector differentiation of Treg cells (Li and Rudensky 2016). The induction of the transcriptional regulator, IRF4 downstream of TCR stimulation promotes effector specific gene programs (Zheng, Chaudhry et al. 2009, Cretney, Xin et al. 2011, Man, Miasari et al. 2013, Huber and Lohoff 2014). Using our novel *Irf4^{GFP}* reporter mice, we tracked the expression of *Irf4^{GFP}* reporter during thymic T cell development and Treg cell ontogeny. Our experiments demonstrate that immature thymocytes (double positive T cell stage) from day-4-old mice expressed low levels of *Irf4^{GFP}* reporter as compared with Foxp3-expressing thymic CD4 T cells suggesting that thymic TCR activation mediates the induction of IRF4. *Irf4^{GFP}* reporter expression was highest in Foxp3-expressing thymic CD4 T cells irrespective of the age of the mouse, in keeping with evidence that increased TCR signals are required for Treg cell selection and differentiation (Moran, Holzapfel et al. 2011). Much to our surprise, we found

that almost all Foxp3-expressing cells from the thymus and spleen of neonates expressed *Irf4*^{GFP} reporter. Given the known requirement for IRF4 in eTreg cell differentiation, it seemed rather surprising that not all thymic Treg cells expressed Blimp-1. These observations indicate that perhaps IRF4 is required both in early Treg cell differentiation and for tonic signalling required to maintain a functional mature Treg cell pool (Vahl, Drees et al. 2014). Notably, IRF4 has been shown to interact with Foxp3 to regulate Treg cell specific gene expression (Zheng, Chaudhry et al. 2009, Fu, Ergun et al. 2012). However, our experiments have shown that tTreg cell development is not perturbed in *Irf4*^{-/-} (data not shown). In contrast, *Irf4*-deficient Treg cells displayed reduced expression of functional Treg cell molecules, such as CTLA4, KLRG1 and ICOS, and accordingly, mice are deficient for eTreg cells. Combined, our data indicate that TCR signals induce the expression of IRF4 and that IRF4 is indispensable for eTreg cell differentiation but not for the development of tTreg cells.

III) T_{FR} cells are a subset of eTreg cells that have a cell-intrinsic requirement for IRF4.

A specialised population of Treg cells that are found within the germinal centre, termed the T_{FR} cells, have been intensively studied over the last decade (Chung, Tanaka et al. 2011, Linterman, Pierson et al. 2011, Wollenberg, Agua-Doce et al. 2011, Sage and Sharpe 2016, Wing, Kitagawa et al. 2017, Clement, Daccache et al. 2019). T_{FR} cells play an essential role in regulating humoral immunity (Sage and Sharpe 2015, Stebegg, Kumar et al. 2018), but whether T_{FR} cells were effectively an eTreg cell subset was unclear. Uniquely, T_{FR} cells maintain the suppressive activity characteristic of Treg cells and the expression of Treg cell signature genes yet they are found within a secondary lymphoid organ. Reminiscent of other eTreg cell subsets such as the visceral adipose tissue Treg cells, T_{FR} cells co-opt transcription factors and signalling pathways characteristic of the germinal centre environment where they function to modulate the activity of T_{FH} and elements of antibody production (Chung, Tanaka et al. 2011, Vaeth, Muller et al. 2014, Botta, Fuller et al. 2017, Clement, Daccache et al. 2019, Liang, Yao et al. 2019). One of the many documented functions of T_{FR} cells is to prevent the expansion of autoreactive B cells generated by somatic hypermutation during an antigen-specific response (Wu, Chen et al. 2016, Botta, Fuller et al. 2017).

As previously mentioned, eTreg cells express Blimp-1 and its differentiation is dependent on the TCR-induced transcription factor IRF4. Given the complete loss of all eTreg cells upon

Irf4 deletion, the mechanisms by which IRF4 functions to regulate T_{FR} cell differentiation has been difficult to determine. First, using our novel *Irf4*^{tdTomato} reporter mice, we showed that T_{FR} cells express high levels of *Irf4*^{tdTomato} reporter, when compared to naïve or CXCR5^{low}PD1^{low} Treg cells. Following influenza virus infection, in three different chimeric mouse models that lack IRF4 expression on Foxp3⁺ cells, we demonstrated that the diminished T_{FR} cell population is reflective of a Treg cell-intrinsic requirement of IRF4 during the effector differentiation of Treg to T_{FR} cells. In addition to data showing impaired expression of Treg cell activation-associated molecules (Cretney, Xin et al. 2011), *Irf4*-deficient Treg cells failed to downregulate migration molecules such as CCR7 and CD62L, relevant for Treg cell migration to secondary lymphoid organs to perform their function. Furthermore, IRF4 is required for the induction of Blimp-1 essential for eTreg cell suppressive function (Cretney, Xin et al. 2011). Our data showed that only a quarter of CXCR5^{high}PD-1^{high}Foxp3⁺ T_{FR} cells express *Blimp-1*^{GFP} reporter. In contrast, T_{FH} cells do not express *Blimp-1*^{GFP}, whose expression is repressed by Bcl6 (Johnston, Poholek et al. 2009). It is likely the *Blimp-1*^{GFP} reporter-expressing T_{FR} cells are the IL-10 producers within the germinal centre, and as such are the bona fide eTreg cells of the germinal centre. Further studies will be required to elucidate this.

Collectively, these data indicate that T_{FR} cells express Blimp-1 and as such, are a subset of eTreg cells, and distinct aspects of T_{FR} cell generation are coordinated by IRF4. Future experiments using 2-photon excitation microscopy may be useful to document the interactions between both *Blimp-1*^{GFP} reporter positive and *Blimp-1*^{GFP} reporter negative T_{FR} cells with T_{FH} and B cells within the germinal centre.

IV) Molecular regulation of T_{FR} cells by IRF4 and c-Maf.

Gene expression analysis of naïve conventional and regulatory T, T_{FH}, T_{FH}-21, and T_{FR} cells identified a specific *follicular signature* gene profile, which reflects a transcriptional program that is common to all follicular CD4 T cells. This signature comprised of genes involved in follicular T cell function and migration. To our surprise, the expression of Foxp3, which defines the Treg cell lineage identity (Gavin, Rasmussen et al. 2007), did not impact the expression of *follicular signature genes*. Our data demonstrated that the loss of IRF4 resulted in the deregulation of not only many genes that are essential for Treg cell activation, migration, function but also genes included in the *follicular signature*. ChIP-sequencing

confirmed that many of these genes are direct targets of IRF4. In particular, we observed Foxo1 to be a target of IRF4. Foxo1 transcriptionally regulates tTreg cell development and Treg cell function (Kerdiles, Stone et al. 2010), and accordingly is critical for naïve T cell survival and quiescence. Based on our ChIP-sequencing result, we performed experiments to determine the role of Foxo1 in T_{FR} cell differentiation. In keeping with the known role of Foxo1 in T cell quiescence, we observed that the absence of *Foxo1* in all peripheral T cells resulted in increased frequencies of T_{FR} cells, spontaneous appearance of T_{FH} cells and expansion of plasma cells. However, due to the variable deletion of Foxo1 in *Foxo1^{fl/fl}pLck^{Cre}* mice, we were unable to recapitulate this result despite three repeat experiments. Future experiments using more definitive mouse strains are required to determine the role of Foxo1 in T_{FR} cell differentiation to substantiate our hypothesis.

ChIP-sequencing of purified Treg cells showed that IRF4 bound to the *Maf* locus, which indicated that IRF4 directly regulated *c-Maf* expression in Treg cells. In line with this, c-Maf expression was significantly reduced in *Irf4*-deficient Treg cells. The transcription factor c-Maf was very recently shown, by others and us, to be critical for the development and function of gastrointestinal tract Treg cells, as well as that of T_{FR} cells (Wheaton, Yeh et al. 2017, Xu, Pokrovskii et al. 2018, Neumann, Blume et al. 2019). Consistent with this data, our *in vivo* results showed a Treg cell-intrinsic defect of T_{FR} cells in *c-Maf*-deficient mice. Interestingly, the specific deletion of IRF4 or c-Maf in Treg cells both resulted in the loss of T_{FR} cells and some dysregulation of the GC response. However, contrary to our expectations, *c-Maf^{fl/fl}Foxp3^{Cre}* mice did not succumb to severe and inevitably fatal autoimmune disease, seen in mice that had a specific loss of IRF4 in Treg cells. Further, unlike the *Irf4*-deficient mice, the Treg cell specific deficiency of c-Maf did not impair the ability of Treg cells to downregulate migration molecules.

Our data demonstrated that in the absence of *Maf* or *Irf4*, genes differentially expressed consisted of those that are required for the T cell effector program. Compared to the deregulation of almost 40% of the *follicular signature* in the absence of *Irf4*, only 11% (59) of the *follicular signature* genes were deregulated in the absence of c-Maf. Strikingly, of these 59 c-Maf-dependent genes, 22% are IRF4-responsive genes, suggesting an overlapping role for both in the regulation of genes essential for the follicular T cell program.

Further investigation is required to unravel the relationship between the regulation of IRF4 and c-Maf and how they cooperatively establish the epigenome of T_{FR} cells. Finally, analysis

of the T_{FR} cell master transcription factor, Bcl6 in the epigenetic programming of follicular T cells would provide additional insights into its relationship with the follicular T cell transcriptome and the possible physical interactions with c-Maf or IRF4.

Collectively, our experiments provide evidence that both c-Maf and IRF4 are not only essential for T_{FR} cell differentiation but cooperate to regulate the factors required to maintain the T_{FR} cell program. Additionally, our data demonstrates that IRF4 plays a central role in regulating the eTreg cell differentiation pathway.

V) Regulation of the chromatin landscape of T_{FR} cells by c-Maf and IRF4

Foxp3 expression is required for commitment into the Treg cell lineage and the induction of the Treg cell transcriptional program (Rudra, deRoos et al. 2012, Lu, Barbi et al. 2017). Mounting evidence, however, indicates that differentiation and functional states of various eTreg cell populations are regulated by distinct transcription factors (Panduro, Benoist et al. 2016, Neumann, Blume et al. 2019, Song, Sun et al. 2020, Vasanthakumar, Chisanga et al. 2020). The network of transcription factors that influence and shape the chromatin landscape of T_{FR} cells, and how epigenetics contribute to the regulation of the transcriptional program is only partly understood. To our knowledge, the chromatin accessibility landscape of T_{FR} cells has not been previously described. Our data demonstrated wide-ranging changes to chromatin accessibility in response to T cell activation. Notably, the three follicular CD4 T cell types, T_{FH}, T_{FH}-21, and T_{FR} cells, clustered closely to each other in PCA compared to their naïve precursors. An interesting observation from our data is that only a small number of *follicular signature* genes identified by RNA-seq can be explained by the organization of accessible chromatin suggesting that physical access to DNA does not directly translate into gene expression.

We propose that IRF4 plays a role in initiating and maintaining accessible chromatin regions in promoter regions allowing for co-binding of chromatin-binding factors and other transcription factors to cooperatively regulate the expression of effector specific genes.

We present data identifying a requirement for IRF4 and c-Maf to maintain some of the chromatin accessibility landscape of follicular T cells. We found that whilst regions of open chromatin allow for transcription factor binding, this did not necessarily result in gene transcription suggesting that other factors are required to regulate the generation of T_{FR} cells.

Conclusion

We started this project based on our interest to further understand the molecular underpinnings of Treg cell differentiation and diversification. Treg cells are critical mediators of immune homeostasis. Its development, maintenance and activation have been studied with great intensity over the last 20 years (Josefowicz, Lu et al. 2012, Ohkura, Kitagawa et al. 2013, Sakaguchi, Vignali et al. 2013, Liston and Gray 2014). Since then, much of the research effort has been devoted to understand the transcriptional regulation of Treg cell differentiation (Luo and Li 2013, DiSpirito, Zemmour et al. 2018). The later discovery of eTreg cells as mediators of tissue immune homeostasis has led to considerable research in the field. The thymus plays a critical role in the supporting the development of these essential mediators. Mapping the ontogeny of eTreg cells has provided valuable insight into the contribution of the thymus to the eTreg cell program and thus highlights the importance of this organ in the maintenance of immune tolerance.

Since the germinal centre reaction is critical to the generation of an effective antibody response and immunological memory, a detailed understanding of the molecular control of T_{FR} cell differentiation and function is fundamental. We have addressed some of the knowledge gaps through examining the role of the TCR-induced transcription factor, IRF4 in the molecular machinery involved in T_{FR} cell differentiation. We observed a layered requirement for transcription factors in eTreg cell diversification where IRF4 is central to eTreg cell differentiation through the promotion of effector specific gene programs in the periphery. A tissue-specific requirement for transcription factors tailored to the germinal centre, in this case, c-Maf was also shown to be critically required for the generation of T_{FR} cells. Our data highlights the importance of transcriptional regulatory networks in coordinating the transcriptional program of T_{FR} cells.

In sum, we have generated a T follicular cell chromatin accessibility map that supports a model of tissue-specific changes associated with a host of genes required for follicular T cell generation. Through the integration of transcriptomic and chromatin accessibility data, we provide a framework for understanding the molecular regulation of T_{FR} cell generation.

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