

The role of Complement and MHC in innate and adaptive immunity cooperation

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'The more you know, the less you need.'

Abstract

Major histocompatibility complex class II (MHC II) molecules are expressed by antigen presenting cells and essential for the development and activation of CD4⁺ T cells. Complement component 3 (C3) is the central component of the complement system and its activation is known to induce protective functions as part of the innate immune system. In this study, we have identified the unknown cooperation of these two central molecules of the adaptive and innate immune system, by describing their covalent association on the surface of conventional dendritic cells (cDCs). This in turn led to their ablation and enriched trogocytosis by marginal zone (MZ) B cells. We therefore describe two novel instances of intersection between the innate and adaptive immune systems, one at the molecular and the other at the cellular level, namely the covalent binding of activated C3 to MHC II and the cellular interaction between cDCs and MZ B cells based on a novel molecular communication axis.

Chapter 3 investigates the biochemical mechanisms of MARCHF1-mediated C3 deposition in cDCs and describes the binding of activated C3 to surface MHC II molecules in scenarios where its surface expression is enriched due to impaired ubiquitination. Biochemical analyses revealed the covalent association of C3dg and C3d, the two smallest C3 fragments, specifically to the N-linked carbohydrate of MHC II α . This novel function of surface MHC II molecules as an acceptor for the covalent association of complement C3 was cDC-specific and also observed in human cDCs from blood samples.

In Chapter 4 we explore the physiological role/consequence of enriched MHC II-dependent C3 fixation on cDCs and identified the specific and significant loss of splenic cDCs, associated with enriched C3 deposition. Furthermore, we identified the appearance of cDC-specific markers at the surface of MZ B cells that lacked the transcriptional expression for these proteins, also associated with enriched C3 deposition.

Chapter 5 demonstrates C3-mediated trogocytosis of cDCs by MZ B cells, in which the latter acquire membrane fractions, as well as surface proteins and peptide-loaded MHC II molecules from cDCs. Importantly this intercellular exchange of membrane proteins was unidirectional and dependent on complement receptor 2 (CR2) expression in B cells, which indicates that C3-mediated trogocytosis is a coordinated cellular process.

In summary, this thesis provides comprehensive analyses on the interplay of innate and adaptive immunity, both on a molecular and cellular level. The data presented in this thesis indicate fundamental new roles for both MHC II and C3, in which surface MHC II serves as an acceptor for covalent C3 binding, which in turn is a driving mediator for close cell-cell interactions between cDCs and MZ B cells and unidirectional trogocytosis.

Declaration

The work presented in this thesis was conducted in the laboratory of Associate Professor Justine D. Minter and Professor Jose A. Villadangos at the Department of Biochemistry and Molecular Biology, Bio21 Molecular Science & Biotechnology Institute, The University of Melbourne. Patrick Schriek was supervised by Associate Professor Justine D. Minter (50 % supervision), Professor Jose A. Villadangos (40 % supervision) and Professor Patrick Reading (10 % supervision) and supported by the Australian Government Research Training Program (RTP) Scholarship. No publications have arisen from this thesis.

This is to certify that

- I. This thesis comprises only my original work towards the PhD except where indicated in the Preface.
- II. Due acknowledgements have been made in the text to all other materials used.
- III. This thesis is less than 100,000 words in lengths, exclusive of figures, tables, bibliographies and appendices.

Patrick Schriek

January 31, 2020

Preface

The contribution of others is acknowledged below, with proportion of original work by the Author indicated.

Chapter 3: Dr. Laura Dagley and Dr. Giuseppe Infusini from the Proteomics Laboratory at the Walter and Eliza Hall Institute of Medical Research performed the mass spectrometry and proteomic analysis in this chapter.

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Chapter 5: B cells from OB1.*Rag*^{-/-} mice and the mAb anti-Clec9A-OVA_{FGD} and anti-Clec9A-OVA_{FGD} (+ isotype controls) were provided by Professor William R. Heath, Department of Microbiology and Immunology, The Peter Doherty Institute, The University of Melbourne.

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Abbreviations

Ab	Antibody
Ag	Antigen
AID	Activation-induced cytidine deaminase
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
BATF3	Basic leucine zipper transcriptional factor 3
BCR	B cell receptor
bHLH	Basic helix loop helix
BM	Bone marrow
BST2	Bone Marrow Stromal Cell Antigen 2
C3	Complement component 3
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Conventional/classical dendritic cell
CDP	Common dendritic cell progenitor
CIITA	Class II transactivator
CLIP	Class II-associated invariant chain peptide
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CpG	CpG oligodeoxynucleotides
CR	Complement receptor
CSR	Class-switch recombination
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCR	CXC chemokine receptor
DAF	Decay acceleration factor
DC	Dendritic cell
DCML deficiency	DC, monocyte B an NK lymphoid deficiency
ds	Double-strand
DTR	Diphtheria toxin receptor
EBF	Early B cell factor
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERAP	ER-associated aminopeptidase
ESAM	Endothelial cell-selective adhesion molecules
ESCRT	Endosomal sorting complexes required for transport
FcR	FC receptor
FDC	Follicular dendritic cell
Flt3(L)	FMS-like tyrosine kinase 3 (ligand)
Foxp3	Forkhead box P3

GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft versus host disease
HAMP	Host-associated molecular pattern
HEL	Hen egg lysozyme
HEV	High endothelial venule
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus
ID-2	Inhibitor of differentiation protein 2
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LC	Langerhans cell
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
mAb	Monoclonal antibody
MAC	Membrane attacking complex
MARCHF	Membrane-associated RING-CH finger
MASP	MBL-associated proteins
MAVS	Mitochondrial antiviral signalling
MBL	Mannose binding lectin
MCP	Membrane-bound cofactor protein
MDP	Macrophage/dendritic cell progenitor
MHC (I/II)	Major histocompatibility complex (class I/II)
MIIC	MHC class II compartment
MIR	Modulator of immune recognition
moDC	Monocyte-derived dendritic cell
MZ	Marginal zone
NFκB	<i>Nuclear factor</i> kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLR	NOD-like receptor
pAb	Polyclonal antibody
PAGE	Polyacrylamid gelectrophoresis
PAMP	Pathogen associated molecular pattern
PD(-L1/2)	Programmed death(-ligand 1/2)
pDC	Plasmacytoid dendritic cell
pMHC I/II	Peptide-loaded major histocompatibility complex class I/II
PolyI:C	polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
RAG	Recombination-activating gene
RING	Really interesting new gene

RLR	RIG-I-like receptor
SCS	Subcapsular sinus
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutations
SLE	Systemic lupus erythematosus
ss	Single strand
TAP	Transporter associated with antigen presentation
TCR	T cell receptor
Tdt	Terminal deoxynucleotidyl transferase
TEC	Thymic epithelial cell
T _{FH}	Follicular T helper cell
Th	T helper cell
tLN	Total lymph nodes
TLR	Toll-like receptor
TM	Transmembrane domain
TNF α	Tumour necrosis factor α
Treg	Regulatory T cell
Ub	Ubiquitin

Table of Contents

Abstract	3
Declaration	5
Preface.....	6
Acknowledgments.....	8
Abbreviations.....	10
List of Figures	16
1 Introduction	19
1.1 THE ADAPTIVE IMMUNE SYSTEM	19
1.1.1 Context.....	19
1.1.2 MHC-restricted antigen presentation	20
1.2 B CELLS	21
1.2.1 B cell development	21
1.2.2 Antigen encounter.....	22
1.2.3 B cell activation	23
1.3 DENDRITIC CELLS	28
1.3.1 Discovery of dendritic cells	28
1.3.2 Subpopulations of dendritic cells.....	29
1.3.3 Human dendritic cells and clinical translation	32
1.3.4 Ontogeny.....	34
1.3.5 Transcription and growth factors of dendritic cells.....	35
1.3.6 Dendritic cell functions in the immune system.....	38
1.4 REGULATION OF MHC II IN DENDRITIC CELLS.....	46
1.4.1 MHC II expression and trafficking	46
1.4.2 Ubiquitination of MHC II by MARCHF Ub ligases	49
1.5 COMPLEMENT C3	56
1.5.1 Overview of the complement system	56
1.5.2 Cleavage of activated complement C3.....	60
1.5.3 Functions of complement C3 in adaptive immunity	63
1.6 RESEARCH AIMS.....	65
2 Materials and Methods	67
2.1 MATERIALS.....	67
2.1.1 Reagents, consumables and other materials	67
2.1.2 Buffers and media	69
2.1.3 Antibodies	71
2.1.4 Mice.....	73
2.2 METHODS	75
2.2.1 Cell culture	75

2.2.2	<i>Purification of primary mouse cells</i>	75
2.2.3	<i>Purification of human blood dendritic cells</i>	78
2.2.4	<i>Flow cytometry</i>	78
2.2.5	<i>Cellular assays and mouse model experiments</i>	83
2.2.6	<i>Biochemical assays</i>	85
2.2.7	<i>Mass spectrometry and proteomics</i>	86
2.2.8	<i>RNAseq</i>	87
3	MARCHF1-mediated C3 deposition in dendritic cells	91
3.1	INTRODUCTION	91
3.1.1	<i>Protein ubiquitination through MARCHF1 Ub ligases</i>	91
3.1.2	<i>The discovery of enriched surface C3 in Marchf1^{-/-} cDCs</i>	92
3.2	RESULTS	94
3.2.1	<i>Validation of enriched C3 deposition on Marchf1^{-/-} cDCs</i>	94
3.2.2	<i>Source of C3 deposited on Marchf1^{-/-} cDCs</i>	98
3.2.3	<i>MHC II as primary substrate for covalent association of C3 in cDCs</i>	99
3.2.4	<i>C3 deposition on other Marchf1^{-/-} immune cells</i>	112
3.2.5	<i>C3 binding to different murine MHC II haplotypes and human HLA</i>	116
3.2.6	<i>MHC II-dependent C3 deposition ex vivo and in vitro</i>	119
3.2.7	<i>MHC II-dependent C3 deposition on in vivo activated WT cDCs</i>	123
3.3	DISCUSSION	126
3.3.1	<i>A new role for MHC II as an acceptor for covalent association of C3</i>	126
3.3.2	<i>Differential binding of C3 to MHC II-expressing cells</i>	128
3.3.3	<i>MARCHF1-mediated internalization of MHC II as a route for C3 uptake</i>	131
3.3.4	<i>Activation and cleavage of C3 bound to MHC II</i>	132
4	The biological role of complement C3 and dendritic cells	136
4.1	INTRODUCTION	136
4.1.1	<i>C3 in adaptive immunity</i>	136
4.2	RESULTS	137
4.2.1	<i>The effect of C3 binding to MHC II on antigen presentation</i>	137
4.2.2	<i>The impact of C3 deposition on cellular composition and dendritic cell homeostasis</i>	139
4.2.3	<i>Characterization of CD11c^{int} CD24^{high} CD8^{int} cells</i>	144
4.3	DISCUSSION	154
4.3.1	<i>C3 binding to MHC II does not impact antigen presentation</i>	154
4.3.2	<i>C3 deposition on cDCs causes their reduction in the spleen</i>	155
4.3.3	<i>Surface expression of cDC markers in MZ B cells</i>	157
5	The role of complement C3 in the trogocytosis between dendritic cells and B cells	163
5.1	INTRODUCTION	163
5.1.1	<i>Trogocytosis</i>	163

5.2	RESULTS.....	165
5.2.1	<i>In vitro</i> trogocytosis of C3-opsonized cDCs.....	165
5.2.2	<i>In vivo</i> trogocytosis of C3-opsonized cDCs	172
5.2.3	Antigen presentation by C3-opsonized cDC1s to B cells.....	175
5.3	DISCUSSION	177
5.3.1	<i>In vitro</i> trogocytosis of C3-coated cDCs.....	178
5.3.2	<i>In vivo</i> trogocytosis of C3-coated cDCs.....	180
6	Final discussion	184
6.1	SUMMARY OF MAIN FINDINGS	184
6.2	THE COVALENT ASSOCIATION OF COMPLEMENT C3 AND MHC II	185
6.3	A NOVEL AXIS OF COMMUNICATION BETWEEN cDCs AND MZ B CELLS	187
	References	192

List of Figures

Chapter 1

Figure 1.1 Ontogeny of DCs with essential growth and transcription factors..	38
Figure 1.2 Intracellular MHC II trafficking and antigen loading.....	48
Figure 1.3 The family of MARCHF Ub ligases and the role of MHC II ubiquitination in dendritic cells..	55
Figure 1.4 Overview of the complement cascade.....	60
Figure 1.5 Summary of successive cleavage of complement C3.....	62

Chapter 2

Figure 2.1 Flow cytometry gating strategies for major immune cells in the spleen.....	79
Figure 2.2 Flow cytometry gating strategies for conventional dendritic cells purified from different organs..	80
Figure 2.4 Flow cytometry gating strategies for human blood dendritic cells..	82
Figure 2.5 Flow cytometry gating strategies for cDCs and B cells from BM chimeric mice and <i>in vitro</i> trogocytosis assay.	82

Chapter 3

Figure 3.1 MARCHF1-deficient cDCs exhibit elevated levels of complement C3 at their surface.	93
Figure 3.2 Validation of enriched C3 surface levels on <i>Marchf1</i> ^{-/-} cDCs.	96
Figure 3.3 Impact of collagenase/DNase I digestion on C3 activation/deposition during cell preparation.	97
Figure 3.4 C3 surface levels on cDCs from mixed bone marrow chimera mice.....	99
Figure 3.5 Surface expression of complement receptors and regulators in WT and <i>Marchf1</i> ^{-/-} cDCs.....	100
Figure 3.6 Enriched MHC II surface levels in MARCHF1-deficient cDCs leads to increased C3 deposition.	102
Figure 3.7 The lack of MHC II ubiquitination leads to enriched MHC II surface levels and C3 deposition in cDCs..	103
Figure 3.8 Enriched MHC II surface levels in UBL3-deficient cDCs leads to increased C3 deposition.....	105
Figure 3.9 MHC II-dependent C3 deposition on the surface of WT cDCs.	106
Figure 3.10 Immunoprecipitation of C3 and MHC II from cDCs with subsequent detection of MHC II and C3 via WB..	109
Figure 3.11 PNGase F digestion of immunoprecipitated MHC II-C3 complexes.....	112
Figure 3.12 MHC II and C3 surface levels across various splenic immune cells from WT and <i>Marchf1</i> ^{-/-} mice..	114
Figure 3.13 C3 surface levels in cDCs from different organs of WT and <i>Marchf1</i> ^{-/-} mice.....	116
Figure 3.14 C3 surface deposition on splenic cDCs expressing different MHC II haplotypes.....	117

Figure 3.15 C3 deposition on human DCs in the blood from healthy donors and HLA-deficient patients.	119
Figure 3.16 Deposition of serological C3 on the cell surface of DCs and B cells through their incubation with serum.....	122
Figure 3.17 MHC II and C3 surface levels in cDCs and B cells upon <i>in vivo</i> activation..	125

Chapter 4

Figure 4.1 Antigen presentation capacity of C3-coated cDC1s.....	138
Figure 4.2 Enumeration of immune cells in the spleen of WT and <i>Marchf1</i> ^{-/-} mice..	141
Figure 4.3 Enumeration of cDCs in the spleen of mice with varying C3 levels..	142
Figure 4.4 Enumeration of cDCs from mixed bone marrow chimera mice..	143
Figure 4.5 C3-dependent appearance of CD11c ^{int} CD24 ^{high} CD8 ^{int} cells in <i>Marchf1</i> ^{-/-} mice.....	144
Figure 4.6 Surface marker phenotype of CD11c ^{int} CD24 ^{high} CD8 ^{int} cells.	146
Figure 4.7 Antigen presentation capacity of CD11c ^{int} CD24 ^{high} CD8 ^{int} cells.....	147
Figure 4.8 Transcriptome profiling of CD11c ^{int} CD24 ⁺ CD8 ^{int} cells.....	149
Figure 4.9 C3-dependent appearance of splenic CD8 ⁺ and CD11c ⁺ B cells..	151
Figure 4.10 Gating of CD11c ^{int} CD24 ⁺ CD8 ^{int} cells for B cell subpopulations.....	153

Chapter 5

Figure 5.1 <i>In vitro</i> trogocytosis assay between WT and <i>Marchf1</i> ^{-/-} cDCs and B cells upon PKH26-staining..	168
Figure 5.2 Acquisition of cDC surface proteins by B cell in <i>in vitro</i> trogocytosis assay.	170
Figure 5.3 <i>In vitro</i> trogocytosis assay between cDCs and CR2-deficient B cells.	171
Figure 5.4 <i>In vivo</i> trogocytosis assay between cDC1s and B cells upon intravenous injection of FITC-anti XCR1 mAb.....	173
Figure 5.5 <i>In vivo</i> trogocytosis of pMHC II (I-Ea ₅₂₋₆₈) between cDC1s and B cells.	175
Figure 5.6 <i>In vivo</i> OB1 B cell activation upon cDC1-targeted immunization with anti-Clec9A-OVA _{FGD}	177

Chapter 6

Figure 6.1 A schematic illustration summarizing the molecular binding of complement C3 to MHC II and its subsequent processing at the surface of dendritic cells and their consequential trogocytosis by B cells in the spleen..	191
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Literature Review

1 Introduction

The first chapter of this thesis introduces the biology of B cells and dendritic cells, the regulation of MHC II surface expression as well as the role of complement C3 in the immune system. The results achieved in this thesis (Chapter3-5) provide new insights into the interplay of all these cellular and humoral components of the immune system and requires their thorough introduction in order to prepare the reader sufficiently for the experimental chapters that bring together these various areas of immunology.

1.1 The adaptive immune system

1.1.1 Context

Immunity refers to the ability of an organism to resist harmful species that would otherwise damage or kill the host, achieved through the complex network of cellular and non-cellular (humoral) components [1]. A commonly used dichotomy categorizes these components into the innate and adaptive immune system, both working together in a complementary fashion to neutralize or eliminate pathogens and curb cancerous cells, while being capable of tolerating the own healthy tissue [2]. The innate immune system acts as a first layer of defense and consists of the rapid but rather unspecific actions of macrophages, neutrophils, natural killer cells and the complement system [3][4]. The more sophisticated adaptive immune system is characterized by slower but exquisitely specific actions that neutralize the infection and/or kill tumorous cells through the generation of pathogen-specific antibodies (Abs) and cytotoxic effector cells [5][6]. Furthermore, an immunological memory is established that provides long-lived protection from reinfections with the same/similar pathogen [7]. Immunotherapies for vaccination and cancer treatment aim to harness the specific actions of the adaptive immune system to prevent or fight diseases [8][9][10]. For this it is critical to understand how the innate and adaptive immune system cooperate to effectively mount a specifically directed immune response. Several key steps and components will be reviewed here, including the basic biology of B cells and dendritic cells (DCs), their presentation and recognition of pathogen-specific molecules (called “antigen”) as well as the role of the major histocompatibility complex class II (MHC II) and the complement system.

1.1.2 MHC-restricted antigen presentation

B cells and T cells provide the two effector arms of the adaptive immune system by generating pathogen-specific antibodies (Abs) and cytotoxic T lymphocytes (CTLs), respectively. For this, each B cell and T cell clone expresses a unique B cell receptor (BCR) and T cell receptor (TCR), respectively conferring each clone a specificity to a certain antigen (Ag). While B cells bind native Ag, T cells require Ag to be processed and displayed in a protein complex called major histocompatibility complex (MHC) [11]. The name MHC derives from its identification as the responsible factor for alloimmune reactions by transplanted donor cells against the host of bone marrow/stem cell and/or organs transplantations, known as graft versus host diseases (GvHD) [12]. The stimulation of CD8⁺ T cells is mediated through MHC class I (MHC I)-restricted Ag presentation while the TCR of CD4⁺ T cells binds to antigenic peptides presented in MHC class II (MHC II), initially discovered by Zinkernagel and Doherty [13][11]. All nucleated cells express MHC I and are able to present endogenous Ag derived from cytosolic self-proteins or viral gene products in MHC I. On the other side MHC II expression is restricted to antigen presenting cells (APCs) which are able to capture exogenous Ag (e.g. endocytosed cell debris or bacteria) to then process and present it on their surface in MHC II [14]. Only B cells, macrophages and dendritic cells (DCs) are considered as professional APCs, as they are able to induce CD4⁺ T cell responses while non-professional APCs, like mast cells, neutrophils and epithelial cells, indeed express MHC II but fail to stimulate naïve CD4⁺ T cells due to the lack of expression of crucial co-stimulatory molecules (i.e. CD86) [15][16][17][18]. Cross-presentation is a process that allows APCs to present extracellular Ag in MHC I (instead of MHC II), predominantly carried out by a certain subset of DCs [see section 1.3.6] [19][20][21]. Complementarily, endogenous or cytosolic Ag can be presented in MHC II (instead of MHC I) through a process called autophagy [22][23].

MHC I and MHC II-restricted Ag presentation has major implications in key stages during immune development and responses, including central and peripheral T cell selection, immune tolerance and the generation of cytotoxic and helper T cells or Abs. DCs are the principle initiator of adaptive immune responses via MHC II-restricted Ag presentation to CD4⁺ T cells. For this DCs tightly regulate the homeostasis of MHC II (+ co-stimulatory receptors) with changes of its biosynthesis, intracellular trafficking and surface expression upon stimulation.

1.2 B cells

B cells provide the humoral effector arm of the adaptive immune system by generating pathogen-specific Abs. For this B cells differentiate into either Ab-producing plasma cells or memory B cells upon acquiring T helper cell signaling through MHC II-restricted Ag presentation [24]. Various B cell subpopulations can be differentiated based on their stage of development and the corresponding surface markers they express (i.e. membrane bound immunoglobulin isotype and co-receptors).

1.2.1 B cell development

The ontogeny of B cells is well described in the literature [11][25]. As for all hematopoietic cells, B cells originate from hematopoietic stem cells (HSCs) in the bone marrow (BM) and fetal liver that give rise to the common lymphoid progenitor (CLP). CLPs progress through pro-B cell, pre-B cell and finally immature B cell stages through the expression of key transcription factors, such as the early B cell factor (EBF), E2a and Pax5, all inducing B cell-lineage specific genes [26][27][28].

During this development immunoglobulin (Ig) heavy and light chain genes undergo rearrangements and random deletions or additions of single nucleotides in order to generate various BCRs with each B cell clone conferring a defined Ag-specificity [29][30]. The rearrangement of these VDJ genes occurs mainly at pro-B cell and pre-B cell stages in the BM [31][32] through the action of recombination-activating gene (RAG-1 and 2) nuclease as well as the DNA repairing enzymes, including Artemis nuclease and terminal deoxynucleotidyl transferase (Tdt) [33][34][35][36][37]. Developed immature B cells in the BM express membrane-bound IgM BCRs [30]. Further genetic editing of the light chains occurs in the case of strongly self-reactive IgM [38][39] or, if this fails, apoptosis-mediated deletion of self-reactive B cells, in order to prevent autoreactivity. In cases where neither depletion nor receptor editing succeeds, self-reactive B cells leave the BM and a silenced state of anergy is induced in the periphery [40][41]. Immature B cells egress from the BM and rapidly reach the spleen through the circulation where they progress through two transitional B cell stages with distinct surface phenotypes: Type 1 transitional (T1) B cells are CD93⁺ CD21⁻ IgD⁻ IgM⁺ and T2 B cells are CD93⁺ CD21⁺ IgD⁺ IgM⁺. The latter give rise to follicular and marginal zone (MZ) B cells in the primary follicles of the spleen [42].

Mature naïve B cells recirculate through secondary lymphoid organs, namely lymph nodes (LN) and the spleen, to scan for Ag. Specialized blood vessels termed high endothelial venules (HEVs) serve as access points into the LN [43][44]. This is coordinated by various adhesion molecules, located on B cells and endothelial/stromal cells in the LN, as well as through chemokine receptor signaling, including CD62L, ICAM1/2 and CCR7/CXCR4 with the corresponding ligands CCL19/21 and CXCL12, respectively [44][45][46]. Within the LN B cells travel through the stromal cell network into B cell follicles [47][11], in which they migrate into the follicular dendritic cell (FDC)-rich center and the outer follicle region in a CXCL13-dependent manner [48][49]. B cells can recirculate into the blood by exiting the LN through the cortical lymphoid sinusoid and efferent vessels, in which sphingosine-1-phosphate has been reported to have a regulatory role [50][51]. The white pulp of the spleen contains T cell zones, B cell follicles and the marginal zone (MZ) [52][53]. Naïve B cells can enter the spleen via central arterioles through the MZ bridging channel into the T cell zone from where they migrate into B cell follicles through CXCR5-signaling [54]. Since follicular B cells do not express sufficient integrins to resist the blood flow in the MZ, they passively migrate through the red pulp and exit the spleen via venous sinuses [55].

1.2.2 Antigen encounter

Unlike T cells that require Ag to be processed and presented by dendritic cells (DCs) in MHC, the BCR of B cells recognizes linear/conformational epitopes of intact Ag [56], independent of MHC-restricted presentation by DCs. For this B cells need to encounter Ag in either secondary lymphoid tissues or in the circulation, which depends on size, dose and immunization route of the given Ag. Although B cell can encounter Ag in the circulation or peripheral tissues, Ag encounter in lymphoid organs is more likely as these tissues are specialized in filtering body fluids to capture circulating Ag and furthermore continuously recruit lymphocytes from the blood in order to shuttle and focus captured Ag into lymphoid follicles [57].

Small (<70 kDa) soluble Ag can drain or freely diffuse through conduits of the subcapsular sinus (SCS) to reach B cell follicles [58][59][60]. In contrast to this larger particulate Ag, such as immune complexes and viruses, cannot diffuse freely into B follicles and are captured by macrophages and DCs within the SCS [61][62][63][64]. SCS macrophages are minimal degradative due to low phagocytic activity and little expression of proteolytic enzymes so that

captured Ag remains intact. Intact Ag on the surface of these macrophages can either be presented to cognate B cells or transferred to non-cognate B cells and follicular dendritic cells (FDCs) for subsequent Ag-specific B cell stimulation [11][62][65][66]. Similar to SCS macrophages, FDCs exhibit low phagocytic activity and can retain intact Ag on their cell surface for days in order to effectively present it to B cells [67]. Display of intact Ag by FDC is critical in the germinal center (GC) reaction, but can also become the initial source of Ag recognition by cognate B cells [66][68]. Migration of B cells, towards FDCs within the follicle is mediated through CXCR5, expressed on B cells and the corresponding ligand CXCL13 is secreted by FDCs [69]. Intact Ag, that does not directly drain into LN, can be captured and transported into the LN through migratory DCs [70], interacting and sharing Ag to homing B cells before entering into the follicles of the LN [71]. In case B cells do not encounter Ag in secondary lymphoid tissue they can exit them and recognize free Ag in the circulation and re-enter the same or other draining LN [72][73]. Similarly, B cells in the spleen constantly encounter free soluble Ag from the circulation without requiring other cells to display them. However, MZ B cells of the spleen migrate back and forth between the MZ and B cell follicles to shuttle opsonized Ag from the blood to FDCs within B cell follicles [74][75][76].

Although B cells can be activated through the BCR-mediated stimulation through free Ag, there is strong evidence that the threshold for B cell activation is reached easier when the cognate Ag is tethered to membranous structures [68][77][78]. A potential explanation is that recognition of membrane Ag causes architectural reorganization of the Ag-BCR synapse between the Ag-carrying cell and the B cell, which leads to a concentration of associated tyrosine kinases within the plasma membrane and consequently enhanced signaling [68][77]. A range of receptors on various cell types have been reported to capture and display Ag for effective B cell activation, including complement receptors 1-4 (CR1-4) [79][80], Fc receptor (FcR) FcγRIIb [81] and the C-type lectin receptor SIGNR1 as well as Clec9A [82][83].

1.2.3 B cell activation

The recognition of Ag via the BCR induces intracellular signaling resulting in the induction of various genes, including immune and co-stimulatory receptors (i.e. MHC II & CD80/CD86), adhesion molecules and pro-survival or cell-cycle related molecules [24]. Contradictory models describe Ag recognition-induced BCR clustering [84] versus that pre-existing BCR clusters dissociating upon Ag binding [85], both initiating intracellular signaling,

predominantly through kinase-mediated phosphorylation (i.e. Syk tyrosine kinase) [86][87][88]. The BCR of naïve B cell consists of two isotypes of membrane bound immunoglobulins, namely IgM and IgD, that differentially respond to Ag by which the B cell's fate is controlled [89][90]. Both IgM and IgD have only small intracellular C-terminal domains and depend on associated co-receptors for effective signal transduction, including Ig α and β as well as CD19 [91][92]. Engagement of signaling through Toll-like receptor (TLR) 7 and 9 as well as complement receptor (CR) 2 has been shown to amplify and modify BCR signalling [93][94][95][96], while ITIM-containing co-receptors, such as CD22 and CD27 act as negative regulators [97]. Importantly, Ag binding to BCR causes internalization of the BCR-Ag complex via clathrin-dependent endocytosis [98]. Internalized Ag is processed through proteolytic degradation in late endosomes and lysosomes for peptide loading and presentation in MHC II to CD4⁺ T cells, which in turn is relevant for further stimulation and development of the cognate B cell (T cell-dependent B cell response) [99][100]. Alternatively, B cell responses can also be T cell-independent, either through co-receptor signalling alone, without the involvement of BCR signalling (type I T cell-independent B cell activation) or through BCR signalling in case of strong Ag affinities, such as through BCR cross-linking (type II T cell-independent B cell responses) [101][102]. Both, T cell-dependent or -independent activation, leads to the proliferation of the respective B cell clone, characterized by its expansion and differentiation.

T cell-independent B cell responses

Certain Ag are capable of inducing B cell responses independent of stimuli from cognate CD4⁺ T helper cells, often referred to as T cell-independent induction of innate-like B cell responses [101][102][103]. Such Ag can either be specific agonists of Toll-like receptors (TLRs), such as lipopolysaccharides (LPS), CpG oligodeoxynucleotides (CpG) or polyinosinic:polycytidylic acid (PolyI:C), classified as type I Ag, or type II Ag, such as polysaccharide with repetitive structures. While type I Ag do not depend on BCR signalling at all, type II Ag, such as dextran and Ficoll, strongly trigger BCR signalling [104][105]. Type II Ag often also activate the complement cascade as well as induce Ag-specific B cell responses with even germinal center (GC) formation, however they generally fail to generate long lasting plasma cells and do not induce the production of high affinity Abs [106]. Instead low-affinity IgM and IgG from extrafollicular B cells and B cells in the splenic bridging channel, known as natural Ab, are

generated in response to type II Ag [107], although memory B cell formation has been demonstrated upon immunization with Ficoll [105].

B1 B cells and MZ B cells (B2) are the main contributors to T cell-independent B cell responses. While B1 B cells derive from a distinct B cell-lineage [108], MZ B cells differentiate from mature naïve B cells in the spleen, where they reside in the MZ. Beside their function in shuttling blood borne particulate Ag, filtered from the circulation, to the follicles [74][75], their rapid differentiation into IgM⁺ plasmablasts and the secretion of large amounts of IgM upon bacterial Ag encounter has been demonstrated [109][110][111][112]. T cell-independent B cell responses are typically short-lived and generate Abs with only low specificity, but they enable a very rapid and intense humoral Ab immune response. Due to these characteristics B-1 and MZ B cells are often considered “innate” B cells and are known to play a critical role in neonates and young infants, as they provide the first line of Ab production at a stage of life where T-dependent Ab responses cannot be generated [113][114].

T cell-dependent B cell responses

The generation of high-affinity Ab requires the stimulation of B cells by CD4⁺ T helper cells that respond to the same Ag and are pre-activated by encounter with Ag presenting DCs. This *linked recognition* is a critical control mechanism to ensure self-tolerance and optimal B cell proliferation [115]. In order to receive help from cognate CD4⁺ T cells, B cells present antigenic peptides in MHC II. This happens at the interface of B cell follicles and T cell zones, called the T-B border. The migration of both T and B cells towards the T-B border is mediated through the change in expression of various chemokine receptors upon TCR/BCR signalling, including CCR7, CXCR3 and EBL2. While B cells downregulate CXCR3 but upregulate CCR3 and EBL2 [11][116][117][118], T cells increase CXCR3 but reduce CCR3 expression [119]. The conjugation between B cells and T cells at the T-B border can last up to one hour [117][120] and involves the interaction of several surface molecules. Both, the interaction between the integrin LFA1 on the T cell surface and ICAM1/2 on B cells, as well as the engagement of pMHC II complexes to the cognate TCR support the stability of the cell-cell interaction [24][121]. B cell-mediated Ag presentation to CD4⁺ T helper cells is also important for their subsequent differentiation into follicular helper T cells (T_{FH}) that subsequently provide help within GCs [120].

Upon receiving help B cells migrate to distinct compartments within the LN and differentiate into either short lived extrafollicular plasma cells or instead, enter GCs to undergo affinity maturation. Using transgenic B cells that express a hen egg lysozyme (HEL)-specific BCR and different HEL mutants with varying BCR affinities Paus *et al.* have demonstrated that Ag recognition strength determines the fate between either extrafollicular plasma cell or GC B cell differentiation [122]. GC B cell differentiation allows class-switch recombination (CSR) and further BCR diversification through somatic hypermutations (SHM) of Ab variable regions, eventually resulting in highly specific Abs after multiple rounds of affinity maturation and clonal selection. The central DNA-editing enzyme in GC B cells for this is the activation-induced cytidine deaminase (AID) [123]. Bcl-6 is a transcriptional regulator and essential in the formation and maintenance of GC B cells [124][125], inducing AID expression but suppressing the DNA-damage responses and thereby allowing SHM [126]. GCs are composed of dark zones in which B cells (centroblasts) undergo extensive proliferation and SHM and FDC- and T_{FH}-rich light zones in which Ag presentation and clonal selection of B cells (centrocytes) with mutated BCRs takes place [52][120][127]. Ab affinity maturation occurs through iterative rounds of SHM and clonal selection when GC B cells transition between the grey and light zone back and forth [128]. Early affinity maturation models describe that clonal selection of centrocytes in the light zone is driven by limited Ag availability. Thereby only a certain amount of Ag on the surface of FDCs is available for B cells to capture, thus depleting B cells with low BCR affinity whilst allowing B cells with higher BCR affinity to survive [129]. More recent imaging of GC has shown that B cells were highly motile whilst probing for many several Ag instead of remaining in stationary contact with FDCs [120][130], indicating that B cells compete for T_{FH} help rather than Ag availability [11]. Victora *et al.* have shown that immunization with anti-DEC205 targeted cognate Ag promotes MHC II presentation and T_{FH}-help acquisition, leading to equal clonal expansion of B cell clones independent from their BCR affinities [131]. Hence, enhanced T_{FH}-help accelerates proliferation and survival of GC B cells and consequently also the rate at which SHM drives affinity hypermutation [132].

Low Ag availability determinates GC responses, with Ag running out or being opsonized with Abs from arising plasma cells, preventing further Ag uptake by GC B cells, which creates a negative feedback loop [133]. GC B cells can either differentiate into memory B cells or plasma cells [134]. GC B cell clones with higher BCR affinity are profound to plasma cell differentiation [135] with interferon regulatory factor 4 (IRF-4) as a central transcription factor in plasma cell

differentiation [136]. Interestingly, GC B cells that have switched to IgE isotype during CSR predominantly become plasma cells rather than memory B cells [137][138], indicating that the Ig isotype influences B cell differentiation. Plasma cells can also develop from early phase B cell activation, but most plasma cells arise from GC B cells after affinity maturation and isotype switching. They are long-lived and migrate into the bone marrow via CXCR4/CXCL12-signalling from where they constantly secrete high amounts of Abs [139]. Due to their constant Ab production plasma cells are characterized by enriched amino acid and sugar metabolism [140].

Activation of B cells through dendritic cells displaying intact antigen

Dendritic cells (DCs), in particular conventional/classical DCs (cDCs), are known to be the principle initiators of T cell-dependent B cell responses by inducing the differentiation of naïve CD4⁺ T cells into various effector T cells through their Ag-specific stimulation. Although SCS macrophages and FDCs are the two major cell types displaying intact Ag to B cells for the initiation of humoral immune responses, numerous studies have demonstrated that conventional DCs (cDCs) can also present Ag to B cells inducing B cell responses in a MHC and T cell-independent manner (reviewed by Heath *et al.* [68]). Early *in vitro* assays carried out co-culturing of Ag-pulsed cDCs and B cells and have demonstrated their direct interaction which led to isotype-switching responses without T cell help [141][142]. Direct B cell-cDC interactions *in vivo* have been demonstrated in the extrafollicular region of LN through two photon microscopy [71], as well as for MZ B cells in the spleen [111], both initiating T cell-independent immune responses. Intact Ag has been detected on the surface of DCs in form of immune complexes through binding to the surface Fc receptor FcγRIIB [81], but more recent immunization approaches aim to target Ag specifically to the surface of DC using Abs [68]. cDC2 in the bridging channels of the spleen can activate transgenic Ag-specific B cells upon immunization with viral nucleoprotein (NP) or hen egg lysozyme (HEL) Ag conjugated to DCIR2-specific Abs, both T cell-independent as well as MHC II and CD86-independent [68][143]. Thereby the chemokine receptor EBL2 plays a critical role for the localization and function of splenic cDCs [144][145]. While these immunization approaches fail to induce GC formation, potentially due to missing adjuvants [68], Ag targeting to the surface of cDC1 via Clec9A-specific Abs has been shown to induce T_{FH} and GC responses with isotype-switching and affinity maturation even in the absence of adjuvants [83][146][147][148]. Interactions

between cDCs and B cells mediated by targeted-Clec9A immunization with hen egg white ovalbumin (OVA) Ag has been intensively investigated by Yu Kato from the laboratory of Professor William R. Heath (University of Melbourne), summarized in his PhD thesis [11].

1.3 Dendritic cells

Dendritic cells (DCs) are a heterogeneous group of hematopoietic immune cells that are specialized in capturing and processing Ag for its presentation to T cells. There are three major subpopulations, namely classical or conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocyte-derived DCs (moDCs) [149][150]. In a broader sense follicular DCs (FDCs) and Langerhans cells (LCs) are often considered non-hematopoietic DCs. Although all of these DC subsets are capable of capturing, processing and presenting Ag to naïve T cells, researchers usually refer to cDCs when talking about DCs (if not specified otherwise) as they have the highest Ag presentation capacity and are the principle initial stimulators of T cell responses via MHC-restricted Ag presentation [151][152][153]. MHC II-restricted Ag presentation by macrophages and B cells serves other purposes, mainly to acquire T cell help [154].

The classification of DC populations is difficult for several reasons: {1} DCs do not possess a distinct, DC-specific phenotypic marker [unlike B cells (e.g. CD19, B220 or BCR) or T cells (e.g. CD3 or TCR)], {2} their phenotype is highly diverse and varies, depending on their localization and activation status, and {3} although all hematopoietic DCs (cDCs, pDCs and moDCs) derive from the bone marrow (BM), no distinct DC precursor has been identified, indicating that the DC lineage is not unified [149][155]. Despite these difficulties, researchers aim to define DC subsets based on ontogeny as well as functional and phenotypical characterizations, including key surface markers, cytokines and transcription factors [reviewed in [155]].

1.3.1 Discovery of dendritic cells

In 1868 Paul Langerhans described cells with dendritic morphology in the human skin [156], today known as dermal Langerhans cells (LCs) [157], but it wasn't until over a century later in 1973 that Steinman and Cohn discovered and defined DCs [158]. Their critical role in the immune system was elucidated decades later and nowadays DCs are recognized as the

principle Ag presenting cells in our body that have the highest capacity to mount an adaptive immune response, implying great potential for immunotherapies. The importance of their discovery was acknowledged in 2011 when Ralph Steinman was awarded the Nobel Prize in Physiology and Medicine [159][160]. The diversity of DCs was initially discovered in the mid-1990s when two subsets of murine DCs were identified based on the expression of CD8 and functional differences [161]. The discovery of DCs with plasma cell phenotype, that produce large amounts of interferon (IFN)- α for T cells priming against viral Ag, led to the classification of plasmacytoid DCs (pDCs) [162]. To distinguish these from Steinman's DCs, the latter were renamed as conventional DCs (cDCs) [155]. The non-hematopoietic follicular DCs (FDCs) were discovered in 1965 by Mitchell and Abbot [163] and play an important role in the lymphoid follicle and the germinal center (GC) by presenting Ag to B cells, contributing to the initiation of humoral adaptive immune responses [67][164][165].

1.3.2 Subpopulations of dendritic cells

Conventional dendritic cells (cDCs)

Murine conventional DCs (cDCs) express high levels of the integrin α X CD11c [166]. Although this surface marker is commonly used for the identification of cDCs, its expression has also been shown on natural killer (NK) cells, macrophages and B cells [167][168][169][170], therefore requiring the use of more than only CD11c as an identification marker for cDCs [171]. Studies with transgenic mice that express the diphtheria toxin receptor (DTR) under the control of the CD11c promotor not only lack cDCs but also macrophages upon administration of diphtheria toxin [172][173], equivocating the observations of impaired CD4⁺/CD8⁺ T cell responses in these mice [174][175]. However, a more targeted approach using the DTR expression under the control of the highly cDC-restricted *Zbtb46* promotor lead to the distinct ablation of cDCs and confirmed their unique role in T cell stimulation [176].

cDCs can be classified based on their migratory behavior: pre-cDCs egressing from the BM can either migrate to secondary lymphoid organs (i.e. thymus, spleen and LN) to become *lymphoid tissue-resident* cDCs or develop into *migratory* cDCs in peripheral tissues including the lung, skin or intestine [155]. While cDCs resident in spleen and LN encounter pathogens/Ag in the filtrate of blood and lymphatic fluid, migratory cDCs patrol the body's periphery for Ag [177][178]. Upon Ag recognition they upregulate the chemokine receptor

CCR7, facilitating their migration into draining LN [179][180][181] to either transfer Ag to LN-resident cDCs [182] or present Ag by themselves for T cell stimulation [183]. Migratory cDCs only migrate into draining LN, so that the spleen only contains tissue-resident cDCs while both migratory and tissue-resident cDCs are present in LN [155][184]. Most migratory cDCs die within the LN but it has been shown that some can exit through efferent vessels to participate in immune regulation and tolerance [155][184][185]. Higher MHC II but lower CD11c surface expression compared to tissue-resident cDCs allows the identification of migratory cDCs in the LN under steady state conditions [155], but this changes dramatically in case of an infection/inflammation, characterized by the strong upregulation of MHC II and costimulatory molecules (i.e. CD86/CD80) and the production of inflammatory cytokines, a process called DC maturation [see section 1.3.6].

In addition to the classification based on their migratory behavior, cDCs can also be subdivided broadly into two subsets with distinct functional and phenotypical properties: cDC1s are CD8⁺ CD11b⁻, while cDC2s are CD8⁻ CD11b⁺ [186][187], both expressing high levels of CD11c and MHC II. This surface phenotype is more reliable for lymphoid tissue-resident cDCs compared to migratory cDCs allowing further sub-classifications of splenic cDC2s based on their surface expression of CD4 [186][188] and ESAM (endothelial cell-selective adhesion molecules) [ESAM^{hi}CD4⁺ vs. ESAM^{lo}CD4⁻] [189][190]. This heterogeneity within the cDC2 subpopulation is further supported by recently published data by Brown *et al.* that have identified two principal cDC2 lineages defined by distinct developmental pathways and transcriptional regulators, including T-bet and RORγt [191]. In principle, the classification of cDC1 and cDC2 also applies to migratory cDCs present in non-lymphoid tissues but implies some differences compared to tissue-resident cDCs and is in general more difficult as their phenotype varies between peripheral tissues and the site of LN drainage. In brief, CD103⁺ migratory cDCs resemble tissue-resident cDC1s (although they do not express CD8) while migratory cDCs expressing CD11b⁺ CD172⁺ share origin and function with tissue-resident cDC2s [192][193][194][195]. Migratory cDC2s are often hard to distinguish from tissue macrophages due to a similar surface phenotype [155]. The functional specialization and differences between cDC1s and cDC2s are discussed in section 1.3.6.

Plasmacytoid dendritic cells (pDCs)

pDCs represent a small subset of DCs. In contrast to cDCs that do not fully differentiate until

reaching the peripheral tissue, pDCs fully mature already in the BM and hence, are present at detectable levels in the circulation of mice as well as in their lymphoid organs. Their role as professional APCs is limited as they are instead important for mediating anti-viral immunity by sensing pathogen-derived nucleic acids and the consequential production of large amounts of type I interferon as antiviral mediators [162][196][197]. Non-activated pDCs show low surface expression of MHC II and co-stimulatory molecules CD80/CD86 but significant levels of Toll-like receptor (TLR) 7 and 9 that allow recognition of foreign nucleic acids [155][162][196][198]. Although it has been shown that pDCs can capture and process soluble Ag to induce T cell responses [199][200][201][202][203], they are inefficient at capturing cell-associated or particulate Ag [203][204]. Activation of pDCs via TLR7/9 signalling leads to a subdued upregulation of MHC II. Additionally, it has been shown that ubiquitination-mediated internalization of MHC II from the cell surface is retained in pDCs upon their activation, which profoundly differs from cDCs [199]. Overall, pDCs only have limited Ag presentation capacity but are important contributors to antiviral immunity as they produce large amounts of type I interferon [196].

Monocyte-derived dendritic cells (moDCs)

Monocytes were originally thought to be the immediate upstream precursors of cDCs based on studies showing *in vitro* differentiation of monocytes into DCs [205]. However, these monocyte-derived DCs (moDCs) are distinct from bona fide cDCs based on functionality and ontogeny. MoDCs occur in high numbers during inflammatory conditions such as acute infections but are present only in low numbers in the intestinal and muscular tissue in steady state [155].

Blood monocytes can be divided into the major subset of Ly6C^{high} CX₃CR1^{low} CCR2⁺ monocytes (Ly6C^{high} monocytes) and a minor group of Ly6C^{low} CX₃CR1^{high} CCR2⁻ monocytes (Ly6C^{low} monocytes) [206]. It is predominantly Ly6C^{high} monocytes that give rise to moDCs in peripheral mucosal tissues in lung and intestine driven by the growth factor GM-CSF (Granulocyte-macrophage colony-stimulating factor) but independent from the growth factor Flt3L (FMS-like tyrosine kinase 3 ligand) [207][208][209]. Under inflammatory conditions Ly6C^{high} monocytes exit the BM via CCR2 signalling (ligand CCL2/7) [210] and migrate into the inflamed tissue via CCR6-CCL20 interaction [211], where they can differentiate into moDCs. From there they either can migrate into draining LN [206][212], where they can (cross)present

exogenous Ag in MHC I and MHC II to CD4⁺ and CD8⁺ T cells, respectively [213][214][215] or stimulate memory CD8⁺ T cells in peripheral non-lymphoid tissues [216]. However, the principle role for Ag presentation remains with cDCs, while moDCs are rather considered monocytes that acquire certain DC-like features with only little efficiency in Ag presentation, depending on the route of Ag uptake [217].

1.3.3 Human dendritic cells and clinical translation

Human dendritic cells

Current knowledge regarding DCs has been predominantly generated through studies using mouse DCs. Human DCs are present in the circulation as fully differentiated cDCs (in contrast to murine cDC) such that blood samples, but also tonsils and skin tissue samples, have been used as a major source for human DCs [218][219][220]. However, clinical translation remains a challenge due to limited sample availability, low abundance of DCs in blood (~0.2%) and significant differences in the surface phenotype between human and mouse DCs [221][222][223]. Human cDCs completely lack expression of CD8 [224], the key surface marker to differentiate mouse cDC1s from cDC2s [161], but flow cytometry and genomic profiling have provided insights into the linkage between mouse and human DCs.

Human blood cDCs are identified as CD11c⁺ and MHC II⁺, while lacking the lineage markers CD3, CD14, CD16, CD19, CD20 and CD56 [221][225][226][227]. Based on these features, two major cDC subsets can be further distinguished: {1} CD11c⁺ CD141⁺ CD1c⁻ (resembling mouse cDC1s) and {2} CD11c⁺ CD141⁻ CD1c⁺ (resembling mouse cDC2s). Blood circulating pDCs are lower in CD11c and MHC II expression but are BDCA2/3⁺ CD123⁺ [223][225][226][227]. Similarly to blood DCs, two main subsets of human cDCs in non-lymphoid tissue (i.e. the skin) and lymphoid tissues, including tonsils, spleen, thymus and LN, have been identified as CD1c/CD1a⁺ CD14⁺ vs. CD141⁺ cDCs [219][228][229][230][231][232]. The linkage between mouse and human DCs is supported by transcriptome profiling that revealed conserved expression profiles between mouse and human pDCs and shared signatures between murine cDC1s and cDC2s with human CD141⁺ and CD1c⁺ DCs, respectively [233]. Furthermore, the expansion of CD11c⁺ CD141⁺ CD1c⁻ cells upon Flt3L treatment [234][235], their expression of XCR1 and Clec9A [83][236][237][238], as well as their ability to cross-present and produce interleukin (IL)-12 [228][236][237][239] strongly indicate their equivalence to mouse cDC1s [224][240]. Additionally, key transcription factors have been shown to be similarly expressed

between human and mouse cDCs with IRF4 (Interferon regulatory factor 4) in cDC2s [241] and IRF8 and BATF3 in cDC1s [239][228].

The importance of DCs in the human immune system and health is demonstrated in patients with immune deficiency syndromes that lead to non-functional or complete absence of tissue and blood DCs. Two major genetically defined syndromes of DC deficiency are described in humans [155][242], predominantly caused by (null)mutations in the *Irf8* gene [243] and a GATA-binding factor 2 mutation that leads to the DCML (DC, monocyte B an NK lymphoid) deficiency syndrome [244]. Both are characterized by the symptoms of increased susceptibility to various viral and bacterial infections as well as enhanced risk for cancer onset [242][245].

Dendritic cells in immunotherapy

Since DCs have been identified as the principle initiators of adaptive immune responses, researchers aim to modulate their stimulatory potential for immunotherapy approaches in the treatment of various cancers and the development of DC-based vaccines [246][247][248][249][250]. Briefly, two main approaches have been pursued: {1} the loading of DCs with the corresponding Ag *ex vivo*, with their subsequent adoptive transfer back into the patient and {2} the injection of Ag conjugated to antibodies for the targeted loading onto the surface DCs *in vivo*.

In the early 1990's the *ex vivo* approaches were successfully tested in mouse models in which loading of DCs with viral and tumorous Ag induced effective CTL responses and immunity [251][252][253]. Anti-tumor CTL response mediated through *ex vivo* antigen-loaded DCs was demonstrated in a human clinical study in which patients with B cell lymphoma successfully showed tumor regression [254], though this approach remains difficult due to low frequency and survival of isolated DCs and limited migration upon adoptive transfer [255]. Although researchers have used various approaches to address these issues, including the expansion of DCs via Flt3L treatment *in vivo* [256] and the generation of DCs from monocytes through GM-CSF culturing [257], targeting DCs with antibody-conjugated Ag *in vivo* avoids these hurdles completely.

In general, antibodies (Abs) conjugated to Ag used for *in vivo* targeting bind specifically to surface molecules exclusively expressed by the cell population of interest, in order to favor Ag capture and presentation and consequently enhance stimulation of naïve T cells. Although

targeting of B cells and macrophages with Ag conjugated to anti-CD169 and anti-CD80 Abs respectively has been shown to form T_{FH} and germinal center (GC)-derived antibody responses [258][259], Ag delivery to DCs *in vivo* has been shown to be the most effective way to enhance immune responses [260]. Anti-CD11c targeted Ag delivery successfully enhanced CD8⁺ and CD4⁺ T cell priming and GC formation in mice [261][262][263], but targeting Ag delivery via anti-CD11c Abs in humans is dubious as CD11c is more widely expressed and less restricted to DCs [255]. Other DC-specific surface markers exploited for targeted antibody-mediated Ag delivery *in vivo* are DEC205 [264], Clec9A, Clec12A [83][146] and XCR1 [265], capable of inducing strong Th1 and CD8⁺ T cell responses [266].

1.3.4 Ontogeny

cDCs, pDCs and moDCs arise from self-renewing hematopoietic stem cells (HSCs) in the BM and fetal liver through the progressive differentiation towards downstream precursors with certain lineage properties [267]. All hematopoietic DCs are short-lived with a life-span of only a few days in case of splenic cDCs and up to three weeks for cutaneous LN cDCs, such that DCs are constantly replenished [188][268]. For a long time there was no evidence for a distinct DC specific lineage and although details still remain unclear, new insights postulate one commonly accepted model: Early stage lymphoid-myeloid stem cells in the BM express Flt3, a critical key component for DC development [see section 1.3.5] [269], followed by the differentiation into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs) [270][271]. Although there is evidence that CLPs can give rise to DCs [272], it has been shown that the majority of DCs derive from CMPs that bifurcate into the common monocyte progenitor (cMoP) and common dendritic cell progenitor (CDP) [273][274]. Some studies describe a macrophage/DC progenitor (MDP) [275][276] but the existence of such an intermediate precursor, emerging downstream from CMP stages, remains controversial [274]. MoDCs arise from cMoPs [277], while CDPs are considered the dedicated precursors for the cDC lineage, generating pre-cDCs, yet quickly losing the potential to spawn pDCs [278][279][280]. The direct pDC progenitor remains elusive but presumably differentiates shortly after CDP formation [281]. In contrast to pDCs that fully develop within the BM, pre-cDCs migrate from the BM into peripheral tissues and locally differentiate into either migratory or lymphoid tissue-resident cDCs [150][282][283][284][285].

Although it is commonly accepted that DCs are myeloid derived, studies have revealed

exceptions: thymic CD8⁺ cDCs seem to have a lymphoid progenitor (shared with T cells) [286][287], pDCs and some thymic cDCs have the potential for Ig heavy chain rearrangement, a characteristic for lymphocytes [288], and the formation of some cDCs and pDCs from CLPs and differentiated T cell progenitors in the spleen has been described [269][289][290][291]. A schematic pathways of DC development with relevant transcription and growth factors is displayed in Figure 1.1.

1.3.5 Transcription and growth factors of dendritic cells

Transcription factors

The differentiation from CPDs and pre-DCs into pDCs or migratory/resident cDC1s (CD8⁺/CD103⁺ cDCs) and cDC2s (CD11b⁺ cDCs) is controlled by several transcription factors at different stages during their development (reviewed by Murphy *et al.* [285]). Transcription factors at early progenitor stages, such as Ikaros and PU.1, affect the expression of large sets of genes for the development of lymphoid and myeloid lineages, while transcription factors at later CDP stages are more DC-specific, including IRF4, IRF8, ID-2 and BATF3 [292]. IFN regulatory factor 4 and 8 (IRF8 and IRF4) are the critical transcription factors regulating the terminal differentiation of cDC1s and cDC2s, respectively [293][294]. IRF8 expressed in CPDs dictates cDC1 development and its activity is promoted by its interaction with BATF3 (Basic leucine zipper transcriptional factor 3) [295][296][297]. Conversely, IRF4, regulated by RelB and to a lesser extent by IRF2, has been shown to be involved in cDC2 development [298][299][300]. As the cDC2 subset is more diverse than cDC1s, their set of described transcription factors is less distinct and involves a larger range of relevant factors, such as such TRAF6 [301] and Notch2 signalling [190], in addition to IRF4, IRF2 and RelB. Furthermore, a subset of CD4⁺ cDC2s has been described that does not derive from CDPs, and is independent of IRF4 [190][241]. However often IRF4 is used to distinguish migratory cDC2s and macrophages in peripheral tissues, as unlike cDC2s macrophages do not express IRF4 [241]. It has been shown that BATF3 is expressed in both cDC subsets but *batf3*-deficiency only abrogates cDC1 development at terminal stages [292][295][297]. The class I basic helix loop helix (bHLH) factor E2-2 binds to conserved DNA motifs, called E boxes, to promote pDC differentiation in early CDP stages and is negatively regulated by the inhibitor of differentiation protein ID-2. Balance between E2-2 and ID-2 determines the choice between pDC and cDC development [292], demonstrated by impaired pDC but enriched cDC1

development in case of increased ID-2 expression [302][303]. Furthermore, IRF8 has been reported to play a role in the differentiation of pDCs at late progenitor stages [304][305].

Growth factors and cytokines

Beside transcription factors several, cytokines and growth factors are relevant for the differentiation and expansion of DC progenitors in the BM as well as the maintenance of differentiated DCs in peripheral and lymphoid tissues.

The most important cytokine controlling DC lineage commitment is Flt3L (FMS-like tyrosine kinase 3 ligand) [289][290][291]. This key regulator is predominantly produced and secreted by endothelial cells and T cells [306] and the corresponding receptor, Flt3 (CD135), is expressed by early DC progenitors, including HSCs, CLPs and CMPs, as well as more direct cDC progenitors, namely CDPs and pre-cDCs [155][278][289][307][308]. Conversely, Flt3 expression is lost in those progenitors that do not develop into DCs [289]. Signalling through Flt3 induces expression of various genes relevant for DC differentiation via the RAS and STAT5 pathway [309]. Interestingly, skin langerhans cells (LCs) and also moDCs do not rely on Flt3L [289], which strongly suggests their independent ontogeny, while *ex vivo* culturing of BM cells with Flt3L leads to DC development (BM-derived DCs) [289]. Flt3 deficiency leads to a loss of DC development and reduction in corresponding progenitors, including MDPs and CDPs [308][310], but remarkably increases serum levels of Flt3L, which indicates a regulated feedback mechanism [155]. Conversely, injection of Flt3L in both humans and mice causes dramatic expansions of DC populations in the circulation and various tissues [256][290][311]. Although Flt3L has critical implications for the development for both cDC subsets, cDC2s were reduced in Flt3L-deficient mice to a lesser extent than cDC1s [308] and Flt3L injection preferentially expanded cDC1s over cDC2s [312], both indicating a stronger relevance for Flt3L in cDC1 development. The role of Flt3L beyond DC lineage commitment remains elusive but is potentially of relevance as the receptor remains highly expressed on the surface of fully differentiated DCs. Furthermore, approximately 25% of all acute myeloid leukaemia (AML) patients show Flt3 mutations [309][313].

The second group of growth factors relevant for DC development includes Csf-1 and Csf-2, also known as macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), respectively. Both are less DC-restricted, as their names indicate, but still play a relevant role in DC development and homeostasis. The receptor for

M-CSF, Csf-1R, is expressed on GMPs and MDPs, but reduced on CDPs and completely lost on differentiated cDCs, indicating a more relevant role in macrophage differentiation [314]. [314]. It is thought that Csf-1R versus Flt3 signalling determines MDP development into either monocytes or CDPs [155][306]. GM-CSF is thought to have more DC-restricted features, as its receptor, Csf-2R, is expressed on fully differentiated cDCs [310][314]. Although GM-CSF is used for the differentiation of mouse and human monocytes and hematopoietic progenitors into cDCs for research and clinical use [205][315][316][317][318], the lack of Csf-2R signalling in mice surprisingly has no major effect on cDC development in spleen and LN [319] but causes a reduction and impaired functions in CD103⁺ DCs in peripheral non-lymphoid tissues [320][321], indicating its role in maintaining cDCs in local tissues. A schematic pathway of DC development with relevant transcription and growth factors is displayed in Figure 1.1.

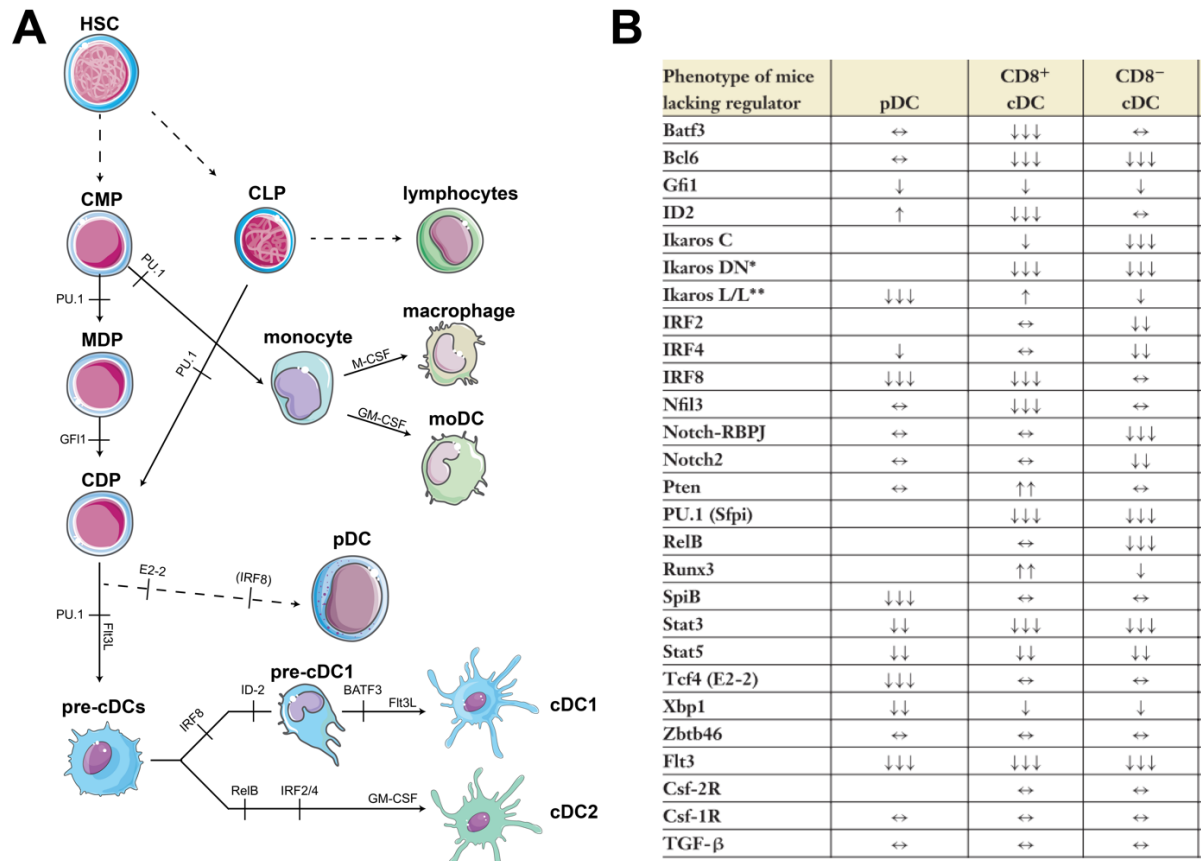


Figure 1.1 Ontogeny of DCs with essential growth and transcription factors. (A) A schematic pathways of DC development. Myeloid and lymphoid progenitors (CMP, CLP) derive from hematopoietic stem cells (HSC) over lymphoid-myeloid stem cells (not shown) into the common dendritic cell progenitor (CDP) in the BM from where pDCs and the diversification of cDC subsets (cDC1 and cDC2) develop. The approximate points at which key transcription factors are first required for DC development (i.e. PU.1 for cDCs, IRF8, ID2 and BATF3 for cDC1, RelB and IRF2/4 for cDC2 and E2.2 for pDCs) are indicated by orthogonal line, while growth factors are indicated along the arrows (based on Merad et al. [155] and Belz and Nutt [322]). **(B)** The table describes the effect of deficiency in various transcription factors in mice on the relative abundancy of pDCs and lymphoid tissue cDC subsets, compared to WT mice. ↓ indicates a reduction in cell numbers; ↑ indicates an increase in cell numbers; and ↔ means no change in cell numbers. The number of arrows is representative of the severity of the phenotype, with ↓↓↓ suggesting an almost complete loss of the population. *Ikaros DN = dominant negative allele; **Ikaros L/L = low-level expression (taken and modified from Merad et al. [155]).

1.3.6 Dendritic cell functions in the immune system

Sensing pathogens

DCs express a range of innate receptors that enables them to sense pathogens through the detection of highly conserved structures/molecules, called pathogen associated molecular patterns (PAMPs). Signalling through these pattern recognition receptors (PRR) is critical for DCs to effectively stimulate T cells as they modulate Ag processing and presentation as well

as expression of essential co-stimulatory molecules and cytokines. Important PRR are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (reviewed in [323]).

The group of TLRs has been studied the most. There are twelve mouse TLRs (TLR1-9 & TLR11-13) all sharing a cytoplasmic signalling domain, known as Toll/IL-1R (TIR) domain and an extracellular, ligand-binding motif containing leucine-rich repeats [324]. Ligand binding causes dimerization of TLRs that brings the intracellular TIR domains in close proximity, inducing proinflammatory cytokine and co-stimulatory molecule expression via the NF κ B and IRAK or TRIF pathways [325][326][327][328][329]. Each TLR has specific cellular localization and binding properties to recognize specific PAMPs, with TLR1, 2, 4, 5, and 6 expressed on the plasma membrane and TLR3, 7, 8, 9, 11 and 13 being present in intracellular vesicles. Mouse DCs express TLR1-9 with a lack of TLR5 and TLR7 in cDC1s and TLR3 in cDC2s [326][330]. TLR2 dimerizes with TLR1 or TLR6 to sense bacterial lipoproteins and peptidoglycans. TLR3 recognizes viral double-strand (ds)RNA, TLR4 binds lipopolysaccharides (LPS), TLR7 and TLR8 are receptors for single-strand (ss)RNA and TLR9 recognizes bacterial unmethylated CpG [327].

C-type lectin receptors (CLRs) are a versatile family of more than 1000 proteins that contain a motif structure called the C-type lectin domain, which binds not only the carbohydrate, lectin, but also proteins, lipids and smaller ligands [331]. Certain CLRs function as PRR in DCs that can mediate signalling through intracellular ITAM (immunoreceptor tyrosine-based activation motif) or ITIM (immunoreceptor tyrosine-based inhibitory motif) domains [332]. Clec9A (DNCR-1) is a CLR exclusive to DCs with high expression in cDC1 and lower levels in pDCs [83], [279]. Signalling through Clec9A is limited but it does function as a scavenger for filamentous actin on necrotic cells to promote the Ag uptake and intracellular trafficking of necrotic cell Ag for (cross)presentation to CD8⁺ T cells [83][333][334][335]. A second important CLR in DCs is DEC205 (CD205) with expression on both cDC subpopulations and intermediate levels on B cells and LCs. Signalling through DEC205 is ITAM/ITIM-independent [336] and it also functions as an endocytic receptor for apoptotic and necrotic Ag [337][338].

Apart from these membrane-bound TLRs and CLRs, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) are cytosolic PRR that sense viral dsRNA and bacterial peptidoglycans, respectively. RLRs activate the mitochondrial antiviral signalling (MAVS) pathway that in turn leads to the induction of NF κ B-dependent production of IL-6 and IL-12

and Type I IFN responses through the transcription factors IRF3 and IRF7 [339]. NOD1 is the central NLR sensing diaminopimelic acid of bacterial peptidoglycans that leads to the interaction with RIP2K kinase and the induction of NF κ B pathway [340].

Antigen uptake

To perform efficient Ag presentation, cDCs need to capture Ag, which they can then process and display on their cell surface in MHC complexes. For this to occur, both migratory as well as tissue-resident cDC1s and cDC2s have developed adjustments allowing them to effectively capture Ag. Antigenic peptides presented in MHC derive from either endogenous or exogenous proteins produced by the presenting cell itself or by other cells [19]. DCs efficiently capture exogenous antigens to process, and then present these for T cell priming. Several endocytic mechanisms allow the uptake of soluble polypeptides, extracellular bacteria, cell debris and apoptotic cells [341]. These include the rather unspecific macropinocytosis during steady state [342], receptor-mediated endocytosis through an array of endocytic receptors (i.e. FcR or C-type lectins) [343] and phagocytosis of larger particles like pathogens or apoptotic cells [188][344]. As professional APCs, B cells and macrophages can also engulf exogenous Ag but primarily only use their antigen-specific BCR and receptor-dependent phagocytosis, respectively, while macropinocytosis is predominantly used by DCs only [345]. Constitutive macropinocytosis in immature DCs has been considered as the primary route of Ag acquisition and hence the basis for Ag presentation and T cell stimulation, though studies fail to specifically inhibit macropinocytosis to ultimately prove this [346].

Antigen processing and loading onto MHC II

Exogenous proteins incorporated into the cell are processed for MHC II-restricted Ag presentation through the gradual degradation along the maturation process of vesicular organelles in which they are carried. Early endosomes mature to late endosomes and lysosomes while phagosomes and macropinosomes fuse with lysosomes and late endosomes. This vesicular maturation process is accompanied by an increase in acidity and proteolytic activity for the digestion of exogenous Ag [341][347]. It has been shown that DCs, compared to macrophages, have lower lysosomal proteases activity and acidification, resulting in slower degradation of Ag, in order to retain its integrity and favor Ag presentation [348]. Endogenous proteins from the cytosol or nucleus can reach lysosomes and late endosomes either through chaperon-mediated microautophagy or through the generation of autophagosomes via

macroautophagy, both enabling the presentation of endogenous Ag in MHC II [23][349]. While Ag presentation in MHC II is restricted to APCs, all nucleated cells constantly present endogenous Ag in MHC I. Misfolded proteins are degraded through the 20S proteasome in the cytosol. A variation of this standard proteasome, called “immunoproteasome” with different cleavage specificities in lymphoid tissues is the a major source of endogenous Ag for MHC I-restricted presentation [350]. Transporter associated with antigen presentation 1 (TAP1) transports cytosolic peptides into the endoplasmic reticulum (ER) where they are further cleaved through the ER-associated aminopeptidase 1 and 2 (ERAP1/2) and then loaded onto MHC I. Peptide-loaded MHC I then reaches the cell surface via the trans-golgi network [351].

Cross-presentation

Cross-presentation is a pathway that enables the loading of exogenous Ag into MHC I, which usually presents endogenous Ag. Although it has been demonstrated for macrophages [352][353] and B cells [354], cDC1s (CD8⁺ CD11b⁻ tissue resident cDCs and CD103⁺ migratory cDCs) are specialized in cross-presentation and most effectively stimulate cytotoxic immune responses against cancerous cells and viruses through Ag-specific priming of CD8⁺ T cells [21][355][356][357]. The cytosolic pathway of cross-presentation requires exogenous Ag to reach the cytosol for proteasomal degradation so that the resulting peptides can access the ER via TAP1-mediated transport to then be loaded onto MHC I [358]. It has been shown that the components Sec61 and p97 of the ER-associated protein degradation (ERAD) pathway are critical for the transport of endosomal Ag into the cytosol by forming multimeric channels [359][360]. In addition to this, it has been shown that phagosomes loaded with exogenous Ag can fuse directly with vesicles that derive from the ER containing MHC I and components of the peptide loading complex [361][362]. Besides the phagosome-to-cytosol pathway an alternative route called the vacuolar pathway does not require the proteasome or TAP and depends on the gradual proteolysis of antigenic proteins within the endocytic system with the direct loading of resulting peptides into MHC I [355][356][363].

Specialization of cDC1 and cDC2 in antigen presentation

cDC1s are specialized in the cross-presentation to CD8⁺ T cells, while cDC2s preferably present antigenic peptides in MHC II to CD4⁺ T cells [364][365][366]. This has been demonstrated in studies in which the ablation of cDC1s, but not cDC2s, led to impaired cytotoxic T cell responses to tumors and intracellular pathogens, while CD4⁺ T cell function was maintained

[297][367][368]. cDC1s have developed several adjustments for efficient cross-presentation of exogenous Ag in MHC I, including the enhanced uptake of cell-associated Ag [369][370][371], a specialized MHC I Ag processing and loading machinery [366], as well as the expression of certain immune receptors and cytokines that mediate their preferred interaction with CD8⁺ T cells [372][373][374]. The chemokine receptor XCR1 is restricted to cDC1s and mediates their migration towards CD8⁺ T cells that produce and secrete the corresponding ligand CXL1 [236][373][374]. IL-12 is mainly produced by cDC1s and is an essential cytokine for CD8⁺ T cell stimulation [375][376]. The presentation of exogenous Ag in MHC I by cDC1s is facilitated through the decreased degradation of endocytosed Ag [348][377], inter alia achieved by the alkalization of phagosomes through Rac2 activity [378]. Furthermore, it has been shown that cDC1s show enriched release of Ag from phagosomes to the cytosol through induced oxidative stress, favoring the phagosome-to-cytosol transport [379].

Although specialized in cross-presentation, cDC1 are able to interact with CD4⁺ T cells and present exogenous Ag in MHC II for their stimulation, as shown for herpes simplex virus 1 (HSV-1) infection [183] and the induction of Th1 responses after adoptive transfer [380]. Furthermore CD40/CD40L-signalling through the interaction with CD4⁺ T cells promotes the production of IL-12 in cDC1s [381][382].

The role of lymphoid tissue resident cDC1s in the LN and spleen in presenting Ag to T cells was initially limited by their localization to the T cell zone of the white pulp. However, more recent studies revealed their presence in the marginal zone (MZ) of the spleen in order to capture blood borne Ag [383][384]. Similarly, tissue resident cDC2s have been identified in the MZ of the spleen and in the interfollicular spaces of LN. While cDC2s in T cell follicles excel in the presentation of soluble Ag, MZ cDC2s are critical for the uptake and presentation of particulate Ag from the circulation [144][385], both to stimulate CD4⁺ T cells and to induce humoral immune responses. The ablation of cDC2 in *Irf4^{flox/flox}* × CD11c-cre mice led to impaired CD4⁺ T cell mediated responses, in particular Th2 responses, as well as the production of IL-4/5 and IL-13 and immunoglobulins (Ig) E and G1 [386][387][388]. Like tissue-resident cDC1s and cDC2s, their migratory counterparts (CD103⁺ and CD11b⁺ CD172⁺ cDCs, respectively) show analogous functions. While migratory cDC1s have been shown to effectively capture and cross-present exogenous Ag in MHC I [183][371][389][390], migratory cDC2s are committed to MHC II-restricted presentation of exogenous Ag to induce Th2 and

Th17 immune responses [214][241][390].

The functional differences between cDC1s and cDC2s are also represented by their expressed TLRs. While cDC1s mainly express TLR3, 11, 12 and 13 to sense viral dsRNA, profilin and bacterial 23S ribosomal RNA [330][391][392][393] for the recognition of intracellular pathogens, cDC2s utilize TLR5 and 7 to sense bacterial flagellin and ssRNA [330][393].

Dendritic cell maturation

Activation of cDCs through signalling via PRR leads to their maturation and conversion from an Ag-capturing to an Ag-presenting mode. Non-activated cDCs efficiently capture Ag predominantly via macropinocytosis [345] but lack effective presentation of antigenic peptides in MHC (pMHC) at the cell surface due to low overall expression of MHC [394][395] and quick endocytosis of surface pMHC [396]. Activation of cDCs leads to the reduction of receptor-mediated endocytosis/phagocytosis of Ag [342][397][398]. Instead, maturation of cDCs causes the induction of enriched expression of new MHC II molecules [199][399], enhanced Ag processing through the acidification and recruitment of cathepsin activity into the late endosome [347][400] and reduced internalization of pMHC from the cell surface [401]. As pMHC II tends to traffic from the endosomal compartment to the plasma membrane [402][403], activation of cDC leads the accumulation and persistence of pMHC II at the plasma membrane in order to enable effective Ag presentation to naïve CD4⁺ T cells [399][404]. Furthermore, posttranslational ubiquitination of MHC II by membrane-associated MARCHF1 Ub E3 ligase is shut down too, preventing further internalization of surface pMHC II molecules (presented in section 1.4.2).

Similarly, activation of cDCs leads to the enriched biosynthesis of MHC I molecules and components of the MCH I presentation machinery [405], as well as an increase and stabilization of surface pMHC I for effective Ag presentation to naïve CD8⁺ T cells [406].

This change of mode from Ag-capturing to Ag-presentation allows cDCs to scan the environment for antigens at steady-state with little Ag presentation to ensure immune tolerance, yet effectively induce T cell responses against specific Ag upon activation.

Dendritic cell-T cell communication via co-receptors

Activation of naïve T cells is mainly triggered through the stimulation of their TCR upon recognition of its cognate Ag presented in MHC on the surface of DCs. However, their differentiation is directed by a range of co-receptors and ligands/co-receptors that are

expressed on the surface of DCs and T cells to modulate T cell differentiation via stimulatory or inhibitory signalling upon interaction (reviewed in [407]).

The key co-receptor CD80/CD86 is expressed by both cDC subsets and interacts with CD28 on naïve T cells, which has been shown to be critical for their differentiation into effector and memory T cells [408][409][410]. In contrast to this stimulatory axis, the interaction of CD80/CD86 with CTLA4 on regulatory T cells (Tregs) and activated T cells has inhibitory effects through various mechanisms. These include dampening TCR signalling through the dephosphorylation of its downstream signalling components [407], a reduction of CD80/CD86 surface expression on DCs via trogocytosis [411] and indoleamine 2,3-dioxygenase (IDO)-expression, which in turn promotes Foxp3 Treg development and suppresses T cell proliferation [412]. Other stimulatory co-receptors expressed by cDCs are ICOS-L and CD70 which promote the differentiation of T cells into T_{FH} and Th1, respectively [413][414][415]. CD40L (CD154) is expressed on activated CD4⁺ T cells and known to provide T cell help for B cell differentiation [416], but it also has been described in the stimulation of cDCs inducing the upregulation of MHC II and CD86/CD80 [417][418], as well as IL-12 production and NFκB pathway for effective cross-presentation [419][420]. The co-inhibitory molecules PD-L1 and PD-L2 are expressed various by immune cells, with a more restricted expression of PD-L2 on DCs and macrophages only, and negatively regulate cytokine production and proliferation of activated T cells through PD-1 [421][422][423].

Dendritic cells in immune tolerance

In addition to their primary role in stimulating T cells, DCs also have modulatory roles in both central and peripheral immune tolerance (reviewed in [424]). It has been demonstrated that the ablation of DCs causes impaired immune tolerance and leads to the onset of autoimmunity [425]. The enriched presence of DCs through the administration of Flt3L can lead to the protection against autoreactivity in diabetes onset [426] but also can cause over-reactivity in lymphocytes and autoimmunity, shown for non-apoptotic (Bim-deficient) DCs [427]. Besides migratory cDCs and pDCs, the most abundant DC subset in the thymus are CD8⁺ resident DCs, that derive from lymphoid precursors [187][186][288][428][429]. CD8⁺ DCs in the thymus are a main contributor for central immune tolerance via presentation of self-antigen, in order to negatively select autoreactive thymocytes [430][431]. Thymic DCs acquire Ag from medullary thymic epithelial cells (mTEC) through trogocytosis of pMHC I and pMHC II

[432]. Conversely, migratory cDCs can reach the thymus via CCR2 and pDCs via CRR9 to modulate immune tolerance via presentation of peripheral self-Ag [186][433][434]. Apart from the negative selection of self-reactive T cells, thymic DCs can also induce Treg development of thymocytes [435]. TGF- β 1⁺ cDCs effectively induce Ag-specific Foxp3⁺ Treg development through the CD27-CD70 pathway [436][437], which in turn regulates autoreactivity. Notably, it has been shown that thymic DCs are not exclusively responsible for Treg differentiation as other thymic cells (i.e. mTEC) can also induce Treg development [432], but differences in Ag uptake and processing properties [438] indicate potential non-redundant roles.

cDCs in peripheral tissues usually present self-Ag at low levels to transiently activated T cells leading an anergic state or their deletion via Bim- and Fas-mediated apoptosis [439][440][441]. Although activation and maturation of peripheral cDCs leads to their migration into draining LN and drastic changes for effective T cell stimulation, it has been described that activated cDCs can also contribute to immune tolerance as Tregs use some of the same signals as naïve T cells, including CD80/CD86 [442][443][444]. Inhibitory co-receptors expressed by DCs, such as CTLA4-CD80/CD86 or PD-1-PD-L1/PD-L2 are also implicated in the induction of peripheral tolerance [445][446]. Similar to thymic DCs, peripheral DCs can induce the development of Foxp3⁺ Tregs via TGF- β [447], with enhanced DC numbers and activation upon depletion of Foxp3⁺ Tregs, indicating a feedback mechanism in which Tregs contribute to peripheral tolerance [448][449]. cDC1 in the MZ of the spleen are responsible for the induction of immune tolerance through the presentation of cell-associated Ag from blood-borne apoptotic cells [450].

1.4 Regulation of MHC II in dendritic cells

MHC II is the central immune receptor in cDCs for the activation of CD4⁺ T cells, which is in turn the basis for the induction of antibody-mediated/humoral immune responses. For this, MHC II homeostasis is tightly controlled through transcriptionally and post-translationally regulation, as well as fine-tuned intracellular trafficking in order to maintain an appropriate balance between immune responses and tolerance.

1.4.1 MHC II expression and trafficking

The functional MHC II complex consists of two polypeptides that are non-covalently associated and stabilized by an oligopeptide bound to the shared peptide binding cleft bridging both chains. α chain (~32-34 kDa) and β chain (~29-32 kDa) are both glycosylated and anchored to the membrane via a transmembrane (TM) domain near the C-terminus. The longer N-terminus of both chains faces the luminal or extracellular space and contains a polymorphic peptide binding cleft that usually binds 11-16 amino acid-long peptides, however it also can bind longer polypeptides [451][452][453].

MHC II expression

In humans MHC II-related genes are clustered in a highly polymorphic and well characterized locus known as human leukocyte antigen (HLA) HLA-DP, HLA-DQ and HLA-DR, while MHC II genes in mice are annotated as H2/I-A and H2/I-E. Both HLA and H2 have different haplotypes of the classical MHC II α and β chain [454][455][456]. Expression of these genes is regulated by the class II transactivator (CIITA) [457]. CIITA does not bind to DNA itself but rather interacts with the DNA-binding proteins, including the RXF complex, CREB and NFI to form the *enhanceosome* that binds to the MHC II SXY module upstream of the HLA/H2 genes [458]. The non-classical HLA/H-2 genes, HLA-DM and HLA-DO in humans and H2/I-M and H2/I-O in mice, are no structural components of the actual MHC II molecule but involved in the Ag loading process and are also under the transcriptional control of CIITA [459][460]. The invariant chain (Ii or CD74) assists and stabilizes the initial assembly of the $\alpha\beta$ heterodimer [461] and resides within the HLA/H2 gene cluster but its expression is not controlled by CIITA [462]. Expression of CIITA itself is controlled by four different promoters (pI, pII, pIII and pIV), distinctively induced in different APCs upon IFN- γ stimulation [463][464].

MHC II assembly and trafficking

Newly synthesized MHC II polypeptides are translocated into the ER through co-translation. Anchored into the membrane through their C-terminal transmembrane domains both chains dimerize and associate with a $\text{I}\alpha$ trimer that acts as a chaperon to assist and stabilize the formation of a nonameric complex consisting of three invariant chains and three $\alpha\beta$ heterodimer [461][465]. The leucine-based sorting motifs of $\text{I}\alpha$ facilitates the exit from the ER to the endosomal compartment. This occurs either directly via the trans-golgi network [466] or indirectly via the plasma membrane, which is clathrin- and AP-2-dependent [467]. The $\text{I}\alpha$ -MHC II complex then enters early endosomes which can mature to late endosome and multi-vesicular bodies, also known as MHC class II compartment (MIIC) [468][469][470]. In non-activated cells (i.e. immature cDCs) the majority of MHC II molecules is located in late endosomes or multi-vesicular bodies/MIIC, where the processing and loading of antigens takes place. In the MIIC cathepsin S and D cleave $\text{I}\alpha$ successively, leaving behind a small peptide called CLIP (class II-associated invariant chain peptide) in the MHC II peptide-binding cleft preventing the binding of premature peptides [471][472][473]. Dissociation of CLIP, positively regulated by HLA-DM/H2-M [474] but suppressed by HLA-DO/H2-O [475][476], is achieved by antigenic peptides with higher affinity to the peptide-binding cleft. The substitution of CLIP results in the formation of stable MHC II complexes loaded with an antigenic peptide (pMHC II), which can be transported in vesicles to the cell surface by fusion with the plasma membrane [395][477][478]. It has been shown that MHC II clusters in CD9-tetraspanin-enriched microdomains at the cell surface, which facilitates efficient Ag presentation [479][480][481].

In steady-state surface pMHC II molecules are quickly internalized from the cell surface via endocytosis which has been shown to be independent of clathrin and dynamin [482][483], but involves Arf6- and Rab35-tubular endosome and lipid rafts [484][485]. This way, pMHC II reaches early endosomes from where it can either get recycled back to the cell surface [486] or remain within endosomes and MIIC in intraluminal vesicles. Trafficking of pMHC II molecules into intraluminal vesicles is mediated by ubiquitination, in which a polyubiquitin chain serves as a recognition motif for the endosomal sorting machinery [487][488][489][490]. Non-recycled pMHC II molecules are either degraded, when endosomes fuse with lysosomes causing a pH drop and activation of proteases [491], or get released as exosomes when endosomes, containing enriched numbers of intraluminal vesicle

loaded with pMHC II, fuse with the plasma membrane [492]. Due to the quick internalization of pMHC II from the surface most MHC II molecules remain within the cell and are not present at the cell surface for effective for Ag presentation [396][493]. However, this changes dramatically upon DC maturation, characterized by the stop of MHC II internalization, a retrograde transport of MHC II molecules from lysosome back to the plasma membrane [494] and a consequential accumulation of MHC II molecules at the cell surface [404]. The molecular mechanism for this drastic change is due to the loss of the oligo-ubiquitin chain in the β chain of MHC II [490][495][496]. A schematic illustration of the intracellular MHC II trafficking and Ag loading is shown in Figure 1.2.

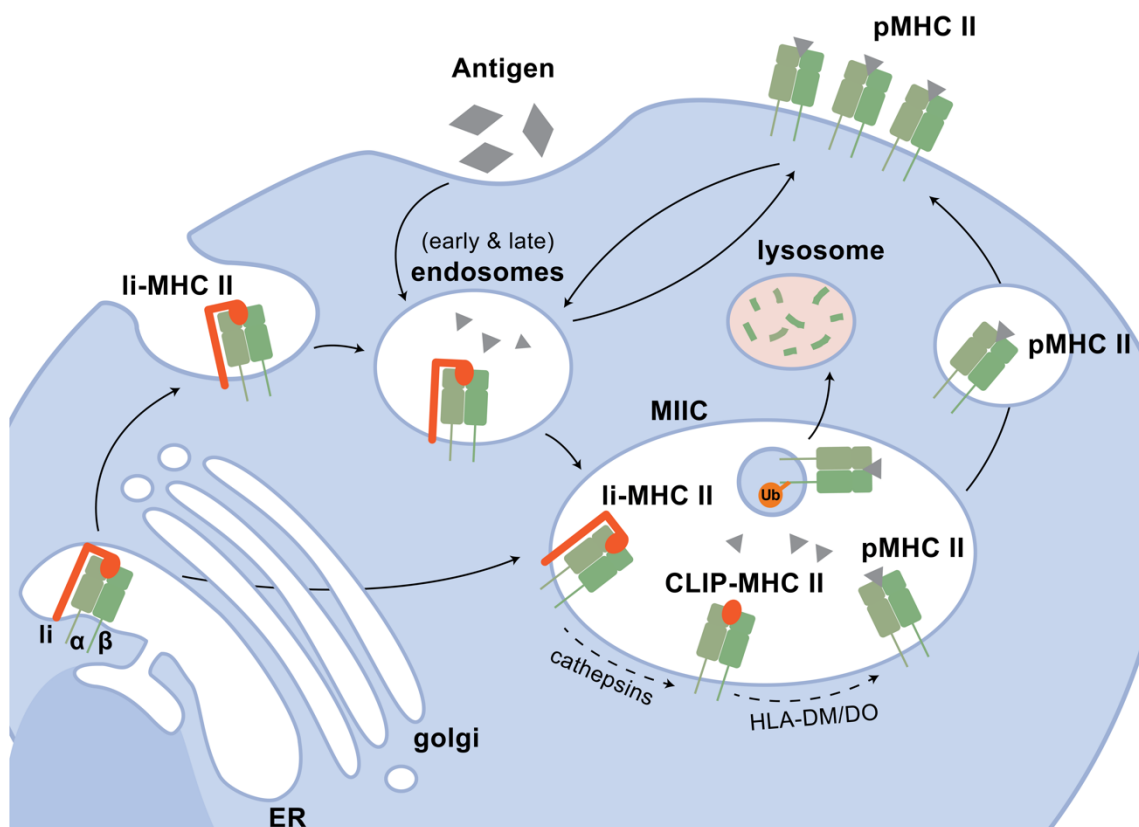


Figure 1.2 Intracellular MHC II trafficking and antigen loading. Newly synthesized MHC II polypeptides (α and β chain) are translocated into the ER and stabilized by their association to each other as well as with the invariant chain Ii, forming nonameric stable complexes. Ii-MHC II complexes are trafficked to the endosomal system, either directly via the trans-golgi network or indirectly via the plasma membrane. Together with endocytosed Ag, Ii-MHC II complexes reach the multi-vesicular bodies or the MHC class compartment (MIIC), in which Ii is sequentially digested by cathepsins down to CLIP (class II-associated invariant chain peptide). Replacement of CLIP with antigenic peptides is assisted by HLA-DM and HLA-DO and peptide loaded MHC II (pMHC II) molecules can reach the plasma membrane via tubular extensions. Surface pMHC II can be endocytosed and recycled back to the cell surface or enter MIIC in intraluminal vesicles, mediated by ubiquitination. Ultimately, ubiquitination of MHC II leads to its trafficking to the lysosomal compartment for its degradation (illustration based on review [497] and [465]).

1.4.2 Ubiquitination of MHC II by MARCHF Ub ligases

Ubiquitination

Ubiquitination is a highly versatile posttranslational modification through the coordinated actions of the three ubiquitin E1, E2 and E3 enzymes/ligases. As a result, ubiquitin (Ub) molecules are attached to substrate proteins via a covalent isopeptide bond between the C-terminal glycine (G76) and an ϵ -amino group of a substrate's lysine residue. Polyubiquitination occurs when several rounds of ubiquitination take place whereby Ub molecules are added to the previous bound Ub, creating Ub-chains of different lengths and conformations. This in turn dictates the protein's location and turn-over depending on the resulting "Ub-code" attached to it, enabling fine tuning of surface expression levels of specific molecules [498][499][500][501]. The versatile group of substrate specific E3 Ub ligases are of particular interest and studied preferentially [502][503].

MARCHF E3 ubiquitin ligases

The Membrane Associated RING-CH Finger (MARCHF, previously known as MARCH) family comprises 11 membrane bound E3 Ub ligases (MARCHF1-11) and is thought to perform immunoregulatory functions via ubiquitination of key surface proteins involved in host immunity, including CD86 and MHC II [503][504][505][506]. The first indication that the ubiquitination of MHC II is enzyme-catalyzed came from the K3 and K5 protein family of viral immunosuppressive membrane ubiquitin ligases [also called modulator of immune recognition (MIR)]. These are encoded by the Kaposi's sarcoma associated herpesviruses and known to reduce MHC I surface expression via ubiquitination in order to suppress the killing of infected cells [507][508][509][510]. Bioinformatical analysis identified eleven mammalian K3 family homologues, eventually annotated as MARCHF ligases [511][512][513]. K3/K5 and MARCHF ligases belong to the *really interesting new gene* (RING) family [514][515] and are determinants of substrate specificity, finalizing the ubiquitination through the recruitment of Ub-conjugating E2 enzymes [502]. All eleven MARCHF ligases contain a N-terminal RING-CH domain which is distinguished from the usual RING domain by the arrangement of its histidines and cysteines in the catalytic core (Cys₄His₁Cys₃ instead of Cys₃His₁Cys₄) [505][516][517][518]. Except for the cytosolic MARCHF7 and 10 ligase the RING-CH domain is followed by two or more transmembrane (TM) domains. While MARCHF1, 2, 3, 4 and 11 have two putative TM domains, MARCHF5 and MARCHF6 have four and 14 TM domains,

respectively [503][504][505]. Phylogenetic analyses resulted in the clustering of MARCHF ligases into four paired groups with high sequence homology: MARCHF1&8, MARCHF2&3, MARCHF7&10 and MARCHF4,9&8 [503][504][505]. The family of MARCHF Ub ligases is illustrated in Figure 1.3A (page 55).

MARCHF1 has been subject of numerous studies and is nowadays the best described MARCHF Ub ligase with major immunoregulatory functions. It has roles in immunity and infection including Treg development [519], inflammation and allergy [520] [521] and host viral defense [522]. The paradigm of MARCHF functionality is the regulation of MHC II and CD86 surface expression in APCs to modulate Ag presentation, extensively investigated and subject of numerous studies [199][489][496][523][524][525]. MARCHF1 and MARCHF8 share approximately 60% sequence homology overall [504][526] and about 90% across their functional RING and transmembrane domains, hence they likely ubiquitinate similar substrates. MARCHF1-mediated ubiquitination of MHC II in haemopoietic APCs is well characterized and it has been shown that the closely related MARCHF8 is not active in these cells but regulates MHC II expression in non-haemopoietic thymic epithelial cells (TECs) for CD4⁺ T cell selection [527][528].

The characterization of the immunological relevance of MARCHF2, 3, 4, 8 and 9 is limited and is even less examined for MARCHF5, 6, 7, 10 and 11 (reviewed in [503]). MARCHF3 was shown to ubiquitinate FcγRIIb [529] and IL-1RL [530] enhancing Ab-mediated immune responses and TNFα-dependent inflammation respectively. Antiviral activity against HIV-1 has been reported for MARCHF8 through the ubiquitination of viral envelope proteins in infected cells, inhibiting viral budding from the plasma membrane [531]. Similar mechanisms have been reported for MARCHF2 in human HIV-1 infected cells [522][532]. Consistent with the model that MARCHF ligases originated from the viral Ub ligases K3 and K5 through the duplication in the host genome, Rigotti *et al.* have demonstrated that MARCHF9 also ubiquitinates MHC I and the MHC I-like molecule CD1a [533]. Surprisingly, the site of this ubiquitination appeared in the Golgi-network in order to promote trafficking to the plasma membrane [533], suggesting immunostimulatory role in cross-presentation [534].

Mechanisms in ubiquitin-mediated MHC II trafficking

Ubiquitination of MHC II occurs at a single and highly conserved lysine in position 225 (K225) of the cytoplasmic tail of its β chain [496][535]. Murine DCs and B cells lacking MARCHF1

expression or expressing a mutant MHC II molecule where the ubiquitin acceptor lysine at position 225 in its β chain is mutated and replaced with an arginine (MHC II K225R^{KI/KI}) show enriched MHC II surface expression [490][495][535]. Enriched MHC II surface expression has also been demonstrated in human moDCs that were deficient in MARCHF1 [536]. The mechanisms of how ubiquitination reduces surface expression of MHC II remain elusive and controversially discussed in the literature. Several studies, using antibody-based surface staining, propose a model of direct internalization from the plasma membrane [490][495][536]. However, this is contradicted in other studies that did not observe altered internalization in cells lacking MHC II ubiquitination but increased recycling of MHC II from intracellular compartments towards the cell surface [489][496][524]. These conflicting observations are further complicated by the fact that MHC II can be endocytosed in a Ub-independent manner via endocytosis through its leucine-based sorting motif [537]. In any case ubiquitination of MHC II causes a reduction in surface levels. Ubiquitinated MHC II is recognized by the endosomal sorting complexes required for transport (ESCRT) and are either recycled back to the cell surface or delivered to late endosomes and lysosomes for degradation [465][503].

MARCHF1-mediated ubiquitination of MHC II

Although MARCHF8 has been described to ubiquitinate MHC II and CD86 in studies with ectopically expressed MARCHF8 [538], knockout mouse models have clearly demonstrated that it is not MARCHF8 but MARCHF1 that is the responsible Ub ligase regulating MHC II and CD86 surface expression in hematopoietic cells, including B cells, DCs and monocytes [199][496][523][527][539], while no changes in MHC II expression were observed in MARCHF8-deficient DCs [527][540]. In general, this demonstrates that overexpression of MARCHF ligases causes ubiquitination of proteins that are not true substrates under physiological conditions. Overexpression of both MARCHF1 and 8 has been shown to regulate several membrane proteins, including MHC I, CD81, CD95, CD44, B7.2 and CD88 [505]. However, our investigations with cDCs lacking MARCHF1 expression showed that surface expression of these proteins was not majorly affected [manuscript in preparation]. As of today, CD86 and MHC II are the only physiological substrates of MARCHF1, verified in primary cells and future unbiased analyses of the impact of MARCHF1 deficiency on membrane protein expression will be required to identify undescribed bona fide substrates.

Ubiquitination of MHC II through MARCHF1 differs between different APCs. For example, B cells and DCs completely shut down MARCHF1-mediated ubiquitination upon activation, whereas pDCs have been shown to retain ubiquitination of MHC II after their activation, allowing further processing of viral Ag [199]. Furthermore, comparison of Ub chains attached to MHC II in B cells and DCs have revealed that B cells overall exhibit shorter polyubiquitination chains than DCs (PhD thesis from Haiyin Liu [541]), presumably causing different distribution of intracellular MHC II with reduced trafficking towards late endosome and multi-vesicular bodies [542].

Detection of endogenous MARCHF1 has technical limitations due to its very low expression, both at protein and mRNA levels, and low stability with a protein half-life of less than 30 minutes [543][544]. MARCHF1 itself is quickly degraded in lysosomes induced through self-regulatory auto-ubiquitination [545] or E2 enzyme UBE2D1-mediated ubiquitination [546]. Besides the ESCRT-associated protein Toll-interacting protein (Tollip), that has been reported to downregulate MARCHF1 levels [547], it is interleukin (IL)-10 that has been described as the main regulator for MARCHF1 expression. IL-10 functions as an immunosuppressant that induces MARCHF1 expression causing a reduction of MHC II surface expression [539][548]. Co-culturing of DCs with Ag specific regulatory T cells (Tregs) promotes MARCHF1 expression and decreases the surface expression of CD86 in DCs through IL-10-dependent stimulation [549]. This indicates an inhibitory immunoregulatory role for IL-10 by inducing MARCHF1-mediated ubiquitination of MHC II and CD86. Controversially, other studies suggest a MARCHF1-independent suppressive function of IL-10 by decreasing transcriptional expression of MHC II and CD86, even in MARCHF1-deficient DCs [550]. Even more controversial, IL-10 treatment in follicular B cells has reportedly caused the opposite effect, where a reduction in MARCHF1 expression increased surface levels of MHC II [551]. While IL-10 leads to a reduction of surface MHCII/CD86 by inducing expression of MARCHF1, activation of APCs causes the opposite. Treatment of DCs and B cells with TLR agonists leads to an increase in cell surface CD86 and MHC II, while expression of MARCHF1 but not MARCHF8 was decreased [199][523][548]. Similar effects were reported in response to exposure to various microbes, associated with a loss of MHC II ubiquitination [552].

MARCHF1 activity can be regulated by CD83. This membrane protein has a single Ig-like domain and a cytoplasmic tail and interacts with MARCHF Ub ligases via its transmembrane domain. In doing so, CD83 outcompetes and sequesters MARCHF1 binding to MHC II and

CD86 to effectively prevent their ubiquitination and to stabilize their surface expression [553]. This demonstrates further mechanisms in the regulation of surface expression of immune receptors as activation of DCs leads to the upregulation of CD83 [554][555]. Mice that lack CD83 or express a non-functional mutated form of it (*CD83^{anu/anu}* mice) reveal reduced surface levels of CD86 and MHC II on DCs and B cells [556]. Moreover, CD83 has been shown to also play a role in the regulation of surface MHC II in TECs, as demonstrated by *CD83^{anu/anu}* mice that harbored fewer CD4⁺ T cells due to a defect in thymocyte selection [527][541][551]. This demonstrates an orchestrated regulation of MHC II surface expression in TECs by MARCHF8 and CD83 [527].

The molecular mechanisms of MARCHF-mediated ubiquitination have been predominantly investigated in overexpression models, proposing the attraction of its substrate via TM domains and a recruitment of E2 enzymes through the catalytic RING-CH domain [514][557]. This is supported by studies in which MARCHF1 failed to ubiquitinate MHC II when its TM domains were exchanged with those of MARCHF9 [545]. Additionally, a cluster of residues in the β chain of MHC II, located at the interface of the plasma membrane and the cytosol, has been identified as a critical recognition sequence for MARCHF8 [558]. A schematic illustration of MARCHF-mediated ubiquitination is displayed in Figure 1.3B (page 55).

Altogether, this hypothesizes that MARCHF1 acts as an immunosuppressive regulator to prevent excessive Ag presentation and uncontrolled T cell stimulation through the downregulation of MHC II and CD86 in steady state, but that this rapidly changes upon DC activation/maturation.

The role of MHC II ubiquitination in the immune system

Studies on the biological implication of MHC II ubiquitination primarily rely on genetically modified mouse models with cells lacking MHC II ubiquitination, rather than constructs where MARCHF1 is overexpressed, a scenario leading to promiscuous Ub ligase activity. With regard to MHC II ubiquitination and its intracellular trafficking, the *Marchf1^{-/-}* phenotype is resembled by the MHC II K225R^{KI/KI} mutant, while CD86 expression is unaltered in these mice.

The effect of impaired MHC II ubiquitination on Ag presentation lacks consensus as studies have reported controversial observations in this. Whilst one could assume that enriched CD86 and MHC II surface expression in DCs implies enhanced stimulation of naïve T cells, a study by Ohmura-Hoshino *et al.* has reported that *Marchf1^{-/-}* cDCs show impaired

CD4⁺ T cell activation [535]. A possible explanation for this is that *Marchf1*^{-/-} cDCs are in a constant activation/Ag-presentation mode, characterised by decreased Ag processing and loading onto MHC II due to its impaired trafficking and turnover. Furthermore, the lack of MHC II ubiquitination results in a lower production of key cytokines for the activation of T cells, such as TNF- α and IL-12 [559]. Another study with BM-derived DCs showed no difference in T cell activation and Ab production between WT and MHC II K225R^{KI/KI} DCs [560], despite MHC II ubiquitination in GC B cells being described as an essential mechanism in the process of Ab affinity maturation [128]. Interestingly, the lack of MHC II ubiquitination can indirectly affect surface expression of other molecules as shown for reduced MHC I surface levels in primary DCs from MARCHF1-deficient and MHC II K225R^{KI/KI} mice, leading to impaired Ag presentation to CD8⁺ T cells [561]. This can be explained by the disruption of MHC-rich lipid rafts and tetraspanin membrane microdomains [562].

Both *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice do not exhibit drastically altered CD4⁺/CD8⁺ T cell populations with equal numbers and proportions/frequencies compared to WT mice [496] [560], which is surprising considering, at least in case of MHC II K225R^{KI/KI} mice, as surface expression of MHC II in the thymic epithelium is critical for T cell selection. However, they do show reduced numbers of FoxP3⁺ Tregs [535], supporting the observations that DCs contribute to immune tolerance through the induction of Treg development in the thymus and periphery (see section 1.3.6) [436][437][447].

The *Marchf1*^{-/-} phenotype resembles the MHC II K225R^{KI/KI} mutant with regard to MHC II ubiquitination, but numerous observations made only in MARCHF1-deficient mice imply other immunoregulatory roles for MARCHF1 beyond MHC II ubiquitination. For instance, MARCHF1-deficient but not MHC II K225R^{KI/KI} mice have been shown to exert systemically higher inflammatory cytokine levels and were more prone to LPS-induced endotoxin shock [520]. Similarly, *Marchf1*^{-/-} mice were more susceptible to the onset of asthma due to increased inflammation and neutrophil influx in the lung upon antigen administration [521].

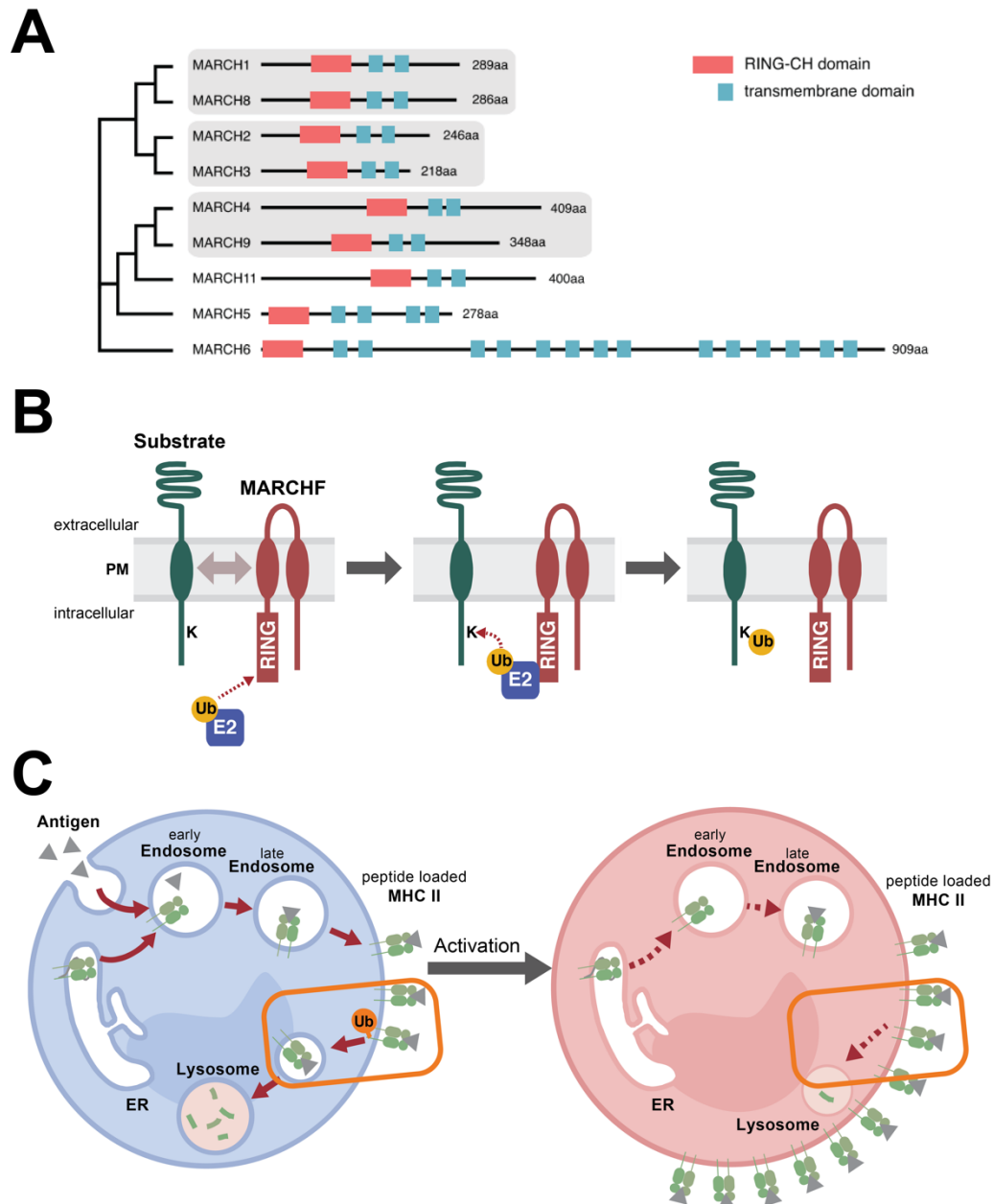


Figure 1.3 The family of MARCHF Ub ligases and the role of MHC II ubiquitination in dendritic cells. (A) Schematic illustration of the membrane-bound murine MARCHF Ub ligases. The RING-CH domain (red boxes) and transmembrane domains (blue boxes) are shown at their respective positions in the protein, with the total size (in amino acid residues) indicated on the right of each protein. MARCH ligases with high homology are grouped in light grey boxes; the figure was taken from Liu et al. [503]. **(B)** Schematic illustration of MARCHF-mediated ubiquitination of membrane-bound substrate proteins. The MARCHF ligase attracts its substrate via TM domain interactions and recruits a Ub-carrying E2 enzyme to its catalytic RING-CH domain. This brings substrate and Ub-carrying E2 enzyme in close proximity which in turn enables the covalent attachment of Ub molecules to a lysine (K) residue in the cytosolic tail of the substrate. **(C)** Schematic illustration of the role of MHC II ubiquitination in non-activated/resting (blue) versus activated/mature (red) dendritic cells. Trafficking of newly synthesised MHC II complexes from the ER to the cell surface is accompanied by Ag loading in early and late endosomal compartments. In non-activated/resting state ubiquitination of peptide-loaded MHC II molecules through MARCHF1 Ub ligases reduces its expression levels at the plasma membrane and leads to its lysosomal degradation, while upon activation MARCHF1-mediated ubiquitination of pMHC II is shut down, causing its accumulation at the cell surface.

1.5 Complement C3

1.5.1 Overview of the complement system

The complement system represents a major component of the humoral innate immune system and comprises of approximately 30 membrane-associated as well as soluble proteins [563][564], predominantly produced by the liver [565][566]. The complement system is considered as one of the oldest form of host defense, with evidence of its origins reaching back more than a billion years [564][567][568]. Its traditional role involves the surveillance and defense against pathogens [569] through various mechanisms, including the clearance of bacteria via direct cell lysis [570], immune shuttling as well as facilitating phagocytosis of pathogens upon their opsonization with complement proteins [571][572][573][574]. However, it has recently become increasingly evident that various complement proteins have regulatory roles in adaptive immunity, involving DC-mediated Ag presentation [575][576] as well as T and B cell responses [577][578][579]. Additionally, researchers aim to modulate complement proteins for therapeutic purposes, including tissue regeneration, the treatment of tumors, inflammatory and degenerative diseases as well as the improvement of Ab-based immunotherapies [580][581][582].

Complement activation

In the absence of infection, most of the complement proteins exist in a non-active state, called zymogen in case of proteases, but become activated upon encountering pathogens through a complex sequence of interactions with pathogen components, proteolytic reactions, and protein-protein interactions in the serum and/or on the surface of bacteria and host cells [563][583]. The central component in this activation cascade is complement component 3 (C3) [584]. All three activation pathways of the complement system converge in the cleavage of C3, leading to its activation and a conformational change that leads to the revealing of a thioester group for the covalent binding to hydroxyl nucleophilic surface structures [585].

Complement activation through the classical or lectin pathway is known to be induced through pathogenic surface structures [586], while activation through the alternative pathway occurs spontaneously during steady state at a low rate, even in the absence of pathogens [587][588]. The classical pathway is initiated through the formation of immune complexes when either IgG or IgM binds to the cell surface. The Fc portions of these immune complexes are then bound by the multimeric C1 complex of the complement system,

consisting of C1q, C1r and C1s molecules. The C1 complex has proteolytic activity and its formation leads to the cleavage of complement C4 and C2 molecules, which then form the C3 convertase, C4bC2a [563][589]. Similarly, the same C3 convertase is induced by the lectin pathway. However, in this case proteolysis of C4 and C2 is carried out by MASPs (MBL-associated proteins), that are present in the circulation as complexes with mannose binding lectin (MBL) or Ficolin. MBL and Ficolin can bind directly to certain carbohydrates on the cell surface of pathogens, which leads to the activation of MASPs and the proteolysis of C4 and C2 to finally form the C4bC2a C3 convertase [563][590]. In contrast to the classical and lectin pathway, a different C3 convertase, namely C3bBb, is formed in the alternative pathway. Basis for this is the spontaneous hydrolysis of complement C3 through the “tick-over” mechanism, which leads to the formation of spontaneously activated C3, often referred to as C3H₂O [591]. C3H₂O has similar characteristics to activated C3, C3b, and although spontaneously activated C3 has limited ability to bind to surface structures, it has been shown to bind and cleave Factor B under the engagement of Factor D [591]. This in turn creates the C3 convertase, C3bBb, which is stabilized by properdin and can activate further C3 molecules through proteolytic cleavage [563][588][592].

All three activation pathways generate C3 convertases for the proteolytic cleavage of C3 to C3b. The fact that C3b can function as a C3 convertase itself through the recruitment of Factor B and D, is the basis for the complement’s amplification feedback loop in which increasing amounts of C3 become activated.

Complement regulation

The formation of the C3bBb C3 convertase is the basis for the complement activation via the alternative pathway but can also lead to the amplification of the complement cascade in case of classical or lectin pathway activation. Given this amplifying activation cascade and additional multitude of effects exerted by the complement system, various regulators are in place to limit or promote complement activation. Negative regulation is especially necessary in case of spontaneous activation through the alternative pathway, as it occurs steadily at a low rate, while complement initiation through the classical and lectin pathway usually requires the presence of pathogens. Central to this is the cleavage of activated C3b through the serine protease Factor I in the presence of various co-factors, such as Factor H, membrane-bound cofactor protein (MCP) and complement receptor 1 (CR1) [593][594][595].

While Factor H and MCP directly assist Factor I in cleaving membrane bound activated C3, CR1 is highly expressed by various phagocytic immune cells and facilitates the clearance of activated C3, bound to cell surfaces or present in immune complexes [571][572]. Furthermore, the decay acceleration factor (DAF, CD55) prevents the formation of the C3 convertase, C3bBb, and therefore also negatively regulates complement activation through the alternative pathway [596]. Dysregulation of the complement system through deficiencies or defects in complement regulators can have severe consequences, which can lead to the onset of autoimmunity, pathological conditions and even disease outcome [592], as seen for systemic lupus erythematosus (SLE), especially in case of Lupus Nephritis [597], sepsis [598] and asthma [599]. The successive cleavage of activated, membrane-bound C3 through the serine protease Factor I is described in more detail in section 1.5.2.

Effect of complement activation

Apart from its formation of the C3 convertase, C3bBb, through the binding and cleavage of Factor B, C3b can also form the C5 convertases, C3b₂Bb and C4C2C3b. The first is considered as the C5 convertase of the alternative pathway as the C3bBb convertase, which initially emerges in the alternative pathway, cannot only bind and cleave further C3 molecules but also can bind an already activated C3b molecule, which leads to the formation of the C3b₂Bb C5 convertase [600]. In contrast to this, the C4C2C3b C5 convertase is generated when activated C3b forms a complex with activated C4b and C2b molecules of the classical or lectin pathway [601]. Cleavage of C5 by either of the two C5 convertase triggers the assembly of the membrane attacking complex (MAC), a multiprotein pore consisting of the complement proteins, C5b and C6-C9, that inserts into the cell surface of bacterial pathogens in order to induce direct cell lysis [602].

The formation of the MAC occurs downstream of the complement cascade, however, various complement fragments generated during cleavage cascade exert various effects, predominantly by modulating other cells. The anaphylatoxins, C3a and C5a, are small fragments generated through the activating cleavage of C3 and C5 respectively. Both are known to act as chemoattractants for mesenchymal stem cells and neutrophils [603][604][605], histamine releasing factors in basophils and mast cells [606], as well as vasodilators to induce smooth muscle contraction [563][607]. Furthermore, their corresponding receptors, C3aR (CD88) for C3a as well as C5aR and C5L2 for C5a, signal

through the G-protein-dependent pathways in various myeloid and non-myeloid immune cells as well as non-hematopoietic cells [608][609][610] to induce the production of various soluble mediators and cytokines [611][612].

In part because of its central role in the initial activation process of the complement system, but also because of its various functions on immune cells upon its successive cleavage through Factor I, C3 is considered as the most pivotal component of the complement system [584]. Activated, membrane-bound C3b is successively cleaved into iC3b, C3dg and C3d. C3b and iC3b predominantly function as opsonizing molecules and facilitate the phagocytosis of apoptotic and pathogenic cells by CR1- and CR3-expressing cells, in which macrophages and also erythrocytes are considered as major contributors [569][572][613][614]. Many implications for the two last fragments of the C3 cleavage cascade, C3dg and C3d, have been reported in the literature, predominantly describing the modulation of B cell-mediated immune responses. The corresponding receptor, CR2 (CD21), is highly expressed by B cells (in particular MZ B cells) and co-ligation of the BCR with CR2 upon its binding of C3dg/C3d is well known to greatly amplify BCR signaling and consequentially B cell activation as well as Ab production [95][615][616]. Furthermore, FDCs are known to use CR2 to retain immune complexes that are associated with C3dg and C3d fragments, in order to prolong Ag presentation to B cells in GC [164][617]. An overview of the complement cascade is illustrated in Figure 1.4.

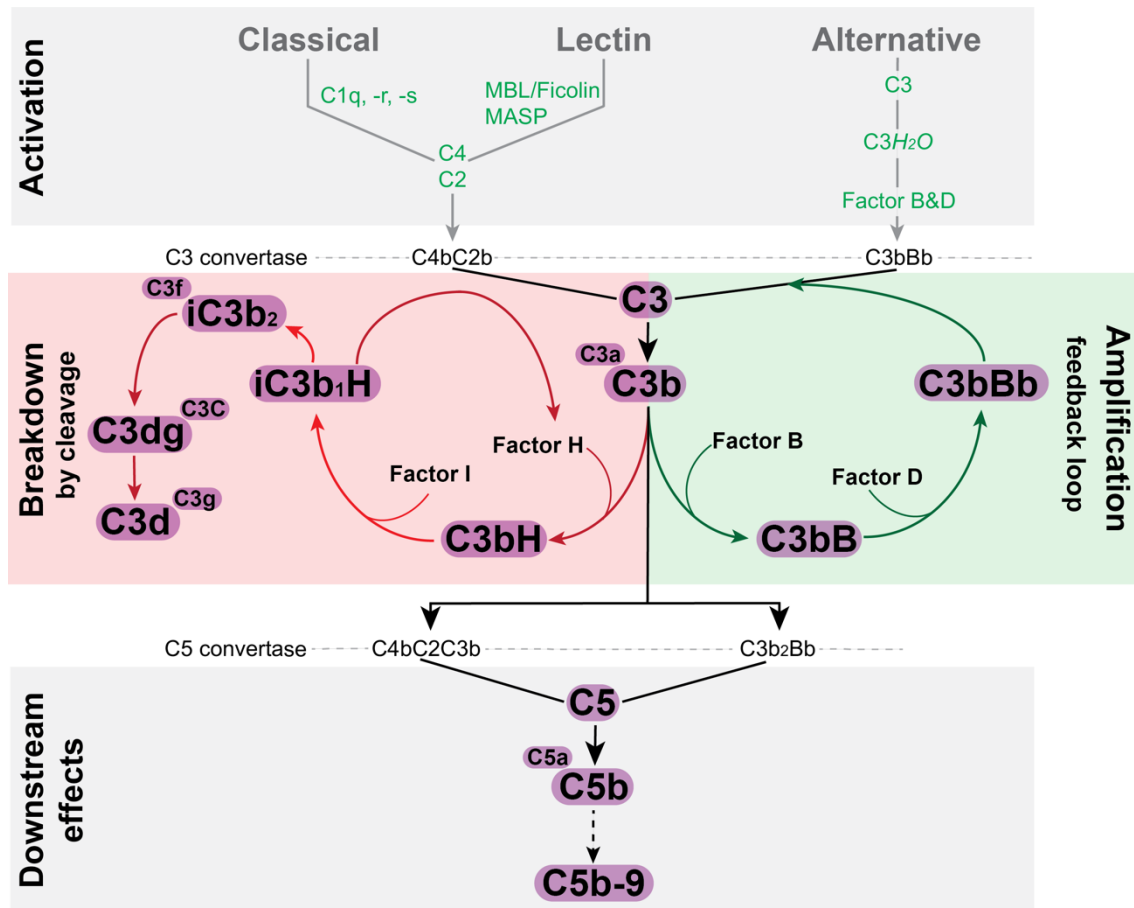


Figure 1.4 Overview of the complement cascade. Complement activation through either the classical, lectin or alternative pathway leads to the formation of C3 convertases which proteolytically cleave further C3 molecules. While complement activation through the classical or lectin pathway is known to be induced through pathogenic surface structures, such as cell surface bound immune complexes or certain carbohydrates, activation through the alternative pathway occurs spontaneously at a low rate during steady state. The outcome of the initial complement activation through the cleavage of C3 molecules depends on the molecular microenvironment, in particular the presence of negative and positive complement regulators that either limit or support further activation. While Factor B and D induce a positive amplification feedback loop, in which more C3 molecules get activated through the generation of more C3 convertases, Factor I and H negatively regulate the complement cascade through the successive cleavage of activated C3b. This dynamic system guarantees controlled complement activation, in which only harmful structures are attacked by the complement system, such as by the membrane attacking complex, consisting of C5b and C6-C9 complement proteins, upon the cleavage of C5 (based on [618]).

1.5.2 Cleavage of activated complement C3

Activation of C3 through its proteolytic cleavage enables the covalent binding to multiple hydroxyl nucleophiles on cell surfaces through the formation of a covalent thioester bond between C3b and its target [585]. Unspecific or spontaneous activation, the latter usually through the alternative pathway, can lead to the binding of C3 to the cell surface, even that of intact and healthy host cells. However, this is described to be shut down strictly through

the action of negative complement regulators, such as of Factor I and Factor H [593][594][595][596][619].

C3 is comprised of an α and β chain (~113 kDa and ~70 kDa, respectively), which are connected by a disulphide bond and non-covalent forces [620]. The thioester site, responsible for the covalent attachment of C3b to cell surfaces, sits protected in a hydrophobic pocket of the α chain in case of non-activated C3, but gets exposed after activation [591]. In the Factor I-mediated breakdown cleavage pathway only the α chain of activated C3b gets successively cleaved, while the β chain stays unaffected. Multiple cuts in the α chain of the membrane-bound C3b through Factor I first generate the small fragment C3f as well as the larger iC3b₁ and iC3b₂ species, followed by C3c and C3dg fragments. Thereby, the bigger C3c fragment (~133.6 kDa) is finally released into the fluid phase, while the smaller fragment C3dg (~38.8 kDa) and C3d (~33.8 kDa) remains bound to the cell surface as both fragments the thioester of the initially activated C3b [619]. The cleavage breakdown pathway of C3, its fragments and their molecular weights (kDa) as well as amino acid residue site (AS) are summarized in Figure 1.5 [621][619].

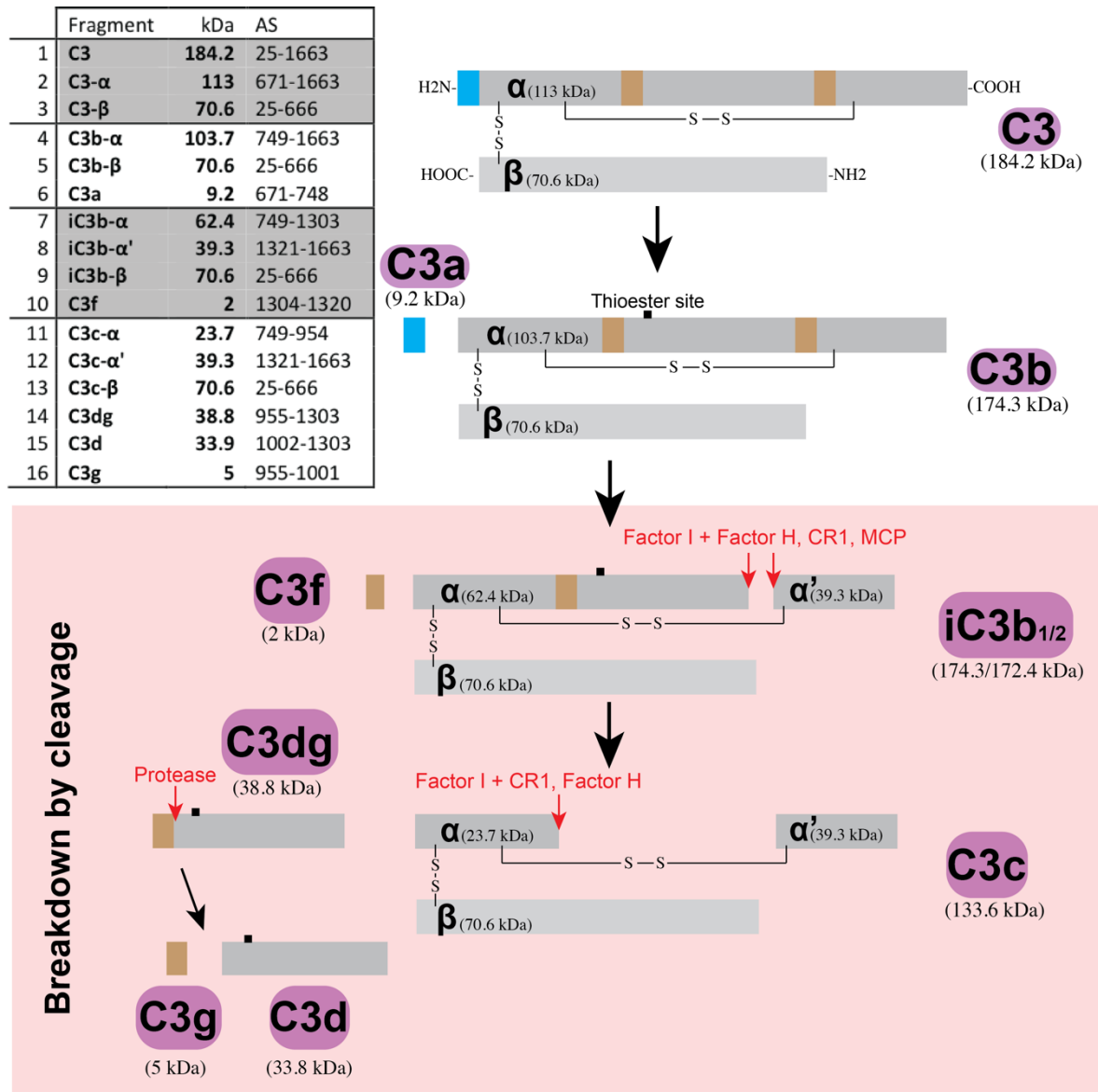


Figure 1.5 Summary of successive cleavage of complement C3. Schematic flow chart showing activation [white background] and inactivation [red background] of complement C3 by cleavage, including a table listing C3 fragments with corresponding molecular weights (kDa) and amino acid residue positions/sites (AS). The murine C3 molecule (P01027) consists of two polypeptides (AS 25-666 β chain and AS 671-1663 α chain + signal peptide AS 1-25 in its β chain) linked by a disulphide bond. Cleavage of C3 for both activation and inactivation occur at several sites in its α chain only. Initial activation of C3 through the cleavage of C3a leads to exposure and activation of the thioester group in the α chain, allowing the attachment of C3b to surface-borne nucleophiles. Further cleavages of surface bound C3b, predominantly through the action of Factor I and H as well as other assisting proteins (such as MCP), leads to the formation of inactivated C3b (i.e. iC3b₁ and iC3b₂) and C3c, leaving behind the thioester containing fragment C3dg, which remains bound to the cell surface. Final cleavage of C3dg generates C3d and C3g (based on [619]).

1.5.3 Functions of complement C3 in adaptive immunity

Although traditionally considered as a humoral effector arm of the innate immune system, numerous recent studies have described regulatory roles of various complement proteins in adaptive immunity (reviewed by Killick *et al.* [622] as well as by Bennett *et al.* [623]). In particular, various species of C3 have been described to modulate adaptive immune responses of B and T cells as well as the Ag presentation by dendritic cells (DCs). This is achieved through C3 acting as a serum effector molecule in the extracellular environment, but also intracellularly as part of a repertoire of complement proteins inside the cell, referred to as the '*complosome*', that has been described to have roles in various intracellular processes, including protein degradation [575][576], cell signaling [624][625] and cell activation (reviewed in [626][627]).

As mentioned before, C3 has several regulatory roles in B cell immunity and humoral Ab responses. Already reported in the early 1990s, the binding of C3d to CR2 in B cells induced co-ligation of various proteins involved in the BCR complex, including with CD19 and IgM, lowering the threshold for B cell activation by up to four orders of magnitude [95][628][629][630]. Furthermore, it has been shown that C3d can also enhance Ag presentation by B cells, which facilitates their acquisition of helper signals through CD4⁺ helper T cells [623][631]. Therefore, C3d is a critical adjuvant for B cells stimulation, especially in conditions where Ag affinity and/or concentrations are low [94][623].

Beside its function as an adjuvant in B cell-mediated immune responses, C3 also has implications in T cell immunity. In contrast to the serum-derived C3 that influences B cell stimulation, C3 impacting T cell responses is thought to be locally produced and functions in an autocrine and paracrine manner, often intracellular [627][632][633][634][635]. Locally produced C3 has been shown to be associated with enhanced activation of the complement system via the alternative pathway and consequential C3aR signaling led to enhanced expression of IL-12 receptor as well as IFN- γ production in T cells [636], favoring Ag presentation by DCs.

Numerous studies have demonstrated an Ag preservative function of complement C3, protecting or prolonging Ag lifetime in order to favor Ag presentation in various settings. FDCs are known to express CR1 and CR2, binding and sequestering C3d-coated immune complexes in lymphoid follicles, which preserves the Ag for long periods of time to promote GC

development [164][617][637][638]. Furthermore, opsonization of free Ag with C3d is known to extend the Ag's half-life [623][639] which has general prolonging effects on Ag presentation by various APCs. The impact of C3 on Ag presentation by cDCs is versatile. Baudino *et al.* as well as Villier *et al.* have described that opsonization of Ag with C3 regulates endocytic transport of apoptotic cell structures within DCs, resulting in enhanced T cell responses. These studies have demonstrated that this is due to reduced intracellular phagosome maturation, associated with C3 opsonization, as MHC II Ag presentation in DCs from C3-deficient mice resulted in an accelerated degradation of endocytosed Ag, causing impaired Ag presentation and T cell proliferation, when compared to DCs from C3-sufficient mice [575][576]. Furthermore, signaling through C3aR in DCs was linked to their increased activation, based on MHC II and CD86 expression [640] and cross-talking between TLRs and various complement receptors have been described to also enhance DC activation [641].

1.6 Research aims

The overall aim of this thesis is to examine the mechanisms and biological consequences of enriched C3 deposition on the surface of cDCs that occurs in the absence of E3 ubiquitin ligase MARCHF1. Prior to this PhD project an unbiased proteomic screen was performed with the aim to identify new MARCHF1 protein substrates and discovered significantly enriched C3 levels on the surface of *Marchf1*^{-/-} cDCs [see section 3.1.2 on page 92].

The specific aims are:

(1) *To elucidate the reasons for MARCHF1-mediated C3 deposition in cDCs*

MARCHF1-mediated surface deposition of C3 is unexpected because C3 is predominantly an extracellular serum protein and therefore presumably inaccessible to cytosolic ubiquitination by MARCHF1. To elucidate the mechanisms for enriched C3 fixation on the surface of cDCs, lacking MARCHF1 expression, this chapter describes the biochemical characterization of this response and identifies the source of C3, the C3 fragments and how these bind to the cell surface.

(2) *To characterize the biological role of the C3 fixation on cDCs*

Beyond its traditionally appreciated protective function in the innate immune system, emerging evidence describe new roles for C3 in modulating adaptive immune responses [see section 1.5.3]. This section aims to evaluate the biological role or consequence of the C3 deposition on DC biology by investigating its effect of functional DC antigen presentation as well as cellular homeostasis in secondary lymphoid tissues.

(3) *To investigate the role of C3 in the interaction between cDC and B cells*

C3 fixation is known to enable cell-cell interactions facilitating phagocytosis of C3 opsonized cells [642][573]. This section investigates the impact of C3 deposition on the interaction between splenic cDCs and marginal zone B cells and explores the physiological relevance resulting from this interaction.

Material and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Reagents, consumables and other materials

Reagents

Table 2.1 Reagents used in this study. This list is a summary of critical chemicals and reagents used in this study. All reagents were purchased from certified manufacturers/suppliers and met general laboratory standards.

Reagent	#cat/ref	Brand/supplier
Ammonium chloride	12125-02-9	Chem-Supply
Bovine serum albumin null (BSA)	A7906	Sigma Aldrich
Bromophenol blue	34725-61-6	Sigma
Coomassie Brilliant Blue R-250	1610436	Bio-Rad
cOmplete™ (protease inhibitor cocktail)	11697498001	Roche
Dimethyl sulfoxide (DMSO)	67-68-5	Life Technologies
Ethylenediaminetetraacetic acid (EDTA)	6381-92-6	Astral Scientific
Fetal calf serum (FCS)*	F9423	Sigma Life Science
Ficoll-Paque™ Plus	17-1440-02	GE Healthcare
GlutaMAX™	35050-061	Gibco
Glycerol	56-81-5	Chem-Supply
IGEPAL® CA-630	9002-93-1	Sigma Aldrich
IMDM	12440	Gibco
Magnesium chloride	7791-18-6	Chem-Supply
Nycodenz®	1002424	Axis shield
Penicillin G sodium salt	P3032	Sigma
Potassium chloride	7447-40-7	Merck
Propidium iodide (PI)	537059	Calbiochem
Normal rabbit serum	011-000-120	Jackson ImmunoResearch
Rat gamma globulin	012-000-002	Jackson ImmunoResearch
Sodium chloride	7647-14-5	Merck
Sodium dodecyl sulphate (SDS)	151-21-3	Amresco
Streptomycin sulphate	S6501	Sigma
Tris (Base)	77-86-1	Astral Scientific
Tween®20	9005-64-5	Chem-Supply
Dimethyl sulfoxide	D2660	Sigma Life Science
β-mercaptoethanol	60-24-2	Life Technologies
CellTrace™ Violet (CTV) stain	C34557	Invitrogen
Ovalbumin egg white (OVA) protein, purified	LS003054	Worthington

*heat-inactivated (56 °C for 30 min)

Consumables, kits and other materials

Table 2.2 Consumables, kits and other materials used in this study.

Product	#cat/ref	Brand/supplier
75 cm ² cell culture flasks	430641U	Corning
96-well U bottom cell culture plates	3799	Corning
Anti-FITC MicroBeads	130-048-701	Miltenyi Biotec
Anti-PE MicroBeads	130-048-801	Miltenyi Biotec
BioMag [®] anti-rat IgG beads	310107	Qiagen
Cell Strainer (40 µm Nylon)	352340	Falcon
ECL Select [™] Western Blotting reagent	17001657	Amersham [™] GE Healthcare
iBlot [®] 2 PVDF Regular/Mini Stacks	IB24001/2	Invitrogen
LS Columns	130-042-401	Miltenyi Biotec
MACS [®] SmartStrainers (70 µm)	130-098-462	Miltenyi Biotec
NuPAGE [®] MOPS SDS running buffer	NP0001	Invitrogen
NuPAGE [™] 4-12% Bis-Tris Plus gels	NW04120	Invitrogen
Pan-DC Enrichment Kit, human	130-100-777	Miltenyi biotec
PKH26 Red Fluorescent Cell Linker Kit	PKH26GL	Sigma Aldrich
RNase-free DNase Set	79254	Qiagen
RNeasy [®] Mini Kit	74104	Qiagen
SeeBlue [®] Plus2 Prestained standard	LC5925	Invitrogen
Sphero [™] blank cal. beads (6.0-6.4 µm)	556296	BD Biosciences

Enzymes

Table 2.3 Enzymes used in this study.

Enzyme	#cat	Brand/supplier
DNase I	10104159001	Roche
Collagenase Type3 CLS3	M3D14157	Worthington
Trypsin Gold, MS grade	V5280	Promega
Trypsin-Versene	27250018/E5134	Gibco/Sigma
PNGase F	P0704S	New England BioLabs

Adjuvants

Table 2.4 Advujants used in this study.

Adjuvant	Brand/supplier
CpG ODN 1668 (CpG) 5'-TCCATGACGTTCTGATGCT-3'	Bioneer
Advax [™] - Delta Inulin (DI)	Vaxine [643][644][645]

2.1.2 Buffers and media

EDTA-BSS. Double distilled water with 150 mM sodium chloride, 4 mM potassium chloride, 12 μ M sodium dihydrogen orthophosphate, 24 μ M disodium orthophosphate, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 5 mM ethylenediaminetetraacetic acid (EDTA); murine cell tonicity balanced. Produced by the Media Preparation Unit (MPU), Peter Doherty Institute, Victoria, Australia.

EDTA-BSS-2%FCS. EDTA-BSS supplemented with 2 % (v/v) fetal calf serum (FCS; Sigma Life Science).

FCS-EDTA. FCS (Sigma Life Science) supplemented with 10 % (v/v) 0.1 M EDTA (Astral Scientific); final concentration of EDTA 10 mM.

KDS-RPMI. RPMI 1640 with 33.6 mM HEPES, 1 μ M sodium pyruvate, 24 mM sodium bicarbonate, produced by the Media Preparation Unit, Peter Doherty Institute, Victoria, Australia.

KDS-RPMI-2%FCS. KDS-RPMI supplemented with 2 % (v/v) fetal calf serum (FCS; Sigma Life Science).

IP lysis buffer. MilliQ water with 1 % (v/v) IGEPAL[®] CA-630 (Sigma-Aldrich), 50 mM Tris (pH 7.5; Astral Scientific), 5 mM magnesium chloride (Chem-Supply) and cOmplete™ protease inhibitor cocktail (Roche).

IP wash buffer. MilliQ water with 0.5 % (v/v) IGEPAL[®] CA-630 (Sigma-Aldrich), 50 mM Tris (pH 7.5; Astral Scientific), 150 mM sodium chloride (Merck) and 5 mM EDTA (Astral Scientific).

Phosphate buffered saline (PBS). MilliQ water with 145 mM sodium chloride, 7.5 mM disodium hydrogen orthophosphate and 2.8 mM sodium dihydrogen orthophosphate; produced by the Media Preparation Unit, Peter Doherty Institute, Victoria, Australia.

3x SDS (reducing) sample buffer. MilliQ water with 240 mM Tris (pH 6.8; Astral Scientific), 6 % (w/v) sodium dodecyl sulphate (SDS; Amresco), 33 % (v/v) glycerol (Chem-Supply), 16 % (v/v) β -mercaptoethanol (Life Technologies) and 0.06 % (w/v) bromophenol blue (Sigma).

Western blot buffer. PBS with 0.1 % (v/v) Tween-20 (Sigma-Aldrich).

Red cell removal buffer (RCRB). MilliQ water with 156 mM ammonium chloride (Chem-Supply), 100 μ M EDTA (pH 8.0; Astral Scientific) and 12 mM sodium bicarbonate (Sigma-Aldrich).

FcR block. 1x PBS with 4 mg/ml anti-CD16/CD32 (FcR) mAb (clone 2.4G2) and 1 mg/ml rat gamma globulin (Jackson ImmunoResearch)

DC media. KDS-RPMI media with 1x GlutaMAX™ (Gibco), 10 % (v/v) FCS (Sigma Life Science), 100 U/ml penicillin and 100 μ g/ml streptomycin (both Media Preparation Unit, Peter Doherty Institute, Victoria, Australia) and 50 μ M β -mercaptoethanol (Life Technologies).

IMDM media. Iscove's Modified Dulbecco's Medium (IMDM, Gibco) + 1x GlutaMAX™ (Gibco), 10% (v/v) FCS (Sigma Life Science), 100 U/ml penicillin and 100 μ g/ml streptomycin (both Media Preparation Unit, Peter Doherty Institute, Victoria, Australia) and 100 μ M β -mercaptoethanol (Life Technologies).

DMEM media. Dulbecco's modified Eagle medium (DMEM) produced by the Media Preparation Unit, Peter Doherty Institute, Victoria, Australia, + 1x GlutaMAX™ (Gibco), 10% (v/v) FCS (Sigma Life Science) and 100 U/ml penicillin and 100 μ g/ml streptomycin (both Media Preparation Unit, Peter Doherty Institute, Victoria, Australia) and 100 μ M β -mercaptoethanol (Life Technologies).

2.1.3 Antibodies

Flow cytometry antibodies

Table 2.5 Flow cytometry antibodies (anti-mouse) used in this study.

Antigen/Specificity	Clone	Isotype	Brand/supplier
B220 (CD45R)	RA3-6B2	Rat IgG2a, κ	Biolegend
BST2 (CD370)	7H11	Rat IgG1, κ	Biolegend
C3	3d29	Mouse IgG2a	Holer <i>et al.</i> [619]
C3	11H9	Rat IgG2a	Novus Biol.
C3	-	polyclonal rabbit Ig	Hycult Biotech (#HP8018)
C3	-	polyclonal goat IgG	MP Biomedical (#55500)
CD11b	M1/70	Rat IgG2a, κ	Biolegend
CD11c	N418	Armenian hamster IgG	Biolegend
CD18	M18/2	Rat IgG2a, κ	Biolegend
CD19	6D5	Rat IgG2a, κ	Biolegend
CD21/CD35 (CR2/CR1)	7E9	Rat IgG2a, κ	Biolegend
CD23	B3B4	Rat IgG2a, κ	Biolegend
CD24	M1/69	Rat IgG2b, κ	WEHI
CD3e	KT3-1.1	Rat IgG2a, κ	WEHI
CD36	HM36	Armenian Hamster IgG	Biolegend
CD4	GK1.5	Rat IgG2b, κ	Biolegend
CD4	RM4-5	Rat IgG2a, κ	Biolegend
CD43	S7	Rat IgG2a, κ	WEHI
CD45.1	A20.1	Mouse IgG2a, κ	Biolegend
CD45.2	104	Mouse IgG2a, κ	Biolegend
CD54	YN1/1.7.4	Rat IgG2b, κ	Biolegend
CD62L	MEL-14	Rat IgG2a, κ	Biolegend
CD64	X54-5/7.1	mouse IgG1, κ	Biolegend
CD86	GL1	Rat IgG2a, κ	BioLegend
CD8a	YTS169.4	Rat, IgG2b	WEHI
CD8a	53-6.7	Rat IgG2a, κ	Biolegend
CD93 (AA4)	AA4.1	Rat IgG2b, κ	BioLegend
Clec12A	5D3	Rat IgG2a, κ	Biolegend
Clec9A (CD370)	7H11	Rat IgG1a, κ	Biolegend
ESAM	1G8	Rat IgG2a, κ	Biolegend
F4/80	F4/80	Rat IgG2b	WEHI
Factor H	-	polyclonal sheep IgG	Abcam (ab8842)
Flt3 (CD135)	A2F10	Rat IgG2a, κ	Biolegend
IgD	11-26c.2a	Rat IgG2a, κ	Biolegend
IgG1	RMG1-1	Rat IgG	Biolegend
IgM	RMM-1	Rat IgG2a, κ	Biolegend
Ly6C	HK1.4	Rat IgG2c, κ	Biolegend
Ly6G	1A8	Rat IgG2a, κ	WEHI
MHC II (I-A & I-E)	M5/114	Rat IgG2b, κ	Biolegend & WEHI
MHC II (I-Ab)	AF6-120.1	Mouse IgG2a, κ	Biolegend
MHC II (I-Ab) + Ea52-68	eBioY-Ae (YAe)	Mouse IgG2b	Thermo Fischer
MHC II (I-Ad)	39-10-8	Mouse IgG3, κ	Biolegend
MHC II (I-Ak)	10-3.6	Mouse IgG2a, κ	Biolegend
NK1.1 (CD161c)	PK136	Mouse IgG2a, κ	BD Biosciences
Siglec H	551	Rat IgG1, κ	Biolegend
Sirp α (CD172a)	P84	Rat IgG1, κ	Biolegend
TCR V α 2	B20.1	Rat IgG2a, κ	WEHI
XCR1	ZET	Mouse IgG2b, κ	Biolegend

Table 2.6 Flow cytometry antibodies (anti-human) used in this study.

Antigen/Specificity	Clone	Isotype	Brand/supplier
CD1c	L161	Mouse IgG1, κ	Biolegend
CD11c	3.9	Mouse IgG1, κ	Biolegend
Lin (CD3/14/16/19/20/56)	OKT3/M5E2/3G8/HIB19/2H7HCD56	Mouse IgG1 & IgG2a, κ	Biolegend
CD141	M80	Mouse IgG1, κ	Biolegend
HLA-DR	LN3	Mouse IgG2b, κ	Biolegend
CD123	6H6	Mouse IgG1, κ	Biolegend
C3	-	Polyconal rabbit IgG	abcam (Ab48342)

Western blot antibodies

Table 2.7 Western blot antibodies used in this study.

Antigen/Specificity		Host	Brand/supplier
MHC II α (H2-A α /I-A α)	JV1	Rabbit (polyclonal)	Biolegend
MHC II β (H2-Ab1/I-A β)	JV2	Rabbit (polyclonal)	Biolegend
C3	HP8012	Rabbit (polyclonal)	Hycult Biotech
Pan-actin	20-33	Rabbit (polyclonal)	Sigma Aldrich

Antibodies used for purification of primary mouse cells

Table 2.8 Immuno-depletion antibody cocktails used in this study.

	Antigen/Specificity	Clone	host	dilution	Brand/supplier
cDC cocktail	CD3e	KT3-1.1	rat	1/200	WEHI
	CD90	T24	rat	1/400	WEHI
	Ly76	TER/119	rat	1/100	WEHI
	Ly6G/Ly6C	RB68C5	rat	1/100	WEHI
	B220 (CD45R)	RA3-6B2	rat	1/400	WEHI
B cell cocktail	CD4	GK1.5	rat	1/200	Biolegend
	Ly76	TER/119	rat	1/200	Biolegend
	CD43	S7	rat	1/200	WEHI
CD4⁺ T cell cocktail	MHC II	M5/114	rat	1/20	WEHI
	Ly76	TER/119	rat	1/100	WEHI
	B220 (CD45R)	RA3-6B2	rat	1/400	WEHI
	CD11b	M1/70	rat	1/500	WEHI
	1A8	Ly6G	rat	1/10	WEHI
	CD8a	YTS169.4	rat	1/25	WEHI
CD8⁺ T cell cocktail	MHC II	M5/114	rat	1/20	WEHI
	Ly76	TER/119	rat	1/100	WEHI
	B220 (CD45R)	RA3-6B2	rat	1/400	WEHI
	CD11b	M1/70	rat	1/500	WEHI
	1A8	Ly6G	rat	1/10	WEHI
	CD4	GK1.5	rat	1/10	WEHI

Fusion antibodies constructs for intravenous injections

Table 2.9 Fusion antibody constructs used in this study. The amino acid sequences corresponding to the epitopes for OT-I (OVA₂₅₇₋₂₆₁), OT-II (OVA₃₂₃₋₃₃₉) OB1 (OVA₆₁₋₆₉) and/or YAc (I-Ea₄₆₋₇₂) were cloned in-frame with the H chain C terminal region of the anti-Clec9A mAb (clone 10B4) via alanine linkers [11].

Name	Antigen	Conjugation	Clone/host	Source/Reference
anti-Clec9A-OVA _{FGD}	Clec9A	OT-I, OT-II, OB1	10B4/rat (mAb)	[83][146][147][148]
anti-Clec9A-OVA _{FGD} -YAc	Clec9A	OT-I, OB1, YAc	10B4/rat (mAb)	Chaminschi & Heath

2.1.4 Mice

Experimental mice were bred and housed under specific pathogen-free conditions in the Bio21 Institute animal facilities, Victoria, Australia, unless otherwise stated. Both male and female mice were used at 6-14 weeks of age and all procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Melbourne and the National Health and Medical Research Council of Australia. All handling as well as experimental procedures were approved by the Animal Ethics Committee at the University of Melbourne (Application 1714238 and 1513472). C57BL/6 mice were utilized for experiments unless otherwise stated. Transgenic/genetically modified mice strains, listed in Table 2.10, were bred as homozygous colonies and genotyped by PCR on a regular basis (every first litter of mating pairs/trios). No siblings/littermate mice were used.

Table 2.10 Transgenic mouse strains used in this study. Mice genetically modified in more than one gene location (i.e. *Marchf1^{-/-}* x *C3^{-/-}*, *Marchf1^{-/-}* x *I-A α ^{-/-}* and MHC II K225R^{KI/KI} x *C3^{-/-}*) were generated through sufficient crosses of respective single K.O. mice.

Strain	Description/Mutation	Background	Source/Reference
<i>C3^{-/-}</i>	Targeted null/knockout through insertion of PGK/Neo cassette	C57BL/6	The Jackson Laboratory 129S4- <i>C3^{tm1Crr}</i> /J; stock #003641 [646]
<i>Cr2^{-/-}</i>	Targeted null/knockout through insertion of PGK/Neo cassette into Exon 1 and 2	C57BL/6	The Jackson Laboratory 129S7(NOD)- <i>Cr2^{tm1Hmo}</i> /J; stock #008225 [578]
<i>I-Aα^{-/-}</i>	Deletion of H2-A α /I-A α leading to loss of MHC II surface complexes	C57BL/6	[647]
<i>Ly5.1</i>	Congenic mice expressing the leukocyte surface antigen Ly5.1 (CD45.1) protein-tyrosine phosphatase receptor type c, a allele (<i>Ptprc^a</i>). All other mice used in this study expressed CD45.2.	C57BL/6	
<i>Marchf1^{-/-}</i>	MARCHF1 RING-CH domain deletion	C57BL/6	[496]
MHC II K225R ^{KI/KI}	Substitution of Lysine (K) at position 225 in the MHC II β (H2-Ab1/I-A β) with Arginine (R)	C57BL/6	[535]
<i>OT-I</i>	CD8 ⁺ T cells express V α 2 and V β 5 TCR transgenes specific for OVA ₂₅₇₋₂₆₁ in I-Ab	C57BL/6	[648][649][650]
<i>OT-II</i>	CD4 ⁺ T cells express V α 2 and V β 5 TCR transgenes specific for OVA ₃₂₃₋₃₃₉ in I-Ab	C57BL/6	[648][649][650]
OB1. <i>Rag^{-/-}*</i>	B cells express transgenic BCR specific to the linear epitope sequence DKLPFGDSI contained in a surface-exposed loop of OVA	C57BL/6	[651][652]
<i>Ubl3^{-/-}</i>	Deletion of <i>Ubl3</i> exons 2-5	C57BL/6	By the MAGEC laboratory (WEHI), manuscript in preparation

*kept in Animal Facility of Peter Doherty Victoria, Australia

2.2 Methods

2.2.1 Cell culture

B16-Flt3L tumor cells

Immortal B16 tumor cells secreting the Flt3L growth factor were grown in 75 cm² cell culture flasks (Corning) in DMEM media. Cells were kept in a humidified incubator with 10 % atmospheric CO₂ at 37 °C and adherent cells were passaged through trypsin versene treatment (MPU) for five minutes at 37 °C and washes in PBS.

DC 1940 cells

MuTu DC1940 cells were a gift from Hans Acha-Orbea [653] and maintained in 75 cm² cell culture flasks (Corning) with IMDM (Gibco) containing 1x GlutaMAX™ (Gibco), 10 % (v/v) FCS (Sigma Life Science), 100 U/ml penicillin, 100 µg/ml streptomycin (both MPU), and 100 µM 2-mercaptoethanol (Life Technologies). Cells were kept in a humidified incubator with 10 % atmospheric CO₂ at 37 °C and passaged at 70 % confluency, using EDTA-BSS+2%FCS, and by pooling adherent and non-adherent populations.

DC1940 cells deficient in *Marchf1* and *H2-AB1* genes were generated via CRISPR/Cas9 technology, prior to this study. In brief, DC1940 cells, expressing the endonuclease Cas9 were lentivirally transduced with plasmid containing small guide RNA (sgRNA) [654], targeting *Marchf1* and *H2-AB1* genes upon induction through incubation with 1 µg/ml doxycycline. Knockout cells were stained with antibodies against surface markers, bulk sorted by flow cytometry and stored in 10 % (v/v) dimethyl sulfoxide (DMSO, Life Technologies) + 90 % (v/v) FCS (Sigma Life Science) in liquid nitrogen according to standard cryopreservation procedures.

2.2.2 Purification of primary mouse cells

Whole splenocyte suspensions

To obtain single-cell suspensions of whole splenocytes, dissected spleens were finely chopped with scissors and then digested in KDS-RPMI-2%FCS containing 140 µg/ml DNase I (Roche) and 1 mg/ml collagenase type 3 (Worthington) for 20 min at room temperature (RT) with constant agitation using a transfer pipette. Cells were filtered through a 40 µm cell strainer (Falcon) and intercellular clusters were disrupted by the addition of further 10 mM EDTA (final concentration, Astral Scientific) under further agitation for 5 min at RT. Red blood cells were lysed using RCRB, and finally cells were washed in EDTA-BSS-2%FCS.

Conventional dendritic cells

Conventional dendritic cells (cDCs) were purified from spleens as previously described by Vremec *et al.* [655]. In brief, single-cell suspensions of whole splenocytes were generated through collagenase/DNase I digestion as stated above (see section purification of '*whole splenocyte suspension*'), excluding lysis of erythrocytes using RCRB. Cells were filtered through a 40 µm cell strainer (Falcon) and low-density splenocytes were isolated by density gradient separation of whole splenocytes with two layers (5+5 mL) of 1.077 g/cm³ Nycodenz® (Axis shield) + 1 mL FCS-EDTA per 4 spleens. Upon centrifugation (2200 g, 20 min, 4 °C), cells in 70-80 % of the upper layer were collected, washed in EDTA-BSS-2%FCS and then resuspended in a depletion cocktail containing the following rat anti-mouse mAbs: anti-CD3 (clone: KT3-1.1), anti-Thy1 (clone: T24), anti-Ly76 (clone: TER119), anti-B220 (clone: RA3-6B2), anti-Ly6C/G (clone: RB6-8C5) [see Table 2.8]. Cells were incubated with the mAb cDC cocktail (10 µl per 10⁶ cells) for 30 min at 4 °C, washed with EDTA-BSS-2%FCS, and then incubated with BioMag® anti-rat IgG-coupled magnetic beads (20 µl per 10⁶ cells; Qiagen) for 20 min at 4 °C with continuous agitation. The cDC-enriched supernatant was then recovered after magnetic depletion. This protocol yields cell preparations with 75-85 % CD11c⁺ MHC II⁺ purity.

cDCs from tissues other than spleen (i.e. thymus, total LN, liver and lung) were isolated through collagenase/DNase I digestion and subsequent Nycodenz® density gradient centrifugation (2+2 ml layers, 1.077 g/cm³) as stated above. Collagenase/DNase I digestion in case of liver and lung tissue was prolonged (45 min instead of 20 min) and red blood cells were lysed using RCRB. All cells were washed in EDTA-BSS-2%FCS to disrupt intercellular clusters.

CD8⁺ conventional dendritic cells (cDC1s) from Flt3L-expanded mice

CD8⁺ cDCs (cDC1s) from mice that have been injected with a B16-Flt3L melanoma cell line that secretes the cDC growth factor Flt3L [656], were purified from spleens as described above (i.e. purification of '*conventional dendritic cells*' from the spleen) with small adjustments due to enriched spleen sizes (i.e. protocol for 2 instead of 4 spleens). Purified cDCs were incubated with PE-conjugated anti-CD8 mAbs (clone: 53-6.7, 1:400 in 10 µl EDTA-BSS-2%FCS per 10⁶ cells) for 30 min at 4 °C, washed in EDTA-BSS-2%FCS, and then incubated with magnetic anti-PE MicroBeads (2 µl per 10⁶ cells; Miltenyi Biotec) for 20 min at 4 °C. The cDC1-enriched

fraction was captured after magnetic depletion of the supernatant. This protocol yields cell preparations with 80-90 % CD11c⁺ MHC II⁺ CD8⁺ purity.

B cells

B cells were isolated from spleens using manual disruption of organs through a 40 µm cell strainer (Falcon). Cells were washed with EDTA-BSS+2%FCS and low-density cells were enriched using centrifugation over a layer of 5ml Ficoll-Paque™ Plus (GE Healthcare), washed with EDTA-BSS-2%FCS and then resuspended in a depletion cocktail containing the following FITC-conjugated rat anti-mouse mAbs: anti-CD4 (clone: GK1.5), anti-Ly76 (clone: TER119), anti-CD43 (clone: S7) [see Table 2.8]. Cells were incubated with the mAb B cell cocktail (1:300 dilution) for 30 min at 4 °C, washed in EDTA-BSS-2%FCS, and then incubated with magnetic anti-FITC MicroBeads (2 µl per 10⁶ cells; Miltenyi Biotec) for 20 min at 4 °C. The B cell-enriched supernatant was then recovered after magnetic depletion using LS magnetic columns and MidiMACS magnet + stand (Miltenyi Biotec). This protocol yields cell preparations with 95-98% CD19⁺ B220⁺ purity.

Transgenic T cells (OT-I and OT-II)

T cells from transgenic OT-I and OT-II mice were isolated from total lymph nodes (LN) using manual disruption of organs through a 40 µm cell strainer (Falcon). Cells were then washed with EDTA-BSS-2%FCS and resuspended in a depletion cocktail containing the following rat anti-mouse mAbs: anti-MHC II (clone: M5/114), anti-Ly76 (clone: TER119), anti-CD45R/B220 (clone: RA3-6B2), anti-CD11b (clone: M1/70), Ly6G (clone: 1A8) and anti-CD4 (clone: GK1.5) in case of CD8⁺ OT-I T cell purification or anti-CD8 (clone: YTS 169.4) in case of CD4⁺ OT-II T cell purification [see Table 2.8]. Cells were incubated with the mAb CD4⁺/CD8⁺ T cell cocktail (1 ml per total LN) for 30 min at 4 °C, washed with in EDTA-BSS-2%FCS, and then incubated with BioMag® anti-rat IgG-coupled magnetic beads (Qiagen) for 20 min at 4 °C with continuous agitation. The T cell-enriched supernatant was then recovered after magnetic depletion. This protocol yields cell preparations with 80-90% CD3⁺ TCR Vα2⁺ CD4⁺ and CD3⁺ TCR Vα2⁺ CD8⁺ purity, respectively.

2.2.3 Purification of human blood dendritic cells

Blood samples from healthy donors were obtained as buffy coats from the Australian Red Cross Lifeblood, Victoria, Australia. Whole blood samples from HLA-deficient patients were collected in EDTA tubes by staff of the Royal Children's Hospital, Victoria, Australia. Peripheral blood mononuclear cells (PBMCs) from blood samples of both healthy donors as well as HLA-deficient patients were enriched using centrifugation over a 15ml layer of Ficoll-Paque™ Plus (GE Healthcare) and subsequent lysis of red blood cells. DCs were then purified from PBMCs with the Pan-DC Enrichment Kit (Miltenyi biotec) according to the manufacturer's instructions. In brief, 10^8 cells were FcR-blocked and incubated with the kit's depletion antibody cocktail and magnetic micro beads in order to depleted Ab-labelled cells using LS magnetic columns and MidiMACS magnet + stand (all Miltenyi Biotec). This protocol yields cell preparations with 5-10% CD11c⁺ HLA-DR⁺.

2.2.4 Flow cytometry

General surface staining and enumerations of cell populations

Cell populations and surface marker expression profiles were identified by flow cytometry using fluorescently labelled antibodies (see Table 2.5). For this, single cell suspensions were stained with fluorescence-labelled antibodies in 50 µl EDTA-BSS-2%FCS in 96-well U bottom cell culture plates (Corning) for 30 min on ice and then washed twice with EDTA-BSS-2%FCS. In case of two-step stainings, such as of biotinylated primary Ab + fluorescently labelled secondary streptavidin, single cell suspensions were first incubated with the primary Ab for 30 min on ice, washed twice with EDTA-BSS-2%FCS and then stained with the corresponding staining panel containing the respective fluorescently labelled secondary streptavidin/Ab. If stated cells FcR-blocked, cells were pre-incubated with 1:50 serum or FcR-block, containing 4 mg/ml anti-CD16/CD32 (FcR) mAb (clone 2.4G2) and 1 mg/ml rat gamma globulin (Jackson ImmunoResearch), before staining with fluorescence-labelled antibodies. For the enumeration of cells via flow cytometry, a defined number of Sphero™ blank calibration beads (6.0-6.4 µm; BD BioSciences) was used as a reference standard for the sample volume. All samples were collected on the LSRFortessa™ (BD BioSciences) and data analysis was performed using FlowJo software (FlowJo LLC) and Prism (Graphpad).

Gating strategies

In all cases cell doublets and dead cells were identified and excluded based on forward and side scatter (FSC and SSC) as well as positive staining with propidium iodide (PI; Calbiochem). Cell populations were then identified based on a combination of surface marker expressions using fluorescently labelled antibodies with appropriate gating strategies outlined below (Figure 2.1 - Figure 2.5).

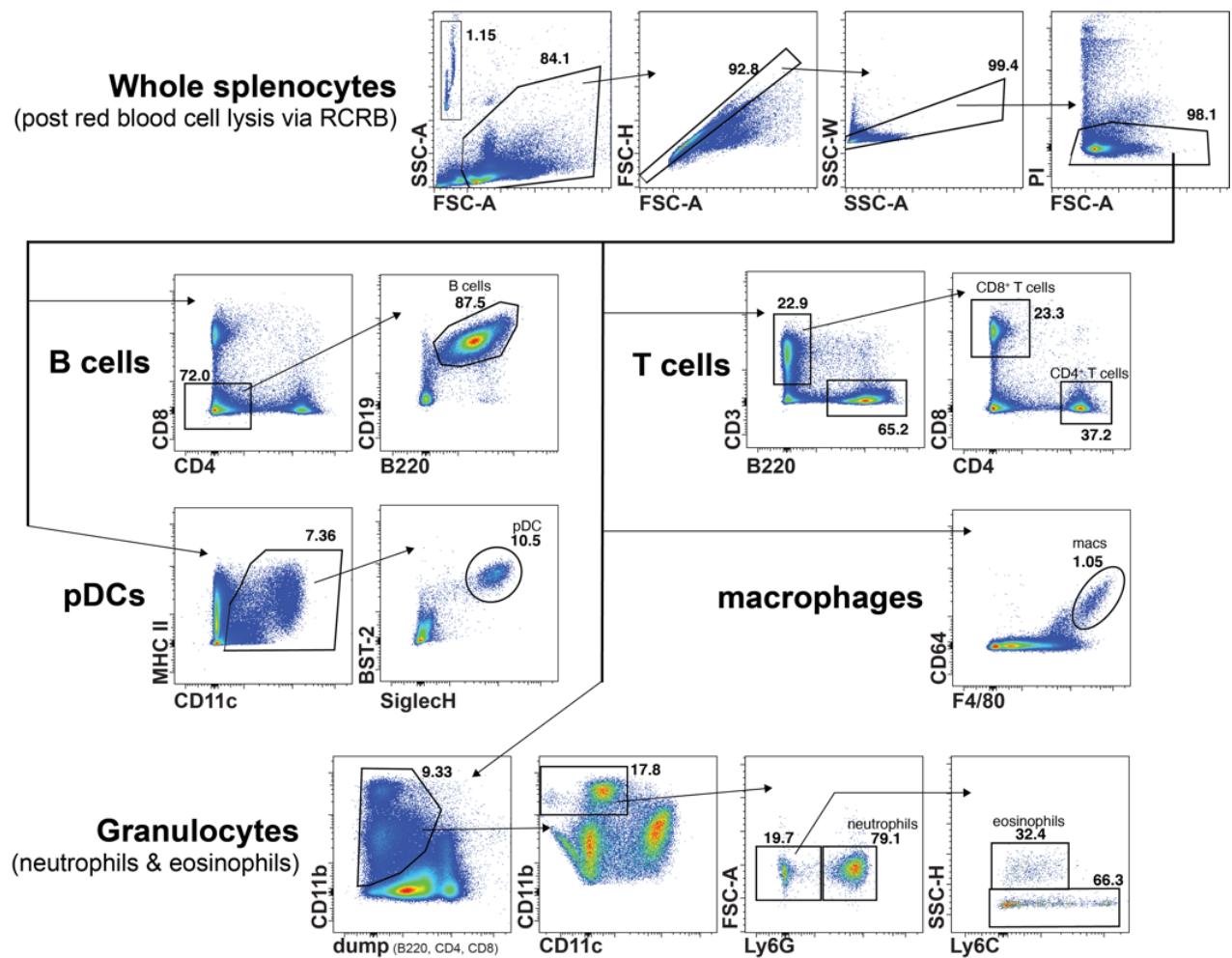


Figure 2.1 Flow cytometry gating strategies for major immune cells in the spleen. Representative flow cytometry gates for the identification of immune cell populations in the spleen. Single-cell suspensions of whole splenocytes were generated through DNase I/collagenase digestion of finely chopped spleens. Cell doublets and dead cells were identified and excluded based on forward (FSC) and side scatter (SSC) as well as positive staining with propidium iodide (PI). B cell populations were then identified as CD4⁻ CD8⁻ CD19⁺ B220⁺, CD4⁺ T cells as CD3⁺ CD4⁺, CD8⁺ T cells as CD3⁺ CD8⁺, pDCs as MHC II⁺ CD11c⁺ BST-2⁺ SiglecH⁺, macrophages as F4/80⁺ CD64⁺ and granulocytes as B220⁻ CD4⁻ CD8⁻ CD11c⁻ CD11b⁺ with neutrophils as Ly6G⁺ and eosinophils as Ly6G⁻ SSC-H^{high} (the latter based on Liyanage *et al.* [657]).

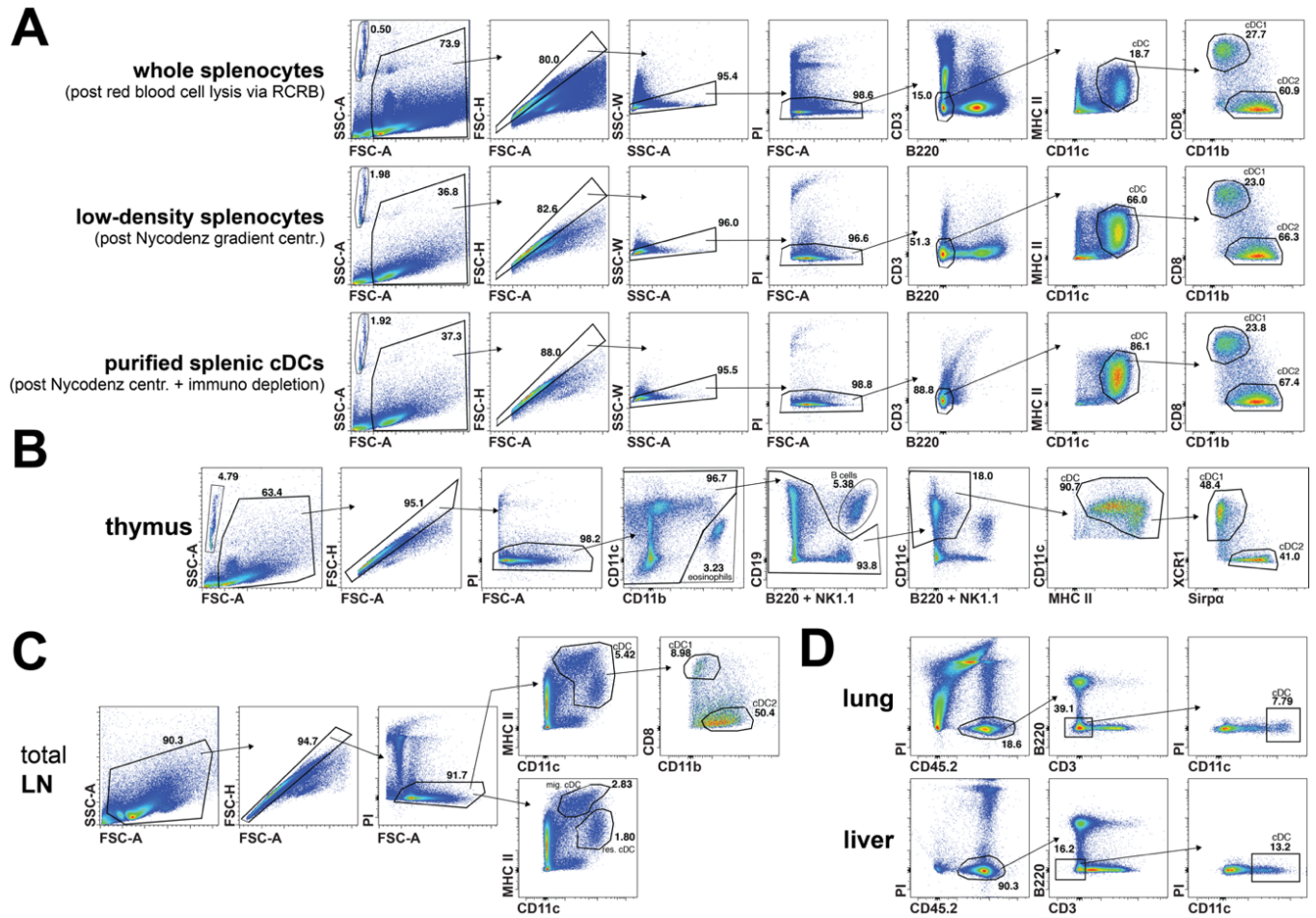


Figure 2.2 Flow cytometry gating strategies for conventional dendritic cells purified from different organs. Representative flow cytometry gates for the identification of cDCs upon purification from different tissues. In all cases cell doublets and dead cells were identified and excluded based on forward (FSC) and side scatter (SSC) as well as positive staining with propidium iodide (PI). **(A)** cDCs from spleens were identified from whole or low-density splenocyte suspensions or after immuno-depletion of non-cDCs from low-density splenocyte suspensions, in all cases as B220⁻ CD3⁻ CD11c⁺ MHC II⁺, with further discrimination of cDC1s as CD11b⁻ CD8⁺ and cDC2s as CD11b⁺ CD8⁻. **(B)** Thymic cDCs were identified as B220⁻ NK1.1⁻ CD11c⁺ MHC II⁺, with further discrimination of cDC1 as Sirpα⁻ XCR1⁺ and cDC2 as Sirpα⁺ XCR1⁻ (based on Ardouin *et al.* [658]). **(C)** cDCs from total lymph nodes (LN) were identified as CD11c⁺ MHC II⁺, with identification of migratory (mig.) cDCs as CD11c^{int} MHC II^{high} and tissue-resident (res.) cDCs as CD11c^{high} MHC II^{int} or with further discrimination of cDC1s as CD11b⁻ CD8⁺ and cDC2s as CD11b⁺ CD8⁻. **(D)** cDCs from liver and lung were identified as CD45.2⁺ B220⁻ CD3⁻ CD11c⁺ [gates for exclusion of cell doublets based on forward (FSC) and side scatter (SSC) are not shown].

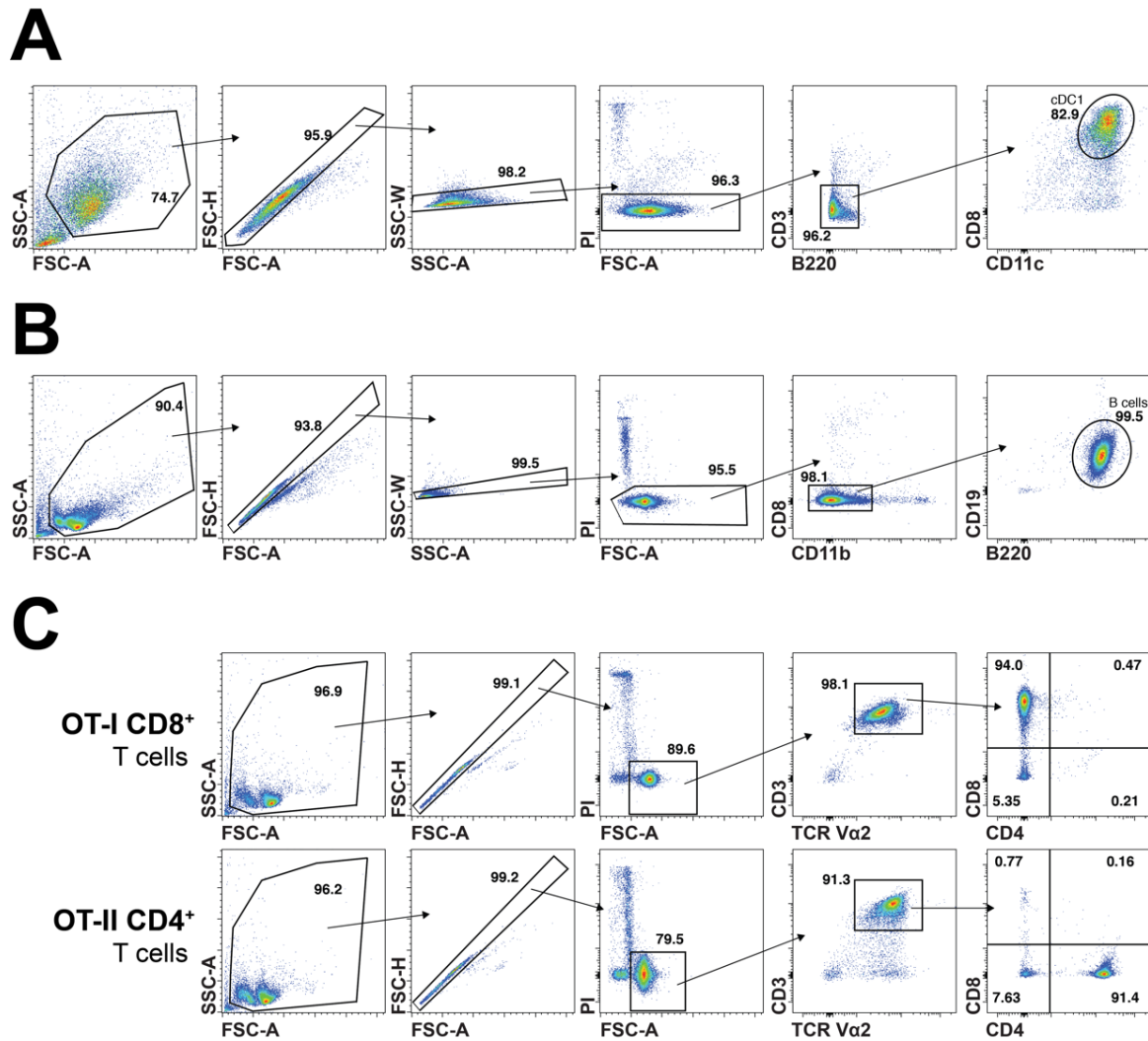


Figure 2.3 Flow cytometry gating strategies for purified cDC1s, B cells and OT-I/II T cells. Representative flow cytometry gates for the identification of purified (A) cDC1, (B) B cells and (C) OT-I/II T cells. In all cases cell doublets and dead cells were identified and excluded based on forward (FSC) and side scatter (SSC) as well as positive staining with propidium iodide (PI). (A) Splenic cDC1s from Flt3L-expanded mice were purified from low-density splenocyte suspensions through immuno-depletion of non-cDCs and subsequent positive selection of CD8⁺ cells, identified as CD3⁻ B220⁻ CD11c⁺ CD8⁺. (B) Splenic B cells were purified from whole splenocyte suspensions through immuno-depletion and identified as CD8⁻ CD11b⁻ B220⁺ CD19⁺. (C) OT-I and OT-II T cells were purified from total LN of transgenic mice through immuno-depletion and identified as TCR Vα2⁺ CD3⁺ with further discrimination based on CD8 and CD4 expression.

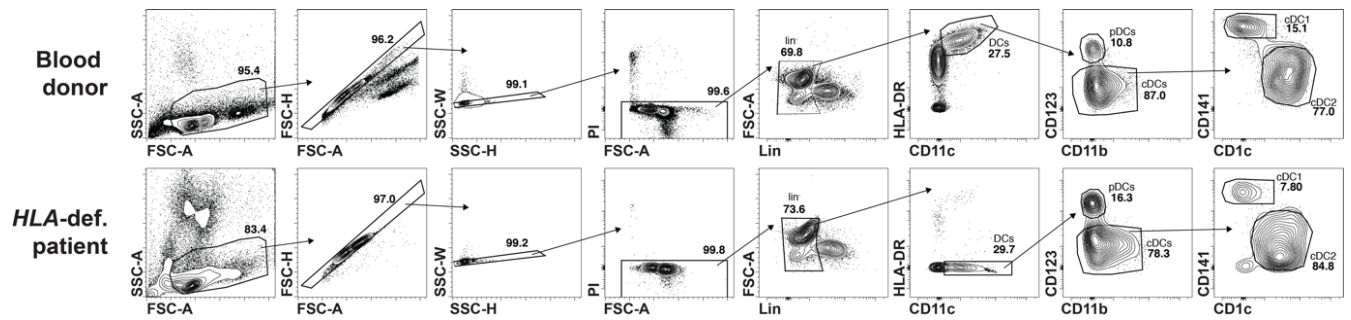


Figure 2.4 Flow cytometry gating strategies for human blood dendritic cells. Representative flow cytometry gates for the identification human blood DCs from healthy donors [top panel] and HLA-deficient patients [bottom panel] after Ficoll-Paque™ Plus centrifugation and lysis of red blood cells followed by DC purification via antibody-bead negative selection with the Pan-DC Enrichment Kit (Miltenyi biotec). Cell doublets and non-viable cells were eliminated based on FSC and SSC plots and propidium iodide (PI) staining. DCs were then identified as lineage⁻ (Lin⁻, including CD3, CD14, CD16, CD19, CD20 and CD56), CD11c^{int-high} and HLA-DR⁺ in case of healthy donors. DCs from HLA-deficient patients were identified similar to DCs from healthy donors, excluding HLA-DR. In both cases, DC subsets were further discriminated as lineage⁻ CD11c⁺ CD11b⁻ CD123⁺ (pDCs), lineage⁻ CD11c⁺ CD123⁻ CD141⁻ CD1c⁺ (cDC2s) and lineage⁻ CD11c⁺ CD123⁻ CD141⁺ (cDC1s).

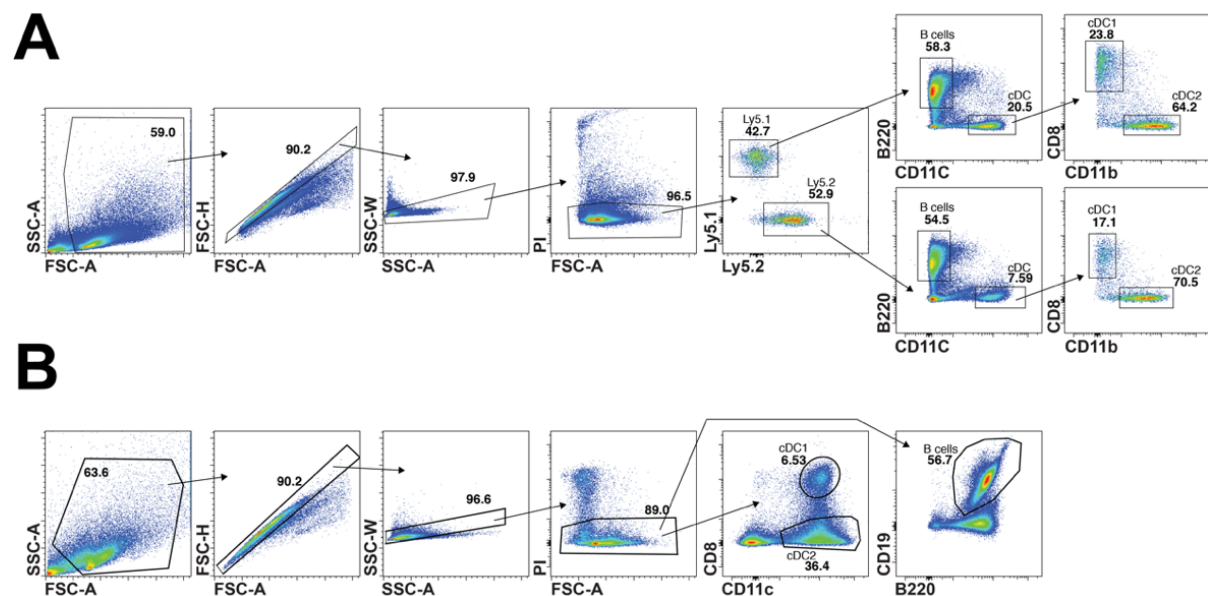


Figure 2.5 Flow cytometry gating strategies for cDCs and B cells from BM chimeric mice and *in vitro* trogocytosis assay. Representative flow cytometry gates for the identification of cDCs and B cells from (A) spleens of mixed bone marrow reconstituted recipient mice and (B) upon 2h co-culturing in *in vitro* trogocytosis assays. Cell doublets and non-viable cells were eliminated based on FSC and SSC plots and propidium iodide (PI) staining. (A) First, cells from mixed bone marrow compartments were distinguished based on Ly5.1 and Ly5.2 expression. Then B cells were identified as B220⁺ and cDCs as CD11c⁺ with further discrimination of cDC1s as CD11b⁻ CD8⁺ and cDC2s as CD11b⁺ CD8⁻. (B) After co-incubation *in vitro* B cells were identified as B220⁺ CD19⁺, cDC1s CD11c⁺ CD8⁺ and cDC2s as CD11c⁺ CD8⁻.

Cell sorting via flow cytometry

Cell sorting was performed on an Influx™ Cell Sorter (BD Biosciences) instrument at the Murdoch Children's Research Institute, Royal Children's Hospital, Victoria, Australia, using up to six fluorochromes (PE, BV510, BV421, FITC, APC and PI). Cells were sorted from low-density splenocytes suspensions, generated through collagenase/DNase I digestion and subsequent Nycodenz® density gradient centrifugation as stated above. Cell doublets and dead cells were excluded based on forward and side scatter as well as positive staining with PI. B cells were sorted based on gates of CD8⁻ B220⁺, cDC1s as CD11c⁺ CD8⁺ and trogocytic B cells as CD11c^{int} CD8^{int}. Influx™ Cell Sorter settings for sort mode were: 1.0 Drop Pure; sheath pressure: 33.0 psi; nozzle diameter: 86 µm. Purity of sorted cells was routinely between 95-99 %.

2.2.5 Cellular assays and mouse model experiments

Injection of B16-Flt3L tumor cells

5x 10⁶ B16 tumor cells, secreting Flt3L [656], were injected subcutaneously in 200 µl 1x PBS between shoulder blades (inter scapula space) using a 25G needle. Tumor development was monitored, and spleens were harvested on day 8-10 days post injection for immune cell isolation (i.e. cDC1s) and analysis.

Generation of mouse serum from whole blood

Whole blood was taken through cardiac punctuation from freshly terminated mice upon CO₂ administration as outlined in the general procedures. Whole blood was left for 30 min at RT and for 16-20 h at 4 °C to let fully coagulate. Serum supernatant was taken upon 10 min centrifugation at 14,000 g and 4 °C and stored at -80 °C.

Generation of mixed bone marrow chimeric mice

Bone marrow recipient mice were irradiated twice at 550 cGy (Rad), 3h apart. Bone marrow from donor mice was flushed out from tibia and femur with KDS-RPMI-2%FCS using a 10 mL syringe and 25G needle and filtered through a 70 µm MACS® SmartStrainers (Miltenyi Biotec). Cells were then washed and resuspended in 1x PBS. For mixed bone marrow chimera experiments, bone marrow cells from two different donor strains were mixed 50:50 and intravenously injected (5x 10⁶ in total per recipient mice) into the recipient mice 1-4 h after irradiation. Recipient mice were injected i.p. with anti-Thy1 (T24) to eliminate radioresistant host T cells the day after irradiation. Mice were reconstituted for at least 8 weeks before use.

Deposition of C3 on cDCs in vitro/ex vivo

1x 10⁶ primary splenocytes or DC1940 cells were incubated in 96-well U bottom cell culture plates (Corning) in 100 µl DC media (without FCS) with 10 % (v/v) serum from WT or C3^{-/-} mice for 15 to 120 min. In case of additional activation 0.5 µM CpG (Bioneer) was added. After incubation cells were washed in EDTA-BSS-2%FCS to disrupt intercellular clusters and analyzed by flow cytometry as described above.

Antigen presentation assay

Purified OT-I/OT-II T cells were labelled with 1 µM CellTrace™ Violet (CTV) stain (Invitrogen) in 1x PBS with 0.1 % (w/v) BSA (10x 10⁶ cells per ml) for 20 min at 37 °C and then washed 5 times with EDTA-BSS-2%FCS. Isolated cDCs were incubated with different amounts (0.05 - 100 µg) of ovalbumin (OVA, Worthington) in 100 µl DC media for 20 min at 37 °C in the presence of 0.5 µM CpG (Bioneer). After washing, 1.5x 10⁴ OVA-pulsed cDCs were incubated with 1.5x 10⁴ CTV-stained OT-I or OT-II T cells in 200 µl DC media in 96-well U bottom cell culture plates (Corning) for 64-72 h at 37 °C. After incubation cells were washed with EDTA-BSS-2%FCS and analyzed by flow cytometry with staining for T cells (i.e. anti-CD3, anti-TCR Vα2 and anti-CD4/CD8). Antigen presentation was determined by the number of proliferated T cells through CTV dilution and numbers were determined by inclusion of Sphero™ blank calibration beads (BD Biosciences).

In vitro trogocytosis assay

In vitro trogocytosis assay was carried out based on a published protocol, established by Daubeuf *et al.* [659] (excluding Ag priming). In brief, 2x 10⁵ B cells and 3x 10⁵ cDCs purified from spleens were co-cultured in 200 µl of DC media in 96-well U bottom cell culture plates (Corning) for 2 h at 37 °C (if not stated otherwise) after quick centrifugation at 150 g to favor cell contact. In case of PKH26 staining either cDCs or B cells were stained with the PKH26 Red Fluorescent Cell Linker Kit before co-culturing according to the manufacturer's instructions (Sigma Aldrich). After incubation cells were washed in EDTA-BSS-2%FCS to disrupt intercellular clusters and analyzed by flow cytometry as described above.

In vivo OB1 B cell activation

OB1-GFP cells were purified from spleens of OB1.*Rag*^{-/-} mice (purity 90-95%) and adoptively transferred into recipient mice (10⁶ cell per mouse) one day prior to immunization with 0.5 mg of anti-Clec9A-OVA_{FGD} mAb (clone: 10B4) or isotype-OVA_{FGD} (clone: GL117) mAbs. 3, 6 or

24 hours after immunization spleens of recipient mice were harvested and B cells were analyzed in whole splenocyte suspension by flow cytometry.

2.2.6 Biochemical assays

Immunoprecipitation of MHC II and C3

Primary cDC1 purified from Flt3L-expanded mice were lysed on ice for 45 min in IP lysis buffer at a concentrations of 10^7 cells/mL, and nuclei were removed by 10 min centrifugation at 14,000 *g* and 4 °C. Lysates were pre-cleared twice by incubation with uncoupled protein-G sepharose beads (Walter and Eliza Hall Institute) in the presence of normal rabbit serum (1:100, serum of host species of used Ab in IP) for 30 min at 4 °C with gentle agitation. To immunoprecipitate MHC II or C3, protein-G sepharose beads pre-coupled with either anti-I-A/I-E (clone M5/115) mAbs (10 µg per 10^7 cells) or anti-C3dg (clone 3d29) mAbs (5 µg per 10^7 cells) were added to the lysate, incubated for 1 h at 4 °C, and finally washed 3 times with IP wash buffer. To eluate MHC II/C3 from protein-G sepharose beads for western blot analysis, beads were incubated in 3x SDS (reducing) sample buffer for 10 min at 95 °C.

PNGase F digestion

Deglycosylation of MHC II molecules, immunoprecipitated from primary cDC1s of Flt3L-expanded mice, was carried out through the incubation with PNGase F according to manufacturer's instructions (New England BioLabs). In brief, washed sepharose G beads with mAb-bound MHC II molecules were incubated with the kit's 1x denaturing buffer for 10 min at 95 °C, cooled down and then incubated with 500 U of PNGase F in 1x GlycoBuffer containing 1 % NP-40 for 1 h at 37 °C. In case serum was deglycosylated, serum samples were diluted 1:100 in MilliQ water and deglycosylation was carried out accordingly. In both cases, reaction was stopped through the addition of 3x SDS (reducing) sample buffer before analysis by western blotting (see below).

Western blotting

For analysis of MHC II and/or C3 by western blotting, serum samples, whole cell lysates or immunoprecipitates from equal cell numbers were incubated in 3x SDS (reducing) sample buffer for 10 min at 95 °C and then separated on a pre-cast NuPAGE™ gel (4-12% Bis-Tris Plus) and then transferred onto a iBlot® 2 PVDF membranes with an iBlot 2 system according to manufacturer's instructions (all Invitrogen). Samples were probed either for C3 through the

polyclonal anti-C3 pAbs from rabbit serum (HP8012, Hycult) or for MHC II through anti-I-A α - or anti-I-A β -specific pAbs from rabbit serum JV1 and JV2 (produced at the WEHI antibody facility) in western blot buffer with 2 % (w/v) BSA, both followed by HRP-coupled secondary antibodies with the appropriate species reactivities. Excess primary and secondary Ab were washed away 3 times in western blot buffer and chemiluminescence was determined using the ECL Select™ Western Blotting reagent (Amersham™ GE Healthcare), acquired on a ChemiDoc MP imaging system (Bio-Rad) and ImageJ.

2.2.7 Mass spectrometry and proteomics

In-gel trypsin digestion

Samples were run on a pre-cast NuPAGE™ gel (4-12% Bis-Tris Plus, Invitrogen) and stained using Coomassie Brilliant Blue R-250 (Bio-Rad). Bands of interest were excised and subjected to reduction, alkylation, and trypsin digestion before mass spectrometry (MS). In brief, excised gel samples were destained in 50 mM ammonium bicarbonate dissolved in 50 % (v/v) acetonitrile, reduced with 10 mM dithiothreitol (DTT) and then alkylized with 55 mM iodoacetamide, each for 30 min at 37 °C. Air-dried gel pieces were incubated with 15 ng/ μ l trypsin (Promega) in 25 mM ammonium bicarbonate for ~16 h at 37 °C and peptides were extracted through incubation with 0.1 % (v/v) formic acid in 60 % acetonitrile.

Mass spectrometry and data analysis

Mass spectrometry and data analysis were carried out by Laura Dagley (Webb laboratory), WEHI Proteomic Laboratory, Victoria, Australia. Extracted peptides (5 μ l in of 0.1% formic acid, 2% acetonitrile) were separated by reverse-phase chromatography on a 1.9 μ m C18 fused silica column (I.D. 75 μ m, O.D. 360 μ m x 25 cm length) packed into an emitter tip (Ion Opticks, Australia), using a nano-flow HPLC (M-class, Waters). The HPLC was coupled to an Impact II UHR-QqTOF mass spectrometer (Bruker) using a CaptiveSpray source and nanoBooster at 0.20 bar using acetonitrile. Peptides were loaded directly onto the column at a constant flow rate of 400 nl/min with buffer A (99.9 % Milli-Q water, 0.1% formic acid) and eluted with a 90 min linear gradient from 2 to 34% buffer B (99.9 % acetonitrile, 0.1 % formic acid). Mass spectra were acquired in a data-dependent manner including an automatic switch between MS and MS/MS scans using a 1.5 second duty cycle and 4 Hz MS1 spectra rate followed by MS/MS scans at 8-20 Hz dependent on precursor intensity for the remainder of

the cycle. MS spectra were acquired between a mass range of 200–2000 m/z. Peptide fragmentation was performed using collision-induced dissociation (CID).

All raw files were analyzed by MaxQuant (1.5.6.5) software using the integrated Andromeda search engine. Experiment type was set as TIMS-DDA with no modification to default settings. Data was searched against the mouse Uniprot Reference Proteome with isoforms and a separate reverse decoy database using a strict trypsin specificity allowing up to 2 missed cleavages. The minimum required peptide length was set to 7 amino acids. Modifications: Carbamidomethylation of Cys was set as a fixed modification, while N-terminal acetylation of proteins, thioester and oxidation of Met were set as variable modifications. Maximum peptide mass [Da] was set at 8000. All other settings in group or global parameters were left as default.

2.2.8 RNAseq

RNA extraction, library preparation and sequencing

Fluorescence-activated cell separation was performed as described above, and cells were sorted into media. Total RNA was extracted from the sorted cells using the RNeasy Mini Kit (Qiagen), including DNase treatment according to manufacturer's instructions.

Quality control, library prep and sequencing were performed by Australian Genome Research Facility (AGRF), Victoria, Australia. Quality was assessed by chip-based capillary electrophoresis using Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano Kit (Agilent Technologies). All samples had a yield of more than 1 µg of total RNA with RNA integrity (RIN) number of more than 9. After rRNA depletion and library preparation according to manufacturer protocols (TruSeq, Illumina), whole transcriptome sequencing using Illumina Hi Seq 2500 (Illumina, San Diego, CA, USA) was performed to obtain around 20 million single-end reads per sample per lane. All samples were run on two separate lanes. Sequencing quality was confirmed using FastQC reporter¹, and all samples passed quality checks (PHRED scores>30).

Data alignment and analysis

100 base pair single end reads were mapped to the reference mouse genome (GRCm38/mm10) using STAR aligner (Version 2.7), and gene-wise counts were obtained using

Subread package (v1.6.2)³. The average library size of 14 million tags with at least one reported unique alignment was greater than 80 % (82-90 % mapping rate) was obtained. As the same sample libraries were run on two separate lanes, we did not find batch effect affecting the sequencing data. Hence the gene count data of samples run in two lanes, data were aggregated and averaged to obtain mean count data per gene across samples.

Differential expression analysis of RNA sequencing data was carried out in Galaxy/Australia (usegalaxy.org.au, Melbourne Bioinformatics) using the differential expression tool (Trinity assembly) or in RStudio (Version 1.1.447) /R (Version 3.6.1) using the edgeR (Version 3.26.6) and limma (Version 3.40.2) packages. For this, genes were first filtered to remove zero counts, and lowly expressed genes if they did not have counts per million (CPM) mapped fragments value of at least 1 in more than four samples. Counts were converted to log2 counts per million and normalized using the trimmed mean of M-values⁶ normalization method in edgeR. Precision weights were applied with the 'voom' function⁷ of the limma package, and a linear model was fitted to each gene correcting for inter-replicate variation. Empirical Bayes moderated t-statistics were used to assess differences in gene expression and determine p-values. Differences in expression levels were evaluated using a linear model on replicates, with samples compared across the different groups. Genes were corrected for multiple testing, and genes having a false discovery rate (FDR) <0.05 using decideTest⁸ function in limma were considered significant.

MARCHF1-mediated C3 deposition in dendritic cells

Abstract

The E3 Ub ligase MARCHF1 is known to participate in the Ub-mediated trafficking of immune receptors in hematopoietic antigen presenting cells. CD86 and MHC II are the only two verified substrates of MARCHF1 in cDCs and their ubiquitination is known to reduce their presence at the plasma membrane. Proteomic screening, that aimed to identify new MARCHF1 protein substrates, confirmed as expected elevated surface levels of CD86 and MHC II in MARCHF1-deficient cDCs but surprisingly identified complement C3 as the most significantly enriched protein in the plasma membrane of *Marchf1*^{-/-} cDCs.

This chapter elucidates the mechanisms for enriched C3 fixation at the surface of MARCHF1-deficient cDCs and describes MHC II as an acceptor for the covalent association of complement C3. Elevated surface expression of MHC II was identified as the reason for enriched deposition of extracellular C3 on the cell surface of *Marchf1*^{-/-} cDCs. The lack of MHC II ubiquitination in cDCs from mutant mice that still expressed MARCHF1 (i.e. MHC II K225R^{KI/KI} and *Ubl3*^{-/-}) was sufficient for enriched C3 accumulation, as elevated numbers of MHC II complexes at the cell surface bound C3 from the extracellular environment. Biochemical analyses revealed the covalent association of C3dg and C3d, the two smallest C3 fragments upon successive cleavage through Factor I, to the N-linked carbohydrate of MHC II α , demonstrating that binding of spontaneously activated C3 through the alternative pathway binds specifically to the surface of cDCs. Furthermore, we identified binding of C3 to surface MHC II does not occur uniformly as MHC II-C3 complex formation was strongest at the surface of splenic cDC1s and cDC2s, followed by cDCs from total lymph nodes, whereas only little C3 deposited on cDCs in other tissues or on the surface of other MHC II-expressing APCs. Investigations with HLA-deficient patients indicated that human C3, similar to its murine counterpart, can bind MHC II molecules on the surface of cDCs.

3 MARCHF1-mediated C3 deposition in dendritic cells

3.1 Introduction

3.1.1 Protein ubiquitination through MARCHF1 Ub ligases

The attachment of ubiquitin (Ub) molecules to intracellular proteins, known as ubiquitination, is a key mechanism in the regulation of various cellular processes, including proteasomal degradation, DNA repair, signal transduction and intracellular trafficking [517][660]. Ubiquitination of proteins is achieved through the coordinated actions of the three enzymes, namely the Ub-activating E1, the Ub-conjugating E2 and the substrate specific E3 Ub-ligase. The versatile group of E3s has been historically grouped into two classes: the RING (*really interesting new gene*)-type and the HECT (*homologous to the E6AP carboxyl terminus*)-type E3 Ub ligases [661], both bringing together substrates and Ub-carrying E2 enzymes in close proximity. While HECT-type Ub ligases typically bind the Ub molecule from the E2-conjugating enzyme to form an E3-Ub thioester intermediate, RING-type E3 Ub ligases rather mediate the direct transfer of Ub molecules to the substrate without the formation of an intermediate E3-Ub state [662]. In both cases Ub is covalently attached to the target protein via an isopeptide bond between its C-terminal glycine and usually a lysine residue of the acceptor substrate. The group of RING-type Ub ligases has more than 600 members and is highly conserved in eukaryotic cells [517].

In order to provide a sophisticated post-translational system that is involved in the regulation of multiple cellular processes, the nature of ubiquitination in a cell is highly versatile and dynamic. First ubiquitination can occur not only with one Ub unit (mono-ubiquitination) but also multiple times in which previous Ub bound to its substrate can itself serve as a substrate for new rounds of ubiquitination, known as poly-ubiquitination [541]. As any of the seven conserved internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal α -amino group of the Ub can serve as a binding site in the process of poly-ubiquitination, differential linkage of Ub molecules can result in various Ub chains with specific architectures [541]. These have distinct molecular structures, referred as the '*Ub-code*', that can be recognized by cellular machineries to signal different outcomes for the substrate protein [501][541][663]. Additionally, established Ub chains can be specifically cleaved through deubiquitinating enzymes (DUB), a group of more than 100 members in the human genome

[664], in order to rescue proteins from proteasomal degradation or change their trafficking path [665].

K63-linked ubiquitin chains on the cytosolic tail of membrane proteins are known to mediate their intracellular trafficking [663]. The list of membrane proteins whose intracellular trafficking is known to be mediated through ubiquitination includes G-coupled protein receptors [666][667], various receptors with intracellular tyrosine kinases [668] and certain channel and transporter complexes [669][670]. MARCHF Ub ligases are known to be involved in the Ub-mediated trafficking of key membrane proteins involved in the immune system [503][504][505][506]. Of these MARCHF1 is by far the best studied MARCHF Ub ligase and its ubiquitination of CD86 and MHC II in APCs is the paradigm of MARCHF-mediated ubiquitination, modulating Ag presentation [199], [494] [521][523]. As of today, CD86 and MHC II are the only two known substrates of MARCHF1 under physiological conditions, verified in primary cells. Overexpression systems have described other substrates of MARCHF1 [505], but this is known to induce promiscuous Ub ligase activity with off target effects and unbiased analyses of the impact of MARCHF1 deficiency on membrane protein expression are required to identify undescribed bona fide substrates.

3.1.2 The discovery of enriched surface C3 in *Marchf1*^{-/-} cDCs

Prior to this PhD project an unbiased proteomic screen was performed with the aim to identify new MARCHF1 protein substrates in cDCs. As cDCs represent only a small fraction of all cells contained in lymphoid organs of normal mice, cDCs were expanded *in vivo* in WT and *Marchf1*^{-/-} mice using a melanoma cell line that secretes the DC growth factor, Flt3L [656]. This led to a significant expansion and consequently increased yield and purity of isolated splenic cDCs, facilitating the feasibility of this proteomic screen. Subcellular microsomal fractionation enriched in plasma membrane (PM) fractions of purified cDCs was achieved through DC-specific surface staining with biotinylated Abs and subsequent purification from post-nuclear supernatants via magnetic immunoaffinity using avidin-coated magnetic beads. PM-enriched fractions were investigated by mass spectrometry and proteomic analysis in order to identify differentially expressed proteins. This has been demonstrated previously to be a reliable approach for the identification of PM proteins that are differentially expressed by splenic cDCs [471][671].

Comparison of the PM sub-proteomes of WT and *Marchf1*^{-/-} cDCs showed that most proteins

were present at similar levels in the two preparations with peptides detected from over 1,000 proteins in total. Of these eight proteins were identified with significant differential expression between WT and *Marchf1*^{-/-} cDC PM ($\log_2$ protein ratio >2 or <-2 , p value <0.001 across four biological replicates). Proteins strongly enriched in the PM of *Marchf1*^{-/-} cDCs included MHC II α (H2-Aa) and MHC II β (H2-Ab1) as well as CD86, confirming the validity of this approach to detect MARCF1 substrates (**Figure 3.1**). Strikingly, the protein that appeared strongest and most significantly overexpressed in the PM of *Marchf1*^{-/-} cDCs was complement component 3 (C3) (**Figure 3.1**). This was unexpected as C3 is known to be an extracellular serum protein and therefore presumably inaccessible to cytosolic ubiquitination by MARCF1. We therefore aimed to validate the outcome of this proteomic screen and characterize C3 binding to cDCs.

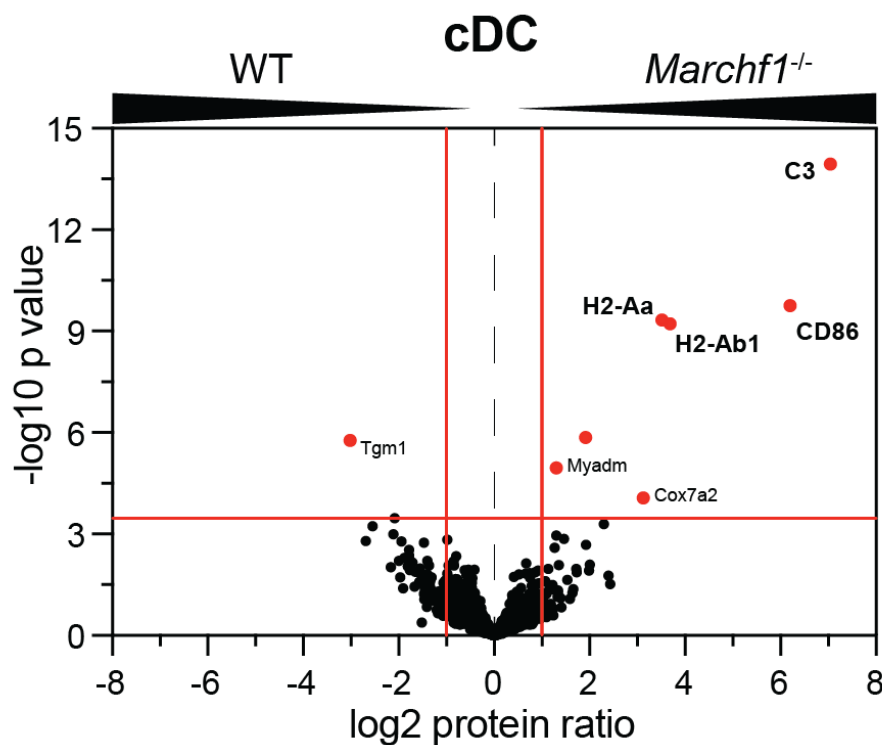


Figure 3.1 MARCF1-deficient cDCs exhibit elevated levels of complement C3 at their surface. Proteomic analysis of plasma membrane (PM) fractions purified from splenic cDCs of FLT3L-expanded WT and *March1*^{f/f} mice. PM-enriched microsomes (PM fractions) were purified from post-nuclear supernatants of biotin-Ab surface stained cDCs via magnetic immunoaffinity using avidin-coated magnetic beads. Equivalent amounts of PM fractions from four biological replicates were analyzed by mass spectrometry and semi-quantitative proteomics. Differentially expressed proteins [red dots] were identified based on four-fold ratio between WT vs. *March1*^{f/f} cDCs (-1 and 1 $\log_2$ protein ratio) and significance across four biological replicates [$p < 0.001$ (expressed as $-\log_{10}$)].

3.2 Results

3.2.1 Validation of enriched C3 deposition on *Marchf1*^{-/-} cDCs

The preceding proteomic screen that discovered enriched C3 surface expression in MARCF1-deficient cDCs was carried out with *in vivo* Flt3L-expanded cDCs [section 3.1.2]. Although several studies have shown that DCs expanded *in vivo* using this approach are phenotypically and functionally equivalent to their counterparts in untreated mice [471][671], we aimed to validate the outcome of the proteomic analyses derived from Flt3L-expanded DCs by using their counterparts from untreated mice.

For this cDCs were purified from spleens of untreated WT, *Marchf1*^{-/-} and *C3*^{-/-} mice to greater than 70% purity (% of alive, single cells) using tissue digestion (collagenase/DNAse I), Nycodenz density gradient centrifugation and Ab-based immuno-depletion of non-cDCs. C3 surface levels were assessed by flow cytometry on both main groups of conventional DCs, namely cDC1s and cDC2s, through two-step staining using a biotinylated anti-C3 mAb (clone 11H9) + streptavidin-APC. As a negative control [fluorescence-minus-one (FMO) control] cells were incubated with streptavidin-APC only, excluding the biotinylated 11H9 anti-C3 mAb.

No C3 was detected on cDCs deficient in C3 with similar gMFI values to the FMO control, demonstrating the specificity of the 11H9 anti-C3 mAb. Some C3 was detected on the surface of splenic WT cDC1s and cDC2s, when compared to the FMO control and *C3*^{-/-} cDCs. Importantly, confirming the results of the proteomic analysis, C3 was present at significantly higher levels on the surface of *Marchf1*^{-/-} cDCs when compared to their WT counterparts, though expression was higher on cDC1s than on cDC2s. (**Figure 3.2A**).

Murine cDCs express a range of Fc receptors, in particular FcγR, that can bind immunoglobulins of different isotypes [672]. To ensure detection of C3 in *Marchf1*^{-/-} cDCs is real and not due to unspecific Ab binding, we blocked Fc receptors on the surface of cDCs before staining with the 11H9 anti-C3 mAb through their pre-incubation with FcR block [including rat gamma globulin + anti-Fc receptor mAb (clone 2.4G2)]. Additionally, we included a biotinylated rat IgG2a isotype control, the same isotype of the biotinylated 11H9 anti-C3 mAb. No C3 signal was detected when *Marchf1*^{-/-} cDCs were incubated with the biotinylated rat IgG2a isotype control + streptavidin-APC (comparable to MFI values of the FMO control), whereas FcR blocking had no considerable effect on the high C3 signal for MARCF1-deficient cDCs, both validating the detection of genuine C3 at the cell surface

(Figure 3.2B).

Expression of C3 was highest on MARCHF1-deficient cDC1s. We further verified high C3 surface levels on these cells by using four different anti-C3 Abs, namely two monoclonal Abs (mAbs) [clone 11H9 & 3d29 (both detecting C3b, iC3b, C3d & C3dg)] and two polyclonal Abs (pAbs) [cat# HP8012 & 55500]. All four Ab confirmed increased C3 surface expression on *Marchf1*^{-/-} cDC1s (Figure 3.2C), with highest C3 signal obtained with Ab clones 11H9, 3d29 and HP8012 (most likely due to signal amplification in two-step staining versus directly FITC-conjugated 55500 pAb). Notably, clone 3d29 anti-C3 mAb was most sensitive in detecting C3 on the surface of WT cDC1s (Figure 3.2C).

Immunoblotting with the HP8012 polyclonal anti-C3 Ab detected various C3 species (~40 kDa, ~45 kDa, ~98 kDa & ~188 kDa) in reducing serum samples of WT and *Marchf1*^{-/-} mice, while no C3 was detected in C3-deficient serum, demonstrating the specificity of the HP8012 anti-C3 pAb in detecting C3 via western blotting (WB). Notably, no significant differences between the serum samples of WT and *Marchf1*^{-/-} mice were detected with the exception of an additional C3 species at approximately 30 kDa in the *Marchf1*^{-/-} samples (Figure 3.2D). C3 detection in reduced cell lysates of WT, *Marchf1*^{-/-} and *C3*^{-/-} cDC1s confirmed enriched C3 levels in MARCHF1-deficient cells, detecting several molecular species of C3 that all correspond, based on size, to the C3 fragments detected in the serum samples with one exception: The most prominent C3 species in *Marchf1*^{-/-} cell lysates had a molecular weight (MW) of approximately 65-70kDa and was not detected in the serum (Figure 3.2D).

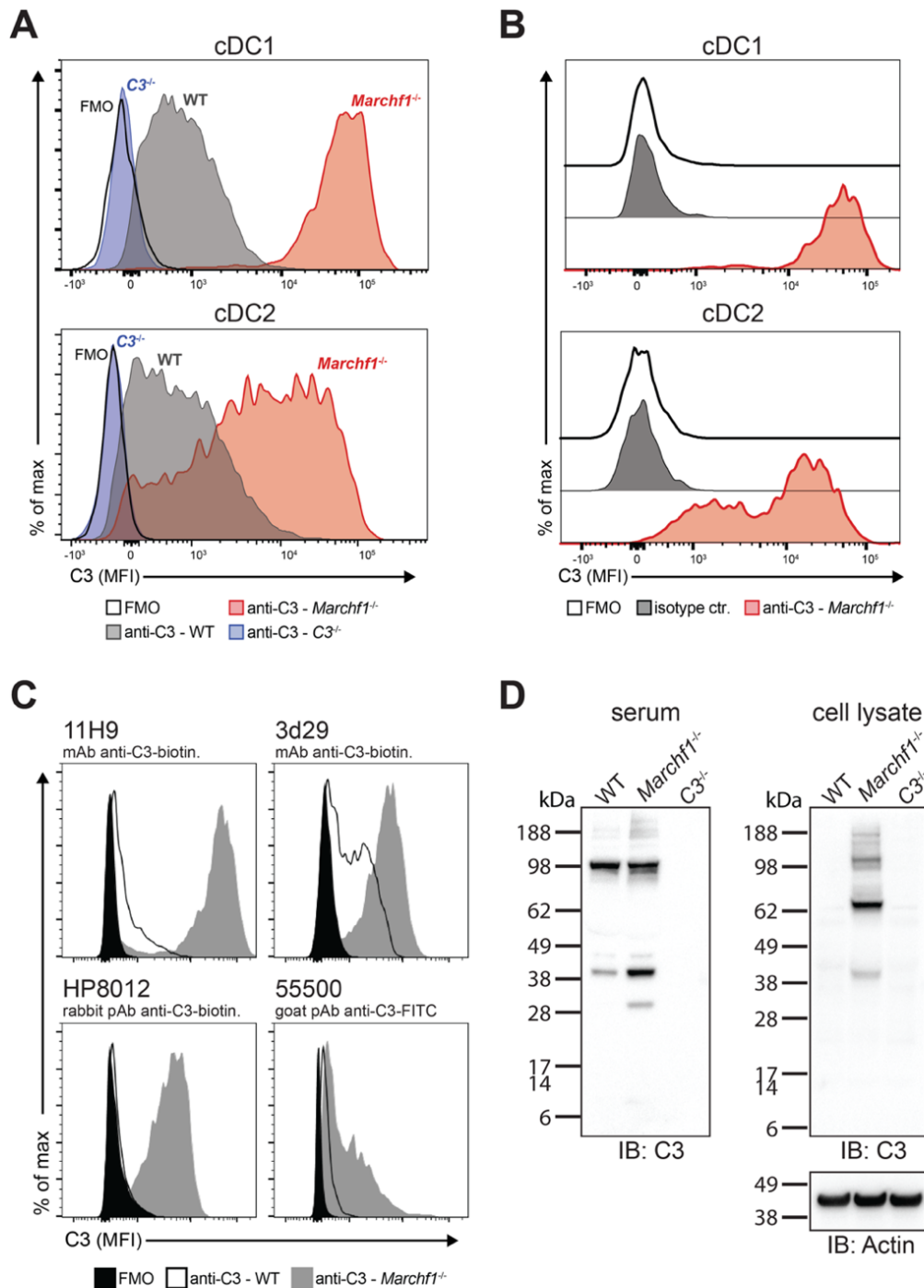


Figure 3.2 Validation of enriched C3 surface levels on *Marchf1*^{-/-} cDCs. (A) Flow cytometry analysis of splenic cDC1s and cDC2s purified from WT, *Marchf1*^{-/-} and *C3*^{-/-} mice, detecting surface C3 via two-step staining with biotinylated anti-C3 mAb (clone 11H9) + streptavidin-APC. For the fluorescence-minus-one (FMO) control cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)]. (B) Detection of APC signal upon incubation of Fc receptor-blocked *Marchf1*^{-/-} cDCs with biotin. anti-C3 rat IgG2a (clone 11H9) or biotin. IgG2a isotype control (+ streptavidin-APC). (C) C3 surface expression in spleen cDC1s from WT and *Marchf1*^{-/-} mice using four different anti-C3 Abs: mAb clones 11H9 & 3d29 (top panel) and pAb cat# HP8012 & 55500 (bottom panel). Abs 11H9, 3d29 and HP8012 were biotinylated and required two-step staining while pAb 55500 was directly FITC-conjugated. (D) Immuno-detection of C3 (pAb HP8012) via western blot in serum samples or cell lysates of cDC1s from WT, *Marchf1*^{-/-} and *C3*^{-/-} mice upon denaturing and reducing SDS PAGE (sec. Ab anti-rabbit IgG-HRP).

We considered the possibility that the detected C3 at the surface of *Marchf1*^{-/-} cDCs was a technical artefact caused at some stage during the cell preparation but did not bind to cDCs *in vivo*. In particular, the enzymatic collagenase/DNase I (C/D) tissue digestion was of concern as it is known to cleave certain cell surface molecules decreasing their expression levels from the cell surface [673]. In order to address the question if C/D digestion causes complement activation and subsequent C3 fixation at the cell surface of cDCs *ex vivo* or if C3 is present at the cell surface without C/D digestion and therefore present *in vivo*, we conducted two approaches: {1} preparing a DC in splenocyte suspensions without any enzymatic digestion but by mechanical disruption of spleens and {2} preparing a single cell suspensions from pooled spleens of WT and *Marchf1*^{-/-} x *C3*^{-/-} mice (50:50) including C/D digestion.

C3 still could be detected on the surface of cDCs in splenocyte suspensions prepared by mechanical disruption only (**Figure 3.3A**), and when preparing DC suspensions including C/D digestion (20 min at 21°C) from pooled spleens of WT and *Marchf1*^{-/-} x *C3*^{-/-} mice (50:50), cDCs derived from the latter (identified by their higher MHC II expression) remained negative for C3 (**Figure 3.3B**), both indicating that C/D digestion was not the reason for high C3 deposition on *Marchf1*^{-/-} cDCs.

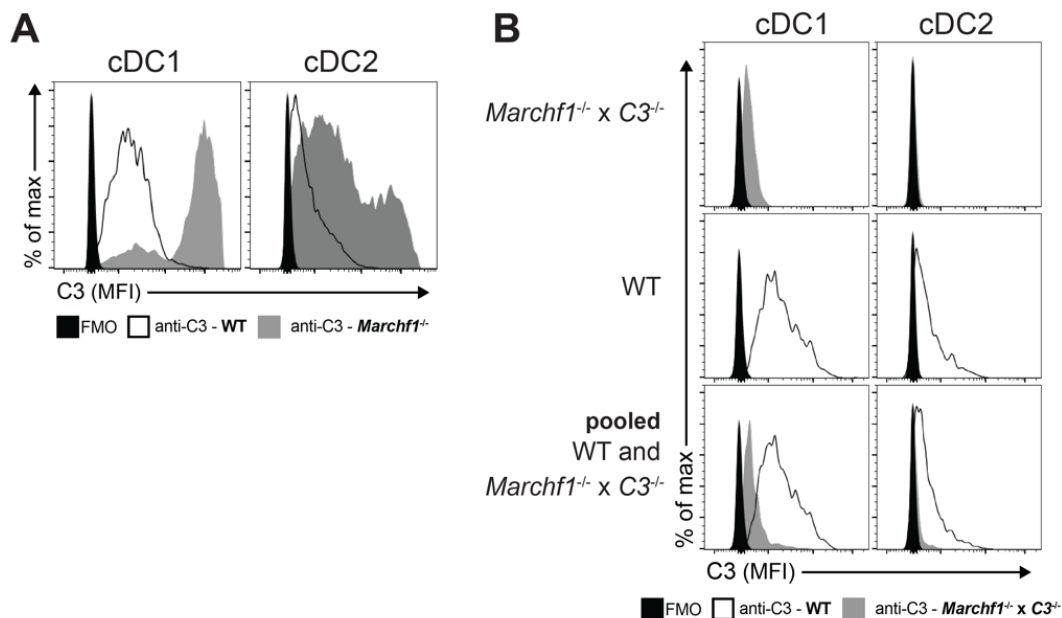


Figure 3.3 Impact of collagenase/DNase I digestion on C3 activation/deposition during cell preparation. (A) C3 surface levels on splenic cDC1s and cDC2s from WT and *Marchf1*^{-/-} mice compared to the corresponding fluorescence-minus-one (FMO) control after mechanical disruption of spleens to create a single cell splenocyte suspension, excluding collagenase/DNase I (C/D) digestion. (B) C3 surface levels on splenic cDC1s and cDC2s after C/D digestion (20 min 21°C) of pooled spleen tissues from WT and *Marchf1*^{-/-} x *C3*^{-/-} [50:50] compared to the corresponding FMO control. For the FMO control in (A) and (B) cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)].

Overall this data clearly validates the observation of enriched C3 surface expression in MARCHF1-deficient cDCs, initially discovered through mass spectrometry and proteomic analyses. Furthermore, we have identified moderate C3 deposition on the surface of WT cDCs, different C3 surface expression levels between *Marchf1*^{-/-} cDC1s and cDC2s and the molecular weight of various C3 fragments present in *Marchf1*^{-/-} cDCs. We also excluded collagenase/DNase I digestion during the preparation of DC suspensions as an artificial or induced cause for C3 activation/deposition.

3.2.2 Source of C3 deposited on *Marchf1*^{-/-} cDCs

Next we sought to identify the source of C3 accumulated on the surface of *Marchf1*^{-/-} cDCs. Circulating C3 is predominantly produced by hepatocytes in the liver and secreted into the circulation resulting in high blood concentration (~130 mg/dl) [565][566]. However, it has been shown that various hematopoietic immune cells, including mast cells, macrophages, T cells and also DCs, can produce C3 themselves (reviewed [632]). In order to assess if the C3 accumulated on the surface of *Marchf1*^{-/-} cDCs was produced by the themselves (originated from hematopoietic cells) or derived from the non-hematopoietic compartment (i.e. hepatocytes) we generated mixed bone marrow (BM) chimeras where *C3*^{-/-} recipient mice were reconstituted with a 1:1 mix of WT and *Marchf1*^{-/-} BM. In these mice, cDCs derived from neither WT nor *Marchf1*^{-/-} BM displayed any C3 on their surface (**Figure 3.4**), indicating the C3 observed on the surface of WT or MARCHF1-deficient cDCs was not produced by the cells themselves but captured from the extracellular environment.

Although immunoblotting for C3 from serum samples of WT and *Marchf1*^{-/-} mice indicated no major differences in the composition of serological C3 (**Figure 3.2B**), *Marchf1*^{-/-} mice might happen to systemically produce or retain larger amounts of C3 or constitutively activate C3 at a higher rate in the circulation. In order to explore if enriched C3 accumulation on cDCs in *Marchf1*^{-/-} mice was systemic and due to cell-extrinsic mechanisms or directly associated with the lack of MARCHF1 within cDCs (cell-intrinsic), we generated mixed BM chimeras where a 1:1 mix of WT and *Marchf1*^{-/-} BM was used to reconstitute WT recipients. In these mice *Marchf1*^{-/-} cDCs had high levels of C3 on their surface (**Figure 3.4**) indicating the cause for C3 deposition was cell-intrinsic, independent of whether the non-hematopoietic compartment was deficient or sufficient in MARCHF1 expression.

Furthermore, this result also confirmed that the difference in C3 detection on WT and

Marchf1^{-/-} DCs was not caused by artifacts introduced during cell preparation or staining, such as C/D digestion but occurs genuinely *in vivo* [see section 3.2.1], as the two cell types were obtained from the same tissue. The conclusion of these experiments is that, in WT mice cDCs deposit C3 of extracellular origin on their surface, and if the cells do not express MARCF1 the amount of C3 that accumulates on their surface increased.

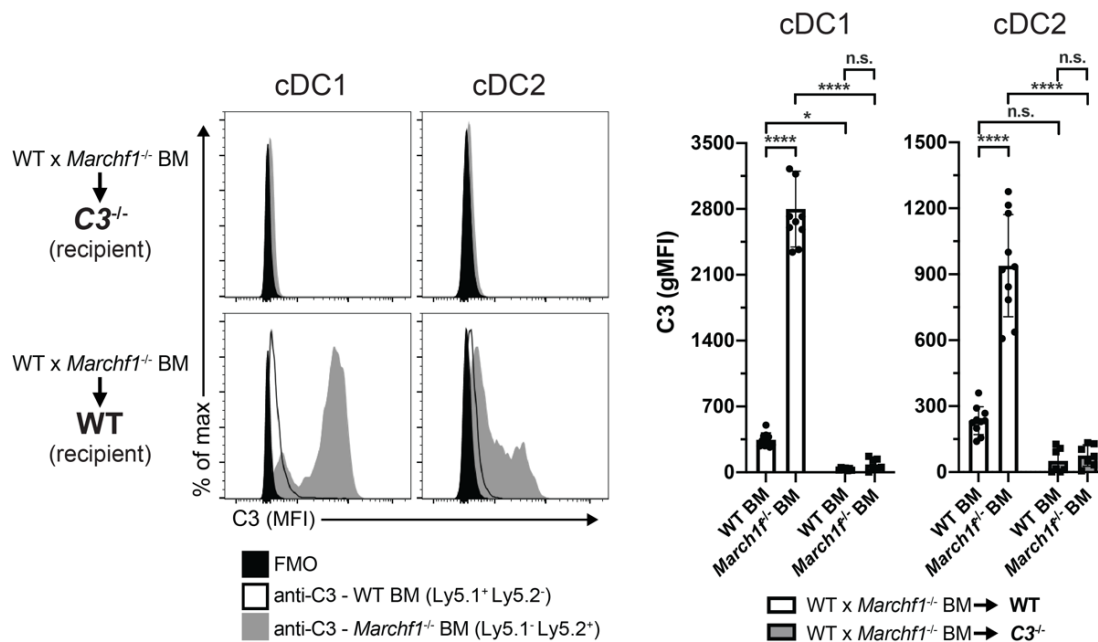


Figure 3.4 C3 surface levels on cDCs from mixed bone marrow chimera mice. C3 surface levels of WT and *Marchf1*^{-/-} cDC1s and cDC2s from mixed bone marrow (BM) chimera WT or *C3*^{-/-} recipient mice, reconstituted with a 1:1 mix of WT (Ly5.1⁺ Ly5.2⁻) and *Marchf1*^{-/-} BM (Ly5.1⁻ Ly5.2⁺) compared to the corresponding FMO control, for which cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)]. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, and bars designating mean $\pm$ SD. Two-way ANOVA followed by Sidak's multiple comparisons test: n.s.: not significant; $P < 0.0332$ (*), < 0.0001 (****).

3.2.3 MHC II as primary substrate for covalent association of C3 in cDCs

Complement receptors and regulators

Although MARCF1-mediated ubiquitination of complement receptors (CRs) has not been described in the literature, a potential explanation for the deposition of C3 on cDCs is that MARCF1 regulates the surface expression of a known C3 binding receptor. There are four major CRs, namely complement receptor 1-4 (CR1-4), all known to bind various fragments of C3 (table in **Figure 3.5**) [674]. We therefore compared the surface levels of CR1-4 as well as

the complement regulators CD55 and Factor H in splenic WT and *Marchf1*^{-/-} cDCs (**Figure 3.5**). While there was no notable difference in the surface expression levels of the components of both complement receptors CR3 (CD11b+CD18) and CR4 (CD11c+ CD18) for cDC1s, *Marchf1*^{-/-} cDC2s showed slightly reduced levels of CD18 (part of CR4). cDC1 lacking MARCF1 exhibited enriched CR1 and CR2 (CD35 and CD21, respectively) and elevated surface levels of CD55 and Factor H (**Figure 3.5**). The membrane bound CD55, also known as complement decay-accelerating factor (DAF), and the soluble Factor H are both complement regulators inhibiting downstream complement activation by preventing the formation of the C3 convertase [596] and assisting the cleavage of activated surface-bound C3 (C3b) [593] [see section 1.5.1 on page 56].

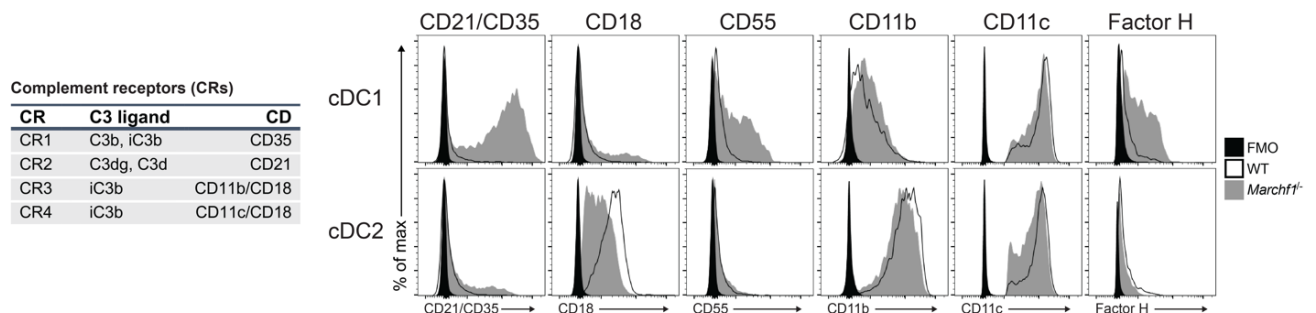


Figure 3.5 Surface expression of complement receptors and regulators in WT and *Marchf1*^{-/-} cDCs. Flow cytometry analysis of splenic cDC1s and cDC2s purified from WT and *Marchf1*^{-/-} mice detecting surface levels of complement receptor CR1-4 as well as complement regulator CD55 and Factor H compared to the corresponding FMO control.

MHC II surface expression strictly correlates with C3 deposition

Elevated surface levels of the inhibitory complement regulators CD55 and Factor H in *Marchf1*^{-/-} cDCs might be a consequence, not a cause of/enhanced C3 deposition in these cells in order to dampen downstream effects of complement activation. Enriched surface expression of CR1 and CR2 was indeed a potential reason for enriched C3 deposition. However, given the outcome of the proteomic screen, that showed MHC II and CD86 were the only strongly increased proteins in the PM of *Marchf1*^{-/-} cDCs (apart from C3 itself) [see section 3.1.2], we investigated the alternative possibility that enriched C3 deposition at the surface of *Marchf1*^{-/-} cDCs was due to high levels of surface MHC II.

For this we generated *Marchf1*^{-/-} mice that also lacked expression of surface MHC II (i.e. *Marchf1*^{-/-} x *I-Aa*^{-/-} mice). While cells from *I-Aa*^{-/-} mice still can express the β chain of MHC II (I-Aβ), deficiency in MHC IIα (*I-Aa*^{-/-}) causes failure in assembly and intracellular trafficking of intact MHC II complexes, causing a complete lack of MHC II surface expression (**Figure 3.6A**). Surface expression of CD86 in cDCs from *Marchf1*^{-/-} x *I-Aa*^{-/-} mice was as high as in *Marchf1*^{-/-} cDCs (**Figure 3.6A**), indicating removal of MHC II surface expression did not affect surface accumulation of other receptors regulated by MARCHF1 ubiquitination. Strikingly, C3 was barely detectable on the surface of both MARCHF1-deficient cDC subsets when these cells also lacked surface MHC II (*Marchf1*^{-/-} x *I-Aa*^{-/-}) with similar gMFI values to the *C3*^{-/-} and FMO control (**Figure 3.6B**). Notably, the signal for C3 deposition on *Marchf1*^{-/-} x *I-Aa*^{-/-} cDCs was below the one of WT cDCs (**Figure 3.6B**), indicating that C3 associates to MHC II on the surface of WT cDCs, and the number of these MHC II-C3 complexes increased significantly when MHC II could not be ubiquitinated.

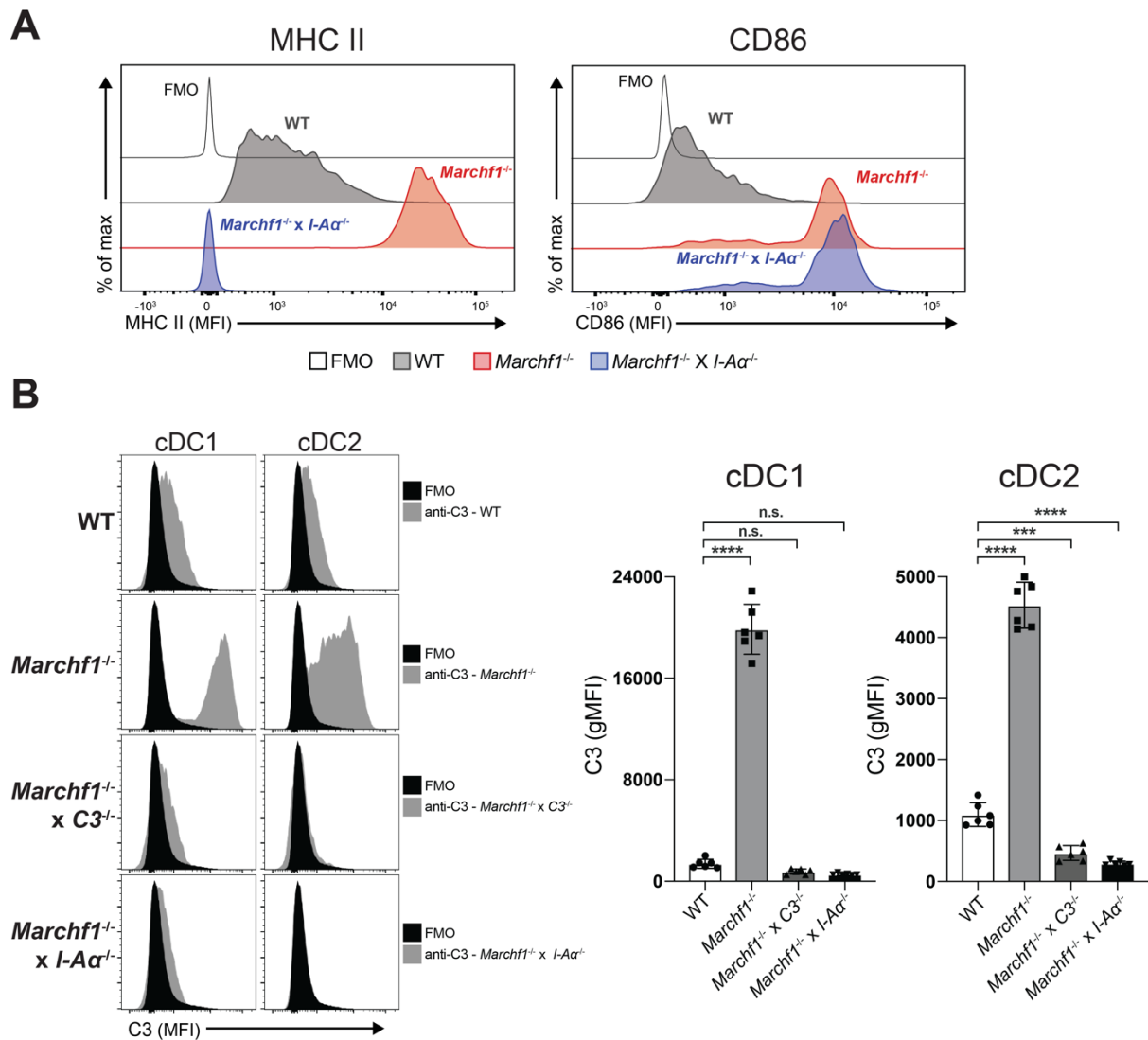


Figure 3.6 Enriched MHC II surface levels in MARCHF1-deficient cDCs leads to increased C3 deposition. (A) Expression levels of surface MHC II and CD86 in splenic cDC1s purified from WT, *Marchf1*^{-/-} and *Marchf1*^{-/-} x *I-A α* ^{-/-} mice compared to the corresponding FMO control. (B) Expression levels of surface C3 on splenic cDC1s and cDC2s purified from WT, *Marchf1*^{-/-}, *Marchf1*^{-/-} x *C3*^{-/-} and *Marchf1*^{-/-} x *I-A α* ^{-/-} mice compared to the corresponding FMO control, for which cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)]. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, bars designate mean $\pm$ SD. One-way ANOVA followed by Dunnett's multiple comparisons test with WT as control, n.s.: not significant; $P < 0.001$ (**); $P < 0.0001$ (****).

To further validate this conclusion, we analyzed cDCs from mice where MHC II β has been replaced with a mutant form that lacks the only Ub acceptor residue (i.e. lysine K225) in the complex of MHC II (MHC II K225R^{KI/KI} mice) [535]. MARCHF1 is functional in cDCs from these mice, as shown by their WT expression of CD86, but as they do not ubiquitinate MHC II, their surface expression of MHC II is as high as in *Marchf1*^{-/-} cDCs (**Figure 3.7A**). The lack of MHC II ubiquitination in cDCs from these mice was sufficient to cause enriched C3 surface signals

comparable to that observed on *Marchf1*^{-/-} cDCs (**Figure 3.7B**) indicating that the increase of MHC II surface expression, not the loss of MARCHF1 per se, was the reason for enriched C3 deposition.

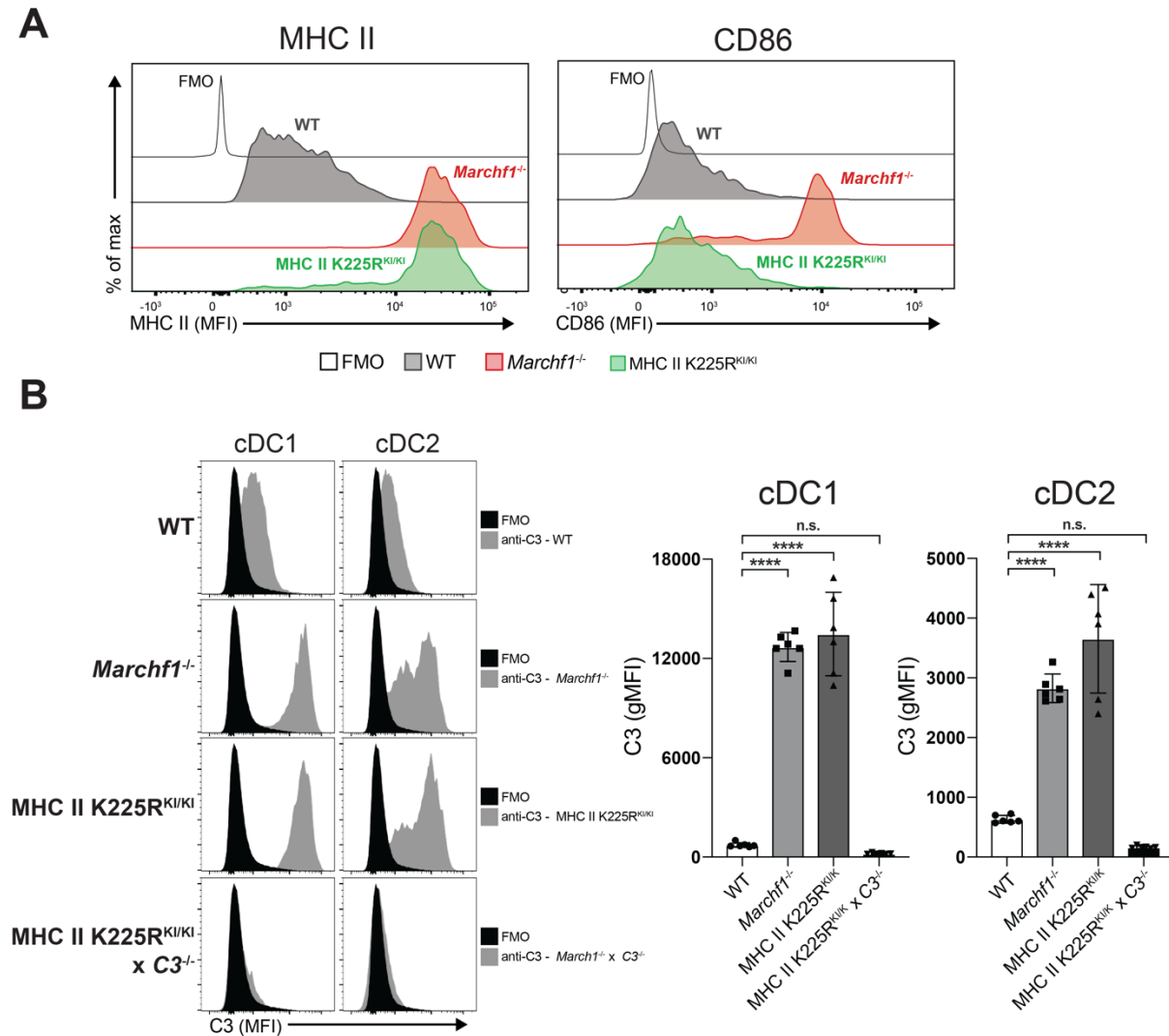


Figure 3.7 The lack of MHC II ubiquitination leads to enriched MHC II surface levels and C3 deposition in cDCs. (A) Expression levels of surface MHC II and CD86 in splenic cDC1s purified from WT, *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice compared to the corresponding FMO control. (B) Expression levels of surface C3 on splenic cDC1s and cDC2s purified from WT, *Marchf1*^{-/-}, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x *C3*^{-/-} mice compared to the corresponding FMO control, for which cells were incubated with streptavidin-APC only [minus biotin, anti-C3 mAb (clone 11H9)]. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, bars designate mean $\pm$ SD. One-way ANOVA followed by Dunnett's multiple comparisons test with WT as control, n.s.: not significant; $P < 0.0001$ (****).

Ubiquitination of MHC II through MARCF1 requires the coordinated actions of various proteins. Besides the actual E1, E2 and MARCF1 E3 Ub ligases, it is thought that further proteins assist the ubiquitination process. A genome-wide CRISPR knockout screen in our group has identified ubiquitin like protein 3 (UBL3) as a new participant in MARCF1-mediated ubiquitination with increased surface expression of MHC II and CD86 in splenic *Ubl3*^{-/-} cDCs [manuscript in preparation]. Although the exact mechanism of action of UBL3 in the MARCF1 ubiquitination machinery remains unsolved, we investigated if enriched MHC II surface expression in splenic *Ubl3*^{-/-} cDCs leads to enriched C3 accumulation on their surface, similar to cDCs from *Marchf1*^{-/-} or MHC II K225R^{KI/KI} mice.

As expected, surface MHC II and CD86 was enriched for *Ubl3*^{-/-} cDCs. The absence of *Ubl3* elicited similar elevation in surface MHC II but less in surface CD86 compared to *Marchf1* deletion (**Figure 3.8A**), indicating a role for Ubl3 haemopoietic cell regulation of surface MHC II and CD86. C3 signals were enriched in UBL3-deficient cDCs compared to WT cDCs, confirming that elevated surface levels of MHC II caused enriched C3 deposition at the cell surface. Similar to *Marchf1*^{-/-} cDCs, UBL3-deficient mice showed higher C3 signals on cDC1s than on cDC2s, though signals were not as elevated as in *Marchf1*^{-/-} cDCs, in particular for cDC2s (**Figure 3.8B**) although MHC II surface expression was similarly high across both mutants.

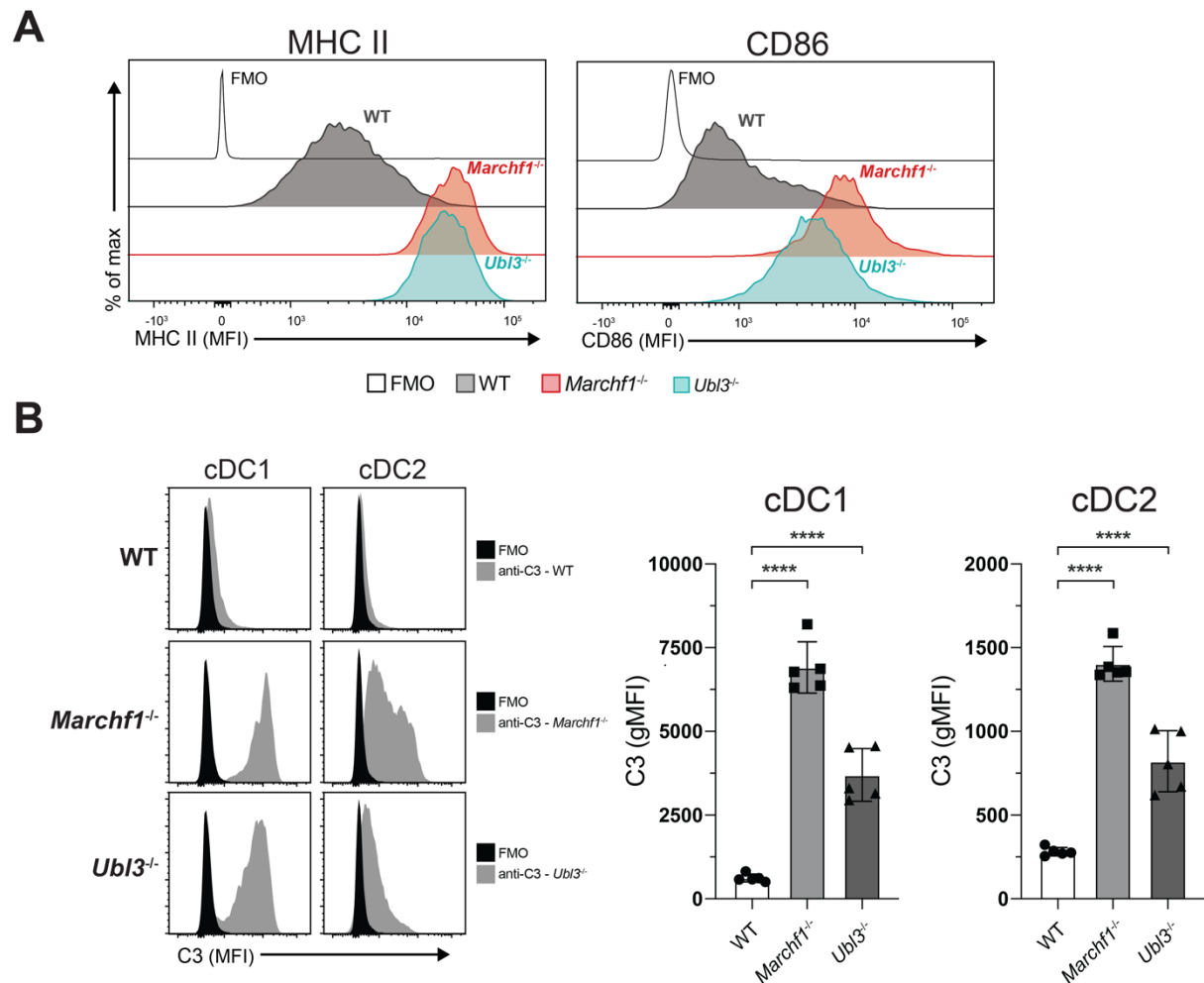


Figure 3.8 Enriched MHC II surface levels in UBL3-deficient cDCs leads to increased C3 deposition. (A) Expression levels of surface MHC II and CD86 in splenic cDC1s purified from WT, *Marchf1*^{-/-} and *Ubl3*^{-/-} mice compared to the corresponding FMO control. (B) Expression levels of surface C3 on splenic cDC1s and cDC2s purified from WT, *Marchf1*^{-/-} and *Ubl3*^{-/-} mice compared to the corresponding FMO control, for which cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)]. Graphs display symbols representing an individual mouse, bars designate mean $\pm$ SD. One-way ANOVA followed by Dunnett's multiple comparisons test with WT as control, $P < 0.0001$ (****).

MHC II-dependent C3 binding to WT cDCs was indicated by the loss of C3 signal in *Marchf1*^{-/-} cDCs that also lacked MHC II expression (*Marchf1*^{-/-} $\times$ *I-A α* ^{-/-}), which was below the C3 gMFI values of WT cDCs (Figure 3.6B). To further validate that C3 binding occurs in WT cDCs where MHC II levels are not artificially elevated, we compared C3 surface expression in WT versus *I-A α* ^{-/-} and *C3*^{-/-} cDCs (Figure 3.9). WT cDC1s displayed small but detectable C3 signals that were significantly reduced for *I-A α* ^{-/-} and absent for *C3*^{-/-} cDC1s. For cDC2s, the trend was similar although the overall C3 signal was much weaker for this DC subset (Figure 3.9). This suggests that C3 binds to the surface of cDCs even under scenarios where MHC II is expressed

at wild-type levels. *I-A α* ^{-/-} cDC1s still exhibited some C3 signal which indicated C3 binding to the cell surface in a MHC II-independent manner. However, in conclusion we have demonstrated that C3 associates with MHC II on the surface of cDCs, with increased complex formation in case MHC II can not be ubiquitinated.

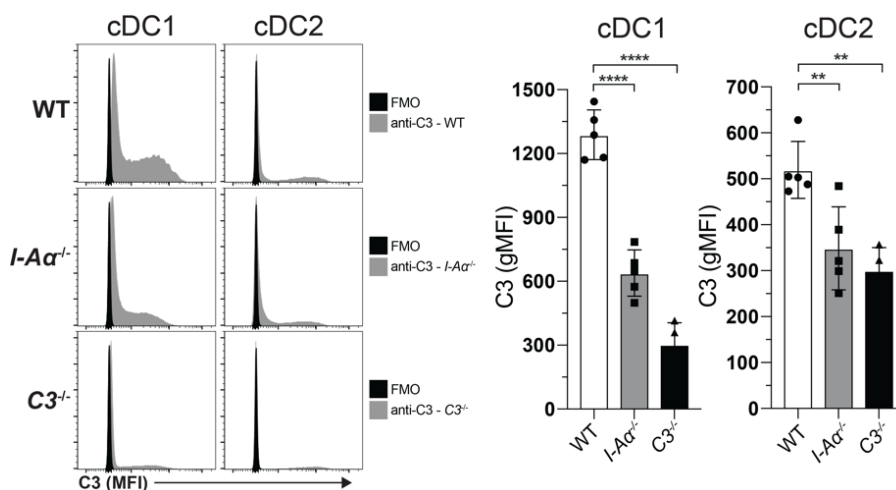


Figure 3.9 MHC II-dependent C3 deposition on the surface of WT cDCs. Expression levels of surface C3 on splenic cDC1s and cDC2s purified from WT, *I-A α* ^{-/-} and *C3*^{-/-} mice compared to the corresponding FMO control, for which cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)]. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, bars designate mean $\pm$ SD. One-way ANOVA followed by Dunnett's multiple comparisons test with WT as control, n.s.: not significant; $P < 0.0021$ (**); $P < 0.0001$ (****).

Covalent binding of C3 to MHC II

Next we sought to characterize the biochemical properties of the MHC II-dependent C3 deposition on the surface of cDCs lacking ubiquitination of MHC II. C3 is the central component of the complement system as all three activation pathways converge in its activation causing a conformational change that reveals a thioester group. This enables the covalent binding to hydroxyl nucleophiles on cell surfaces forming a covalent thioester bond between C3 and its target [584][585][591].

In order to address the question if C3 binds covalently to MHC II via its thioester group we immunoprecipitated MHC II complexes ($\alpha\beta$ heterodimer via the mAb clone M5/114) from cDC1s purified from Flt3L-treated mice that were WT, MHC II K225R^{KI/KI} or deficient in MARCF1, MHC II (*I-A α*), C3 or both MARCF1 and C3, and detected MHC II α , MHC II β , and C3 by WB after denaturing and reducing SDS-PAGE (**Figure 3.10A**). Immuno-detection using anti-*I-A α* - and anti-*I-A β* -specific pAbs from rabbit serum JV1 and JV2, respectively detected

the ~30-35 kDa alpha and beta chain of MHC II in all samples except for *I-A α* ^{-/-}, that exhibited no signal for MHC II α (JV1) and a reduced signal for MHC II β (JV2). Both of the MHC II signals (anti-I-A α - and anti-I-A β) were stronger in the immunoprecipitates from cells that lacked MHC II ubiquitination (i.e. *Marchf1*^{-/-} and MHC II K225R^{KI/KI}) due to disrupted turn-over and enriched surface expression of MHC II complexes, although not only surface but also intracellular MHC II was immunoprecipitated. Interestingly, a ~65-70 kDa protein that reacted with the anti-I-A α (JV1) and anti-C3 (HP8012) Ab, but not with the anti-I-A β (JV2) Ab, was identified in the immunoprecipitates from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} cDC1s but not from any other sample (**Figure 3.10A**). This indicated a covalently linked protein construct between C3 and MHC II α in cDC1s with constitutively high surface levels of MHC II.

Mass spectrometry analysis of the ~65-70 kD protein extracted from the SDS-PAGE gel lane loaded with the MHC II immunoprecipitates from *Marchf1*^{-/-} cDC1s confirmed the presence of complement C3, which was absent from equivalent gel regions of the lanes loaded with samples from *C3*^{-/-} or *Marchf1*^{-/-} x *C3*^{-/-} cDC1s (**Table 3.1**). All detected C3 peptides in this mass spectrometry approach derived from the amino acid region 960-1171 of the C3 protein, suggesting that the C3 fragment bound to MHC II α was C3dg and/or C3d (**Table 3.1**). This was further verified through re-analyses of the data from the initial proteomic screen comparing of the PM sub-proteomes of WT and *Marchf1*^{-/-} cDCs [see section 3.1.2], which revealed that 25 out of 27 detected C3 peptides derived from the region of C3d/C3dg (**Table 3.2**).

C3dg and C3d are the last two C3 fragments of its successive cleavage cascade through Factor I, both retaining the thioester that is relevant for the covalent binding to cell surfaces. The MW of C3dg and C3d is ~39kDa and ~34kDa, respectively while MHCII α is ~30kDa [see Figure 1.5 and section 1.5.2 on page 62]. A complex between these two proteins would migrate at ~65 and ~70kDa, respectively which is indeed the approximate MW range in which this protein was detected. The appearing double-band for the C3-MHC II α complex (~65-70kDa) in the detection with the anti-I-A α pAb (JV1) (**Figure 3.10A**) might correspond to C3d and C3dg bound to MHC II α , while the anti-C3 pAb only detected only one of these complexes (C3d-MHC II α or C3dg-MHCII α). Of note, the proportion of MHC II α that was associated with C3 was small, as indicated by the weak signal rendered in the WB with the anti-I-A α pAb (JV1) by the ~65-70 kDa I-A α -C3 complex compared to that produced by free (~30-35 kDa) I-A α . Furthermore, the ~65-70 kDa C3dg/C3d-MHC II α protein construct was most likely detected before in the WB from the straight cell lysates of *Marchf1*^{-/-} cDC1s (without

immunoprecipitation of MHC II), in the validation process of enriched C3 deposition (**Figure 3.2D**). It was the only detected C3 species that did not correspond, based on size, to the C3 fragments detected in the serum samples.

In order to validate our conclusion, we reversed the immunoprecipitation/WB experiment and immunoprecipitated C3 (via mAb 3d29) from WT, *Marchf1*^{-/-} and *C3*^{-/-} cDC1s from Flt3L-expanded mice with subsequent detection of MHC II α and C3 (**Figure 3.10B**). Again, only in the immunoprecipitates from *Marchf1*^{-/-} cDC1s a protein construct of ~65-70 kDa was detected with both an anti-C3 and anti-I-A α Ab, confirming the presence of a C3-MHC II α protein construct. Interestingly, several C3 fragments in the range of >100 kDa were detected in the immunoprecipitates from *Marchf1*^{-/-} cDC1s but not associated to MHC II (**Figure 3.10B**), indicating MHC II-independent binding/presence of larger C3 fragments in MARCF1-deficient cDCs.

Overall, we therefore concluded that cDCs accumulate MHC II-C3dg/C3d complexes on their cell surface, with higher amounts of these complexes when surface expression of MHC II was enriched due to the lack of its ubiquitination.

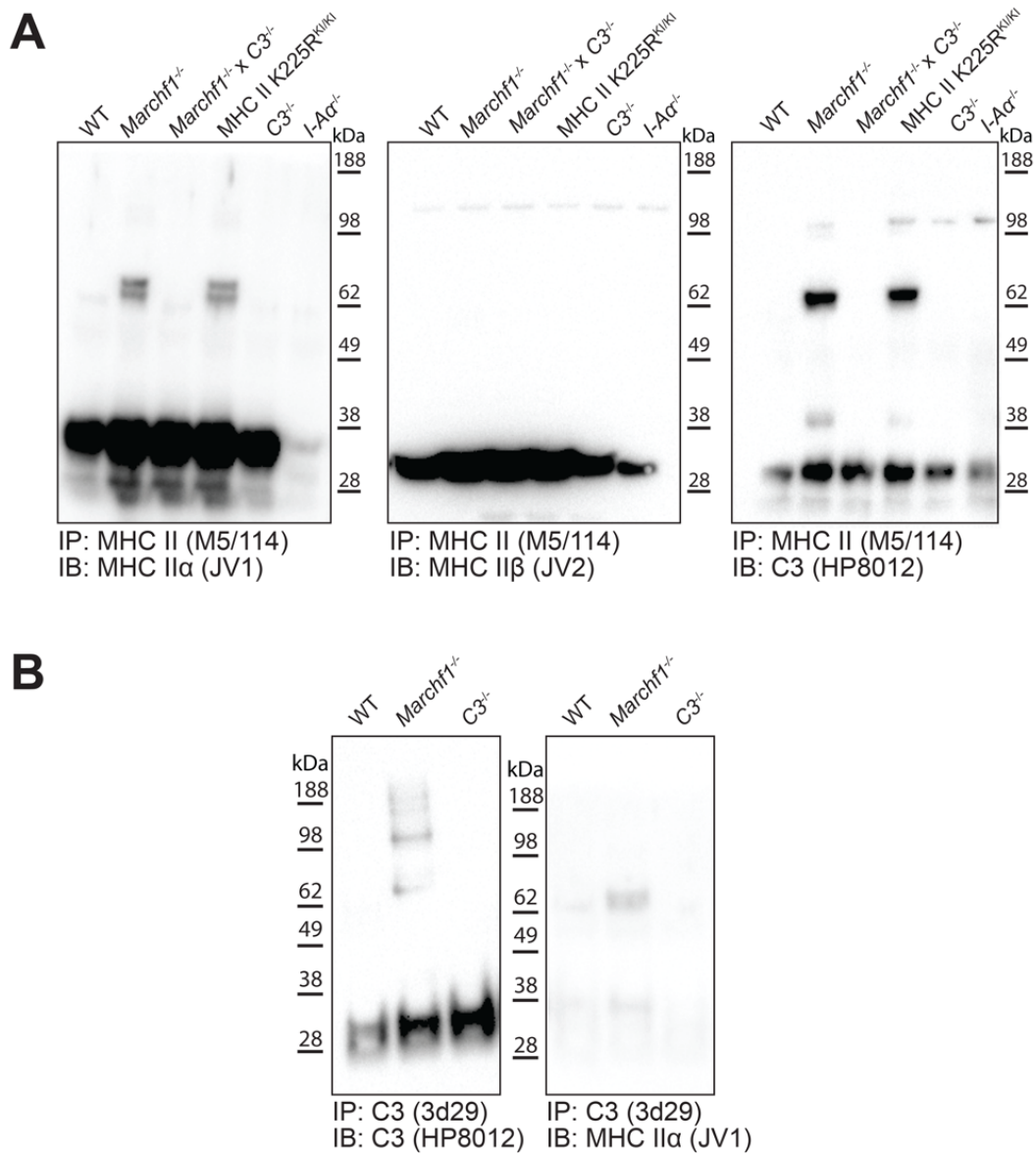


Figure 3.10 Immunoprecipitation of C3 and MHC II from cDCs with subsequent detection of MHC II and C3 via WB. (A) Western blot analysis after immunoprecipitation (IP) of MHC II from cell lysate of cDC1s purified from Flt3L-treated WT, *Marchf1*^{-/-}, *Marchf1*^{-/-} × *C3*^{-/-}, MHC II K225R^{KI/KI}, *C3*^{-/-} and *I-Aα*^{-/-} mice. Immunoprecipitated fractions were analyzed by denaturing and reducing SDS-PAGE with subsequent immuno-detection of MHC IIα [left], MHC IIβ [middle] or C3 [right]. **(B)** Western blot analysis after immunoprecipitation (IP) of C3 from cell lysate of cDC1s purified from Flt3L-treated WT, *Marchf1*^{-/-} and *C3*^{-/-} mice followed by immuno-detection of C3 [left] or MHC IIα [right] upon (reducing & denaturing) SDS PAGE. Western blots for **(A)** and **(B)** are representative of at least two independent experiments.

Table 3.1 C3 peptides detected by MS upon immunoprecipitation of MHC II from *March1*^{-/-} cDC1 + extraction of gel lane (~65-70 kDa region) of SDS PAGE.

#	peptide	AA position	C3 fragment	charge	modification
1	VDVPAADLSDQVPDTSER	960-979	C3g	2;3	Unmodified
2	IILQGSPVVQMAEADVDGER	980-999	C3g	3	Unmodified
3	RQEALIELIK	1042-1050	C3d	3	Unmodified
4	RQEALIELIKK	1042-1051	C3d	3	Unmodified
5	QEALIELIK	1043-1050	C3d	2	Unmodified
6	QEALIELIKK	1043-1051	C3d	2;3	Unmodified
7	KGYTQQLAFK	1051-1060	C3d	3	Unmodified
8	GYTQQLAFK	1052-1060	C3d	2	Unmodified
9	VFSLAANLIAIDSHVLCGAVK	1085-1105	C3d	3	Unmodified
10	DICEGQVNSLPGSINK	1156-1171	C3d	2	Unmodified

Table 3.2 C3 peptides detected by MS upon subcellular fractioning of *March1*^{-/-} cDC PM (from previous MS analysis, see section 3.1.2).

#	peptide	AA position	C3 fragment	charge	modification
1	IFTVDNLLPVGK	150-162	C3 beta chain	2	Unmodified
2	VVIEDGVGDAVLTR	292-305	C3 beta chain	2	Unmodified
3	VDVPAADLSDQVPDTSER	960-979	C3g	3; 2	Unmodified
4	DVPAADLSDQVPDTSER	961-979	C3g	3; 2	Unmodified
5	IILQGSPVVQMAEADVDGER	980-999	C3g	3; 2	Unmodified
6	GSPVVQMAEADVDGER	984-999	C3g	2	Unmodified
7	SPVVQMAEADVDGER	985-999	C3g	3; 2	Unmodified
8	VVQMAEADVDGER	987-999	C3g	2	Unmodified
9	MAEADVDGER	990-999	C3g	2	Unmodified
10	QEALIELIK	1043-1050	C3d	2	Unmodified
11	KGYTQQLAFK	1051-1060	C3d	2	Unmodified
12	GYTQQLAFK	1052-1060	C3d	2	Unmodified
13	SSAYAAFNNRPPSTWLTAYVVK	1063-1084	C3d	3	Unmodified
14	VFSLAANLIAIDSHVLCGAVK	1085-1105	C3d	3	Unmodified
15	QKPDGVFQEDGPVIHQEMIGGFR	1112-1134	C3d	4; 3	Unmodified, Gln->pyro-Glu
16	EADVSLTAF	1138-1146	C3d	3; 2	Unmodified
17	EADVSLTAFVLIALQEAR	1138-1155	C3d	2	Unmodified
18	RQEALIELIK	1142-1050	C3d	3; 2	Unmodified
19	VLIALQEAR	1147-1155	C3d	2	Unmodified
20	DICEGQVNSLPGSINK	1156-1171	C3d	2	Unmodified
21	AGEYIEASYMNLQR	1172-1185	C3d	2	Unmodified
22	PYTVAIAGYALALMNK	1186-1201	C3d	3; 2	Unmodified
23	TVAIAGYALALMNK	1188-1201	C3d	3; 2	Unmodified
24	LEEPYLGK	1202-1209	C3d	2	Unmodified
25	ALLALLLK	1237-1244	C3d	2	Unmodified
26	DFDSVPPVVR	1245-1254	C3d	2	Unmodified
27	DLNMDVSFHLPSR	1291-1303	C3d	3	Unmodified

C3 binding to MHC II α via its carbohydrate

Considering activated C3 binds covalently to any exposed hydroxyl nucleophiles through its revealed thioester, it has been described as an opsonin with broad-spectrum binding activity [585]. However, various studies have indicated preferences in binding of C3 to specific carbohydrates of certain proteins, such as of itself, complement C4 and immunoglobulins [675][676][677][678]. Our results of the immunoprecipitation experiments clearly demonstrated the covalent attachment of C3 specifically to MHC II α but not to its β chain. Both polypeptides of MHC II are glycosylated and human as well as mouse MHC II α exhibits a conserved asparagine at position 118 (Asn¹¹⁸) that functions as an acceptor site for *N*-glycosylation [679]. We therefore examined the possibility that C3 binding to MHC II α occurs via its extracellular N-linked glycan chain, for which we immunoprecipitated the MHC II-C3 complex from Flt3L-expanded cDCs of WT, *Marchf1*^{-/-} or MHC II K225R^{KI/KI} mice and digested the immunoprecipitates with PNGase F (Peptide:N-glycosidase F) before detection of MHC II α and C3 via WB (**Figure 3.11**). PNGase F is an amidase that effectively removes carbohydrates from glycoproteins through the specific hydrolytic cleavage of asparagine (Asn)-linked oligosaccharides with two or more sugar moieties [680][681][682].

As observed previously, immunoprecipitated MHC II (via mAb M5/114) with subsequent immunoblotting detected a protein construct of ~65-70 kDa from only MARCF1-deficient cDCs by both anti-MHC II α (JV1) and C3 (HP8012) pAbs. PNGase F treatment of immunoprecipitated MHC II caused a mass shift of approximately 6 kDa of the detected MHC II α chain in all samples (~35 to ~29 kDa). This was likely due to the cleavage of its N-linked glycosylation chain from the conserved asparagine. Interestingly, the ~65-70 kDa MHC II-C3 complex was no longer detected by the anti-I-A α pAb (JV1) upon PNGase F treatment (**Figure 3.11A**), indicating binding of C3dg/C3d (~39kDa and ~34kDa, respectively) to MHC II α via its N-linked glycan chain.

Detection of C3 (HP8013) associated with immunoprecipitated MHC II from MARCF1-deficient cDCs revealed a mass shift of several bands upon PNGase F treatment (**Figure 3.11B**). Because multiple mass shifts were observed the outcome of the PNGase F digestion in this setting was not clear. However presumably three bands reduced their MW by approximately 30 kDa (~110 to ~80 kDa, ~100 to ~70 kDa and ~70 to ~40 kDa). This suggested that several C3 cleavage fragments were covalently bound to the glycan chains of proteins captured in the immunoprecipitation of MHC II and removed after PNGase F digestion. Shifts

other than 30 kDa after PNGase F treatment might indicate C3 binding to glycosylation chains of other proteins than MHC II and/or binding of C3 fragments other than C3d/C3dg. The interpretation of this was further complicated by the fact that C3 itself is differentially glycosylated at multiple sites [683][684][685][686], which could be seen by the mass shift of the serological ~45 kDa C3 species upon PNGase F digestion (**Figure 3.11C**).

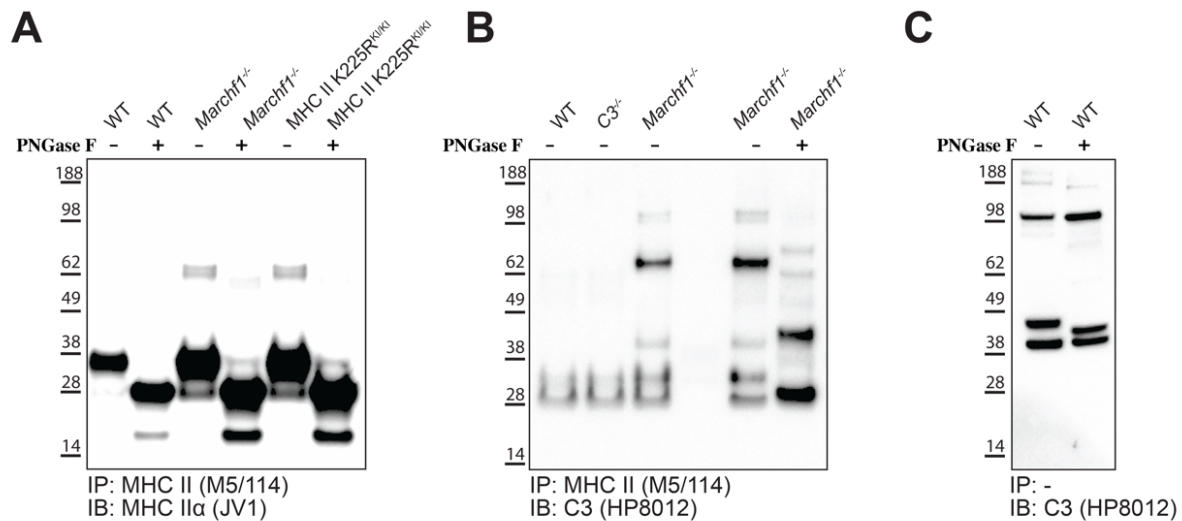


Figure 3.11 PNGase F digestion of immunoprecipitated MHC II-C3 complexes. (A) and (B) Immunoprecipitation (IP) of MHC II from cell lysates of cDC1s purified from Flt3L-treated WT, *Marchf1*^{-/-}, MHC II K225R^{KI/KI} or *C3*^{-/-} mice followed by incubation with and without PNGase F (+/-) and subsequent immuno-detection of (A) MHC IIα or (B) C3 by WB upon reducing & denaturing SDS-PAGE. (C) Immuno-detection of serological C3 via Western Blot from WT upon PNGase F digestion.

3.2.4 C3 deposition on other *Marchf1*^{-/-} immune cells

The biochemical characterization of enriched C3 deposition in *Marchf1*^{-/-} cDCs identified the covalent binding of activated C3 (i.e. C3d and C3dg) to the α chain of MHC II, specifically directed to its extracellular N-linked carbohydrate. Not only cDCs but also other antigen presenting cells (APCs), in particular B cells, macrophages and pDCs, express MHC II and regulate its intracellular trafficking and surface expression via MARCF1-mediated ubiquitination. We therefore followed up the question if accumulation of C3 is enriched at the surface of other MHC II expressing APCs when lacking MARCF1. For this we assessed MHC II and C3 surface levels side-by-side in cDCs, B cells, macrophages and pDCs as well as non-APCs, namely T cells and the granulocytes neutrophils and eosinophils, purified from the spleen of WT and *Marchf1*^{-/-} mice (**Figure 3.12**).

MARCF1-deficiency caused enriched MHC II surface expression in all splenic APCs (i.e. cDC1s, cDC2s, B cells, macrophages and pDCs) compared to their MARCF1-sufficient WT

counterpart, demonstrating an immune-regulative role for MARCHF1 in these cells. *Marchf1*^{-/-} B cells, cDC1s and cDC2s showed all similarly enriched MHC II surface levels, whereas macrophages and pDCs only displayed little increase in surface MHC II (**Figure 3.12A**). Interestingly, equally increased MHC II surface expression between MARCHF1-deficient cDC subsets and B cells did not lead to an equal increase in C3 surface levels across these cells, with cDC1s exhibiting the highest C3 surface deposition, followed by cDC2s (bimodal shape) and only little C3 signal in MARCHF1-deficient B cells. Macrophages and pDCs exhibited moderate increase in MHC II surface expression when lacking MARCHF1 but only little C3 deposition (**Figure 3.12A**).

Neither WT nor MARCHF1-deficient CD4⁺ and CD8⁺ T cells exhibited any significant MHC II or C3 surface levels, indicating no role for MARCHF1 in these cells and no MHC II-dependent C3 deposition. Low levels of MHC II were detected at the surface of spleen neutrophils and eosinophils in WT spleen which were considerably increased for *Marchf1*^{-/-} cells. Although the role in Ag presentation of these non-professional APCs (i.e. granulocytes) is controversially discussed in literature, more recent studies clearly have demonstrated their potential to induce Ag-specific T cell responses via MHC II-restricted presentation [687][688][689][690]. Conclusive to this, we here clearly have demonstrated that neutrophils and eosinophils regulate MHC II surface expression via MARCHF1-mediated ubiquitination [manuscript in preparation]. Surprisingly, C3 surface levels were considerably enriched in MARCHF1-deficient neutrophils and eosinophils when compared to WT cells, indicating MHC II-dependent C3 accumulation at their surface, though not to the same extent as in cDCs (**Figure 3.12B**).

While splenic cDC1s are considered as a homogenous subpopulation of cDCs, heterogeneity within the cDC2 subpopulation has been described with two main subgroups of cDC2s [191], based on their surface expression of CD4 [188], [691] and ESAM [189], [190] [ESAM^{hi}CD4⁺ versus ESAM^{lo}CD4⁻]. As the C3 expression profile (histogram) for splenic cDC2s exhibited a bimodal shape (**Figure 3.12A**), we assessed C3 surface deposition on splenic CD4⁺ versus CD4⁻ cDC2s (**Figure 3.12C**). While CD4⁺ cDC2s showed a more homogenous expression profile, CD4⁻ cDC2s retained a bimodal C3 histogram, indicating a homogeneously high C3 deposition in CD4⁺ cDC2s but heterogenous C3 accumulation in CD4⁻ cDC2s (**Figure 3.12C**).

Overall this data suggests that equally enriched surface expression of MHC II in APCs does not

cause equal levels of C3 accumulation across these cells and that C3 preferably binds to MHC II of cDCs, in particular of cDC1s.

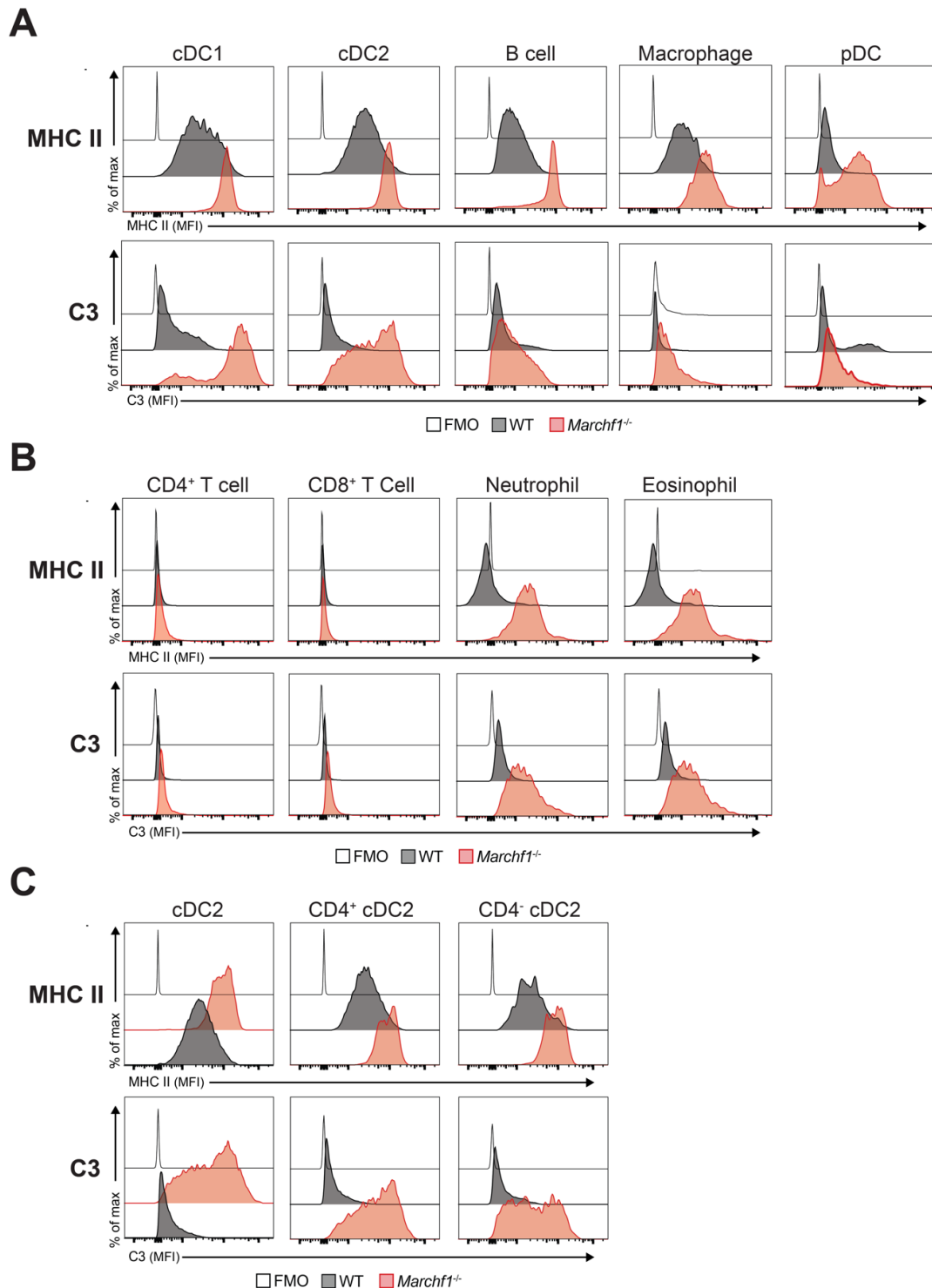


Figure 3.12 MHC II and C3 surface levels across various splenic immune cells from WT and *Marchf1*^{-/-} mice. (A)-(C) Flow cytometry analysis of surface expression of MHC II and C3 for (A) professional antigen presenting cells (i.e. cDC1s, cDC2s, B cells, macrophages and pDCs), (B) T cells (CD4⁺ and CD8⁺) and granulocytes (neutrophils and eosinophils) and (C) CD4⁺ and CD4⁻ cDC2s purified from the spleen from WT and *Marchf1*^{-/-} mice compared to the corresponding FMO control, for which in case of C3 detection cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)].

C3 is highly abundant in the circulation with a blood concentration of approximately 130mg/dl [565][566] from where it can reach peripheral tissues. Its localization and activation has been reported in various organs, including the intestine, lung, LN and kidney [692][693][694][695]. To gain a broader overview of the C3 deposition on cDCs in MARCHF1-deficient mice, we screened for C3 surface expression on cDCs from other tissues than the spleen including total lymph nodes (tLN), thymus, lung and liver (**Figure 3.13A**). While no increase in C3 levels between WT and *Marchf1*^{-/-} cDCs from liver and lung was detected, with only little C3 surface expression overall, lymphoid tissue cDCs from spleen, tLN and thymus all showed increased C3 surface expression when lacking MARCHF1. However, C3 accumulation was strongest on MARCHF1-deficient cDC1s in the spleen, followed by splenic cDC2s. This C3 staining pattern was similar in lymph node cDCs, though lower compared to splenic cDCs, while cDCs in the thymus showed lower C3 levels and similarly enriched signals between *Marchf1*^{-/-} cDC1s and cDC2s (**Figure 3.13A**).

C3 accumulation was highest of splenic *Marchf1*^{-/-} cDCs. As cDCs in the spleen are exclusively tissue-resident [155][184], we asked if C3 deposition varies between migratory and tissue-resident cDCs and therefore assessed C3 surface levels in cDCs from tLN of WT and *Marchf1*^{-/-} mice with differentiation of tissue-resident and migratory cells based on their MHC II and CD11c surface expression [155] (**Figure 3.13B**). Migratory WT cDC1s and cDC2s (CD4⁺ vs. CD4⁻) showed higher C3 surface levels when compared to their tissue-resident counterpart. Conversely, C3 expression was higher in tissue resident *Marchf1*^{-/-} cDC1s than in migratory cells, while no difference for cDC2s was observed (**Figure 3.13B**).

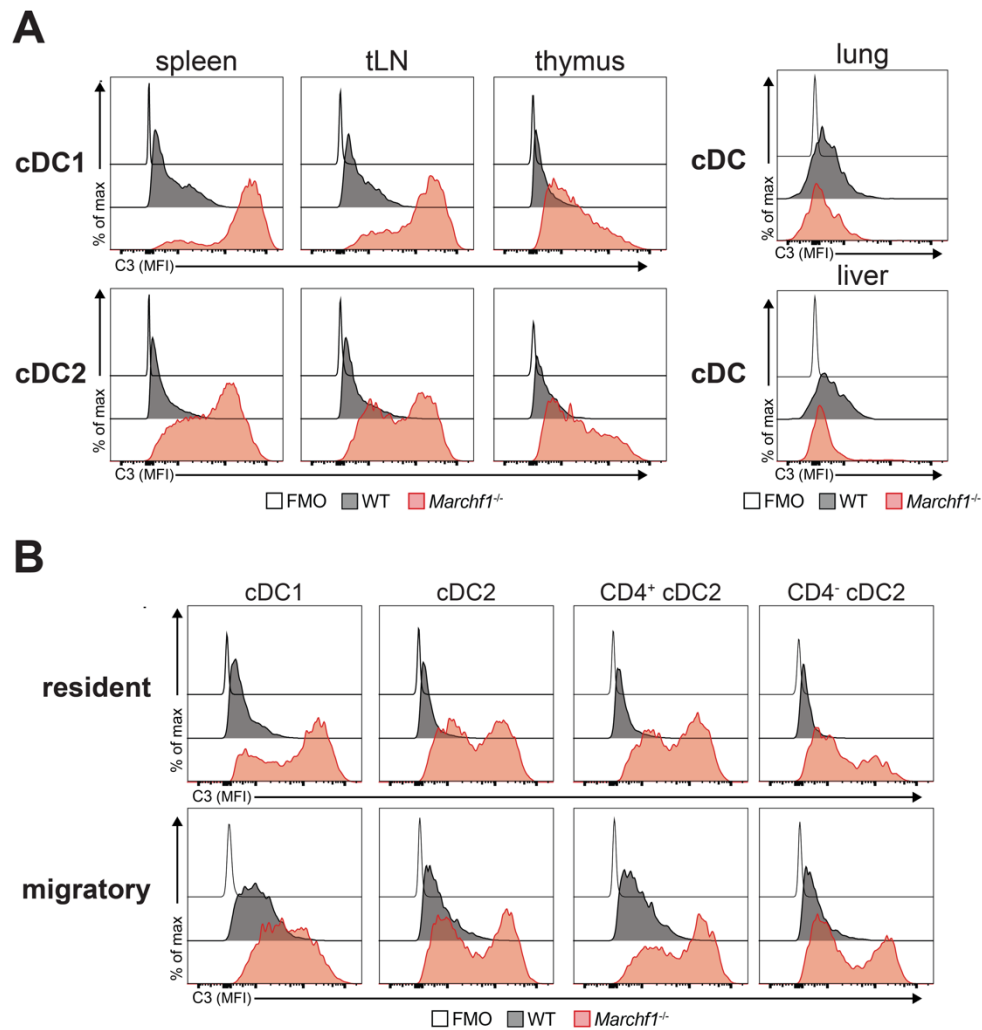


Figure 3.13 C3 surface levels in cDCs from different organs of WT and *Marchf1*^{-/-} mice. **(A)** Flow cytometry analysis of C3 surface expression in cDCs from spleen, total lymph nodes (tLN), thymus, lung and liver of WT and *Marchf1*^{-/-} mice. **(B)** C3 surface expression in tissue-resident and migratory cDC1s and cDC2s (CD4⁺ vs. CD4⁻ cDC2s) in tLN from WT and *Marchf1*^{-/-} mice. For the corresponding FMO controls in **(A)** and **(B)** cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)].

3.2.5 C3 binding to different murine MHC II haplotypes and human HLA

The genes for the different MHC molecules are arranged in clusters on chromosome 6 in humans (HLA genes) and on chromosome 17 in the mouse (H-2 or I genes). MHC molecules are highly polymorphic and the cluster region contains several loci and subclasses of each MHC gene. While there are more than 800 alleles for the human MHC I and MHC II genes in the human population, mouse strains used in laboratories are usually homozygous and have a unique MHC haplotype, designated by a small letter [696].

C3 binds to different murine MHC II haplotypes

The data previously shown were generated from primary cells isolated from C57BL/6 mice which express I-A^b MHC II molecules (and I-E^bα). To assess if C3 also binds to other murine MHC II haplotypes, we generated F2-backcrosses of MARCF1-deficient (C57BL/6 background) and BALB/C or C3H mice in order to obtain *Marchf1*^{-/-} cDCs that homozygously express I-A^d MHC II and I-A^k MHC II, respectively (**Figure 3.14**). Flow cytometry analysis verified that generated MARCF1-deficient C57BL/6, BALB/C and C3H mice exclusively expressed the I-A^b, I-A^d or I-A^k haplotype of MHC II, respectively (**Figure 3.14A**). MARCF1 deficiency in cDCs from all three mouse strains displayed similarly enriched C3 levels, indicating equal binding of C3 to I-A^b, I-A^d and I-A^k MHC II haplotypes (**Figure 3.14B**). This was expected since the I-A^b, I-A^d or I-A^k haplotypes share high sequence homologies and importantly all display the conserved asparagine for N-linked glycosylation, targeted by C3 binding.

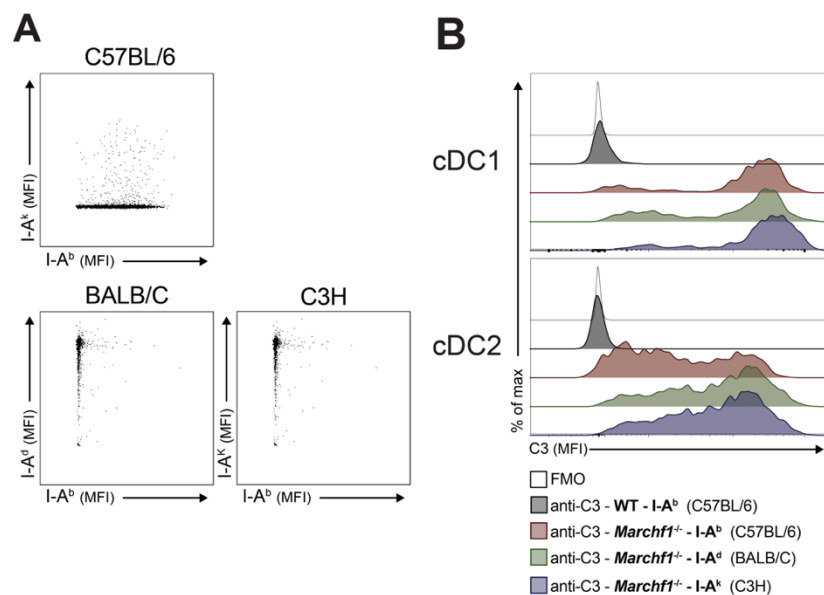


Figure 3.14 C3 surface deposition on splenic cDCs expressing different MHC II haplotypes. (A) Surface expression of MHC II haplotypes I-A^b, I-A^d and I-A^k in splenic cDCs from WT and *Marchf1*^{-/-} mice backcrossed to C57BL/6, BALB/C or C3H mice. (B) Expression levels of surface C3 on splenic cDC1s and cDC2s purified from WT and *Marchf1*^{-/-} expressing the MHC II haplotype I-A^b, I-A^d or I-A^k compared to the corresponding FMO control, for which cells were incubated with streptavidin-APC only [minus biotin. anti-C3 mAb (clone 11H9)].

C3 deposition on human blood cDCs

Next, we examined whether C3 binding to MHC II occurs at the surface of human DCs. Three DC subsets can be distinguished in human blood [226], [227]: {1} lineage (lin)⁻ CD11c^{int-high}

CD123⁻ CD141⁺ (cDC1s), {2} lin⁻ CD11c^{int-high} CD123⁻ CD141⁻ CD1c⁺ (cDC2s) and {3} lin⁻ CD11c^{int-high} CD11b⁻ CD123⁺ (pDCs), with lin defined as CD3⁺ CD14⁺ CD16⁺ CD19⁺ CD20⁺ and CD56⁺. In order to assess C3 deposition on these three cell types we purified DCs from healthy blood donors and HLA-deficient patients in two independent experiments. Both HLA-deficient patients had a 362A>T mutation in RFXANK, a molecule involved in MHC II transcription [697] and completely lacked HLA expression. PMBCs were enriched from whole blood samples via Ficoll density gradient centrifugation and subsequent lysis of red blood cells. DCs were then purified from PMBC suspensions through surface staining on non-DCs with biotinylated antibodies and subsequent negative selection via immunoaffinity using avidin-coated magnetic beads. Purified DCs were blocked with whole rabbit serum (1:50) and surface C3 was detected in a two-step staining with biotinylated rabbit anti-human C3 pAb followed by streptavidin-APC. Appropriate controls ensured specific C3 detection, including staining of DCs without primary anti-human C3 pAb (**Figure 3.15A**). Low, but significant surface C3 was detected on all DCs. The highest level was detected on cDC2s followed by pDCs and cDC1s. DCs from HLA-deficient patients displayed significantly reduced C3 expression on cDC2s but not on cDC1s or pDCs (**Figure 3.15B**).

This experiment has three limitations: {1} only two HLA-deficient donors were analyzed, {2} HLA-deficient donors and the healthy controls were not age-matched and {3} we cannot discard the possibility that reduced C3 deposition on the surface of HLA-deficient DCs was a secondary consequence of lack of HLA expression or therapeutic treatments aimed at improving the immunocompetence of the patients. Nevertheless, this data indicated that, like its mouse counterpart, human C3 can bind MHC II molecules on the surface of cDCs.

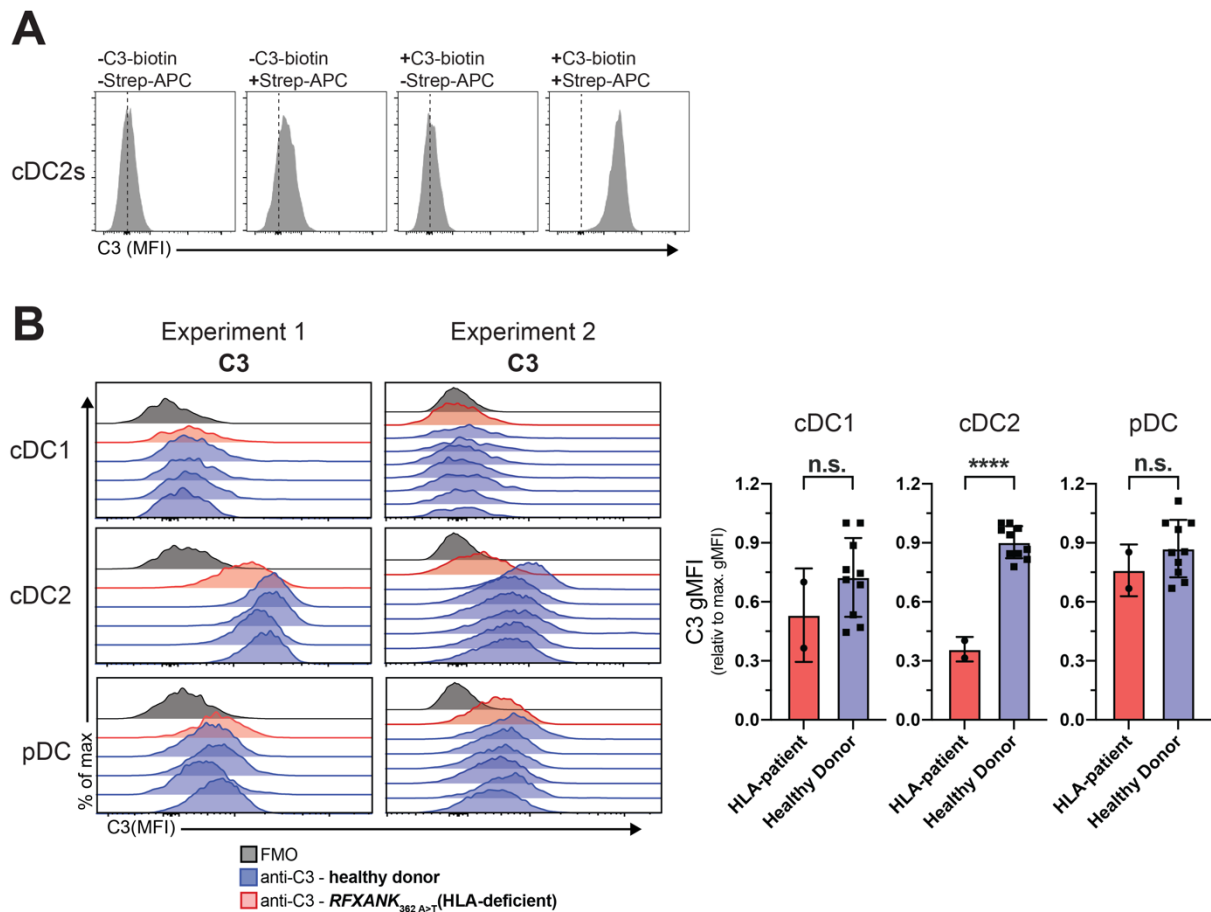


Figure 3.15 C3 deposition on human DCs in the blood from healthy donors and HLA-deficient patients. (A) Biotinylated anti-C3 pAb controls were conducted to ensure specific detection of C3 on human blood cDCs, including incubation of purified cells without any Ab [first histogram], with streptavidin-APC only [second histogram], with biotinylated anti-C3 pAb only [third histogram] or with both biotinylated anti-C3 pAb + streptavidin-APC [last histogram]. (B) C3 surface expression of DCs purified from blood samples of healthy donors [blue] and two different HLA-deficient patients [red] in two independent experiments. Cells were blocked with whole rabbit serum (1:50) and surface C3 detected with biotinylated rabbit anti-C3 pAb compared to FMO controls, for which cells were incubated with streptavidin-APC only (minus biotin. anti-C3 pAb). Graphs display pooled data (normalized to highest gMFI values) from the two experiments, with each symbol representing an individual blood sample, and bars designating mean $\pm$ SD. Two-way ANOVA followed by Sidak's multiple comparisons test: n.s.: not significant; <0.0001 (****).

3.2.6 MHC II-dependent C3 deposition *ex vivo* and *in vitro*

Incubation of primary DCs with C3-containing serum

In vitro complement activation and C3 deposition on cellular surfaces *ex vivo* has been demonstrated through the incubation of cells with serum samples, containing complement proteins [698][699][700][701]. We therefore examined if C3 accumulation is elevated on primary cDCs with high surface MHC II when incubated with serum. For this DCs with varying

surface MHC II and C3 levels from WT, *Marchf1*^{-/-} x *C3*^{-/-} and *Marchf1*^{-/-} mice were incubated in splenocyte cell suspensions with WT or C3-deficient mouse serum for 15–120 min and C3 surface expression in both cDCs and B cells was subsequently measured by flow cytometry (**Figure 3.16A**).

WT cDC1s and cDC2s showed only little increase in C3 surface levels when incubated with WT mouse serum in the time course of 15 to 120 min, whereas B cells quickly bound high amounts of C3 at their cell surface within the first 15 min but then steadily reduced C3 surface levels, presumably due to internalization. As expected, no C3 deposition was observed when cells were incubated with serum from *C3*^{-/-} mice, indicating genuine C3 deposition and detection when cells were incubated with WT serum. A similar pattern was seen for cells from *Marchf1*^{-/-} x *C3*^{-/-} mice with the exceptions that cells initially had no C3 expression and remained, as expected, negative for C3 when incubated without or *C3*^{-/-} serum. cDC1s from *Marchf1*^{-/-} x *C3*^{-/-} mice showed no C3 deposition when incubated with WT serum for 15–120 min, although cells were high in MHC II surface expression. This indicated that simple incubation of cDCs with C3-rich serum did not recapitulate the C3 binding to MHC II at the cell surface of cDCs in MARCF1-deficient mice. *Marchf1*^{-/-} cDCs already exhibited highly deposited C3 at their surface when isolated from mice and further incubation with or without serum for 120 min did not affect high C3 surface levels (**Figure 3.16A**).

Incubation of DC1940 with C3-containing serum

In addition to the investigations of C3 deposition on primary cDCs, we sought to reproduce C3 binding to MHC II *in vitro* using the dendritic cell line MuTu DC1940 [653][702]. This was of particular interest as our group has established a sophisticated CRISPR/Cas9 platform to generate gene mutations in this cell line with a small guide RNA (sgRNA) library of more than thousand targets (described in PhD thesis from Haiyin Liu [541]), providing the potential to further characterize the MHC II-C3 complex formation on the cell surface. To examine if C3 binding to the cell surface of these cells *in vitro* depends on surface MHC II levels, we deleted the gene *Marchf1* or *H2-AB1* by transducing MuTu DCs with gene-specific sgRNA. As these cells express the endonuclease Cas9, this enabled *Marchf1* or *H2-AB1* deletion via doxycycline-inducible sgRNA (*isgMarchf1* and *isgH2-AB1*). Negative control cells were generated expressing an irrelevant sgRNA targeting human (not mouse) Bim (*isgBim*).

Established mutant cells were incubated with WT or C3-deficient mouse serum for 60 min in

the presence or absence of CpG. Oligonucleotides, in particular CpG, have been described as a potent complement activator, through direct interactions with complement components in a sequence-specific or CpG backbone-dependent manner [703][704]. As expected, we observed elevated surface MHC II expression in *isgMarchf1* MuTu DCs relative to *isgBim*, while *isgH2-AB1* cells displayed no significant MHC II surface levels, indicating successful gene deletions. Incubation of cells with WT but not *C3^{-/-}* serum led to the deposition of C3 on the cell surface with similarly increased surface levels between *isgBim* and *isgH2-AB1* but slightly more increased C3 signals in *isgMarchf1* MuTu DCs. The presence of CpG caused, as expected, increased surface expression of MHC II in *isgBim* MuTu DCs but had no significant effect on C3 accumulation at the surface of any of the tested mutant DC1940 cells (**Figure 3.16B**).

Although *isgMarchf1* MuTu DCs exhibited remarkably elevated surface MHC II, their incubation with serum led to similar or only slightly enriched C3 deposition on their cell surface when compared to both *isgBim* and *isgH2-AB1* cells, indicating that, similarly to our experiments with primary cells, simple incubation of cells with C3-rich serum did not recapitulate the C3 binding to MHC II at the cell surface of cDCs in MARCF1-deficient mice.

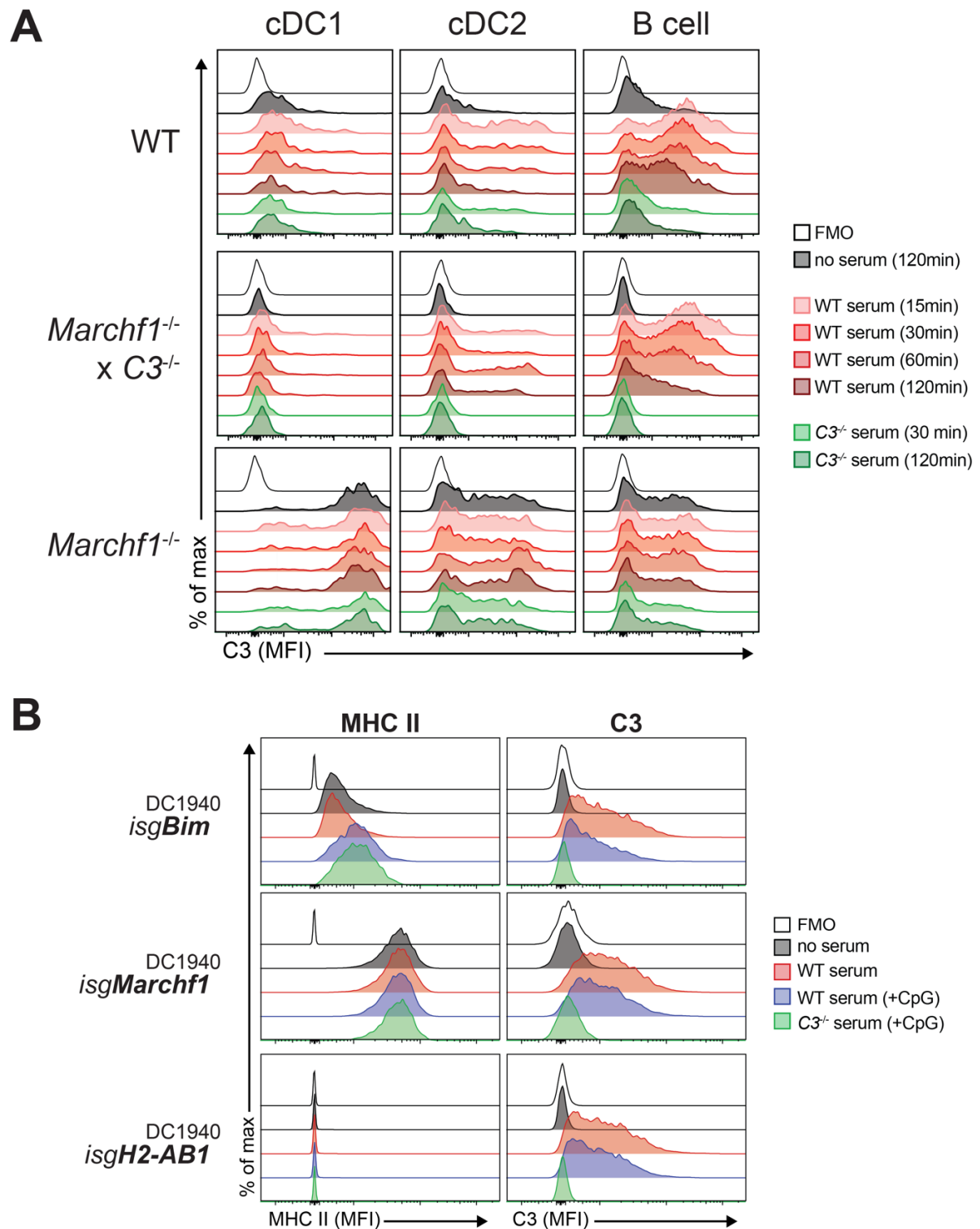


Figure 3.16 Deposition of serological C3 on the cell surface of DCs and B cells through their incubation with serum. **(A)** C3 surface levels on primary cDCs and B cells purified from WT and *I-A α* ^{-/-} mice upon incubation with WT or *C3*^{-/-} serum for 15-120min. **(B)** Surface expression levels of MHC II and C3 in MuTu DCs with CRISPR/Cas9 deleted *Bim*, *Marchf1* or *H2-AB1* genes upon incubation with CpG and WT or *C3*^{-/-} serum for 60min. For the corresponding FMO control in **(A)** and **(B)** cells were incubated with streptavidin-APC only [minus biotin, anti-C3 mAb (11H9)].

3.2.7 MHC II-dependent C3 deposition on *in vivo* activated WT cDCs

We have described in different mutant mice and human blood samples that the level of MHC II surface expression in cDCs determines the amount of C3 accumulating at their plasma membrane by capturing and covalently binding C3. While no C3 was detected in MHC II-deficient cDCs, WT cDCs expressed little surface C3 but MHC II-C3 complex formation was remarkably increased in cDCs with enriched MHC II surface expression due to impaired or omitted MHC II ubiquitination. Activation of DCs, e.g. through signalling via PRR, leads to their maturation and conversion from an Ag-capturing to an Ag-presenting mode, characterized by a significant increase of surface expression of MHC II due to its induced expression [199], [399] and reduced internalization from the cell surface [401]. We therefore tested if activation of WT DCs through the administration of TLR agonists induces C3 deposition on their cell surface through the upregulation of surface MHC II. For this we intravenously injected CpG into WT and MHC II-deficient (*I-A α ^{-/-}*) mice in order to activate DCs *in vivo*, as attempts to recapitulate MHC II-dependent C3 deposition on DCs *in vitro* and *ex vivo* failed. MHC II and C3 surface expression on splenic cDCs and B cells was examined by flow cytometry, 12-16 h post injection of CpG (**Figure 3.17A**).

cDCs and B cells from WT mice both strongly upregulated MHC II surface expression in response to CpG injection, while cells from *I-A α ^{-/-}* mice remained, as expected, negative for MHC II surface levels (**Figure 3.17A**). CpG has been described as a potent complement activator, through direct interactions with complement components and also indirectly through TLR signalling [703][705][704]. Intravenous injection of CpG caused a slight increase in surface levels of C3 in cDC1s and cDC2s and a decrease in C3 surface expression in B cells. However, C3 deposition in response to CpG administration was equal between WT and *I-A α ^{-/-}* cDCs, demonstrating no MHC II-dependent C3 accumulation in this setting (**Figure 3.17A**).

Delta Inulin (DI) is a derivatized isoform of β -D-[2 $\rightarrow$ 1] poly(fructo-furanosyl) α -D-glucose polymers, an insoluble and crystallized polysaccharide of the natural occurring inulin. It is currently used as a potent adjuvant (Advax™) in human clinical trials enhancing humoral and cellular immune responses [643]. DI has been described as a strong complement activator, triggering the alternative pathway [643][644][645]. We therefore assessed C3 deposition on splenic WT cDCs and B cells in response to increasing amounts of intravenously injected DI [0.05mg, 0.1mg, 0.5mg and 1mg] (**Figure 3.17B**). cDC1s and cDC2s but not B cells

moderately upregulated surface MHC II in a dose-dependent manner upon DI injection, though not to the same extent as in response to CpG (**Figure 3.17A**). Only subtle increase in C3 surface levels was detected on both cDC subsets, whereas B cells clearly exhibited enriched accumulation of C3 at their cell surface depending on the amount of DI injected (**Figure 3.17B**).

Overall *in vivo* activation of cDCs through intravenous injection of TLR9 agonist CpG or complement activator DI into WT mice did not cause similarly high C3 surface expression levels compared to mutant cDCs with constitutively high surface MHC II (e.g. *Marchf1*^{-/-} or MHC II K225R^{KI/KI}).

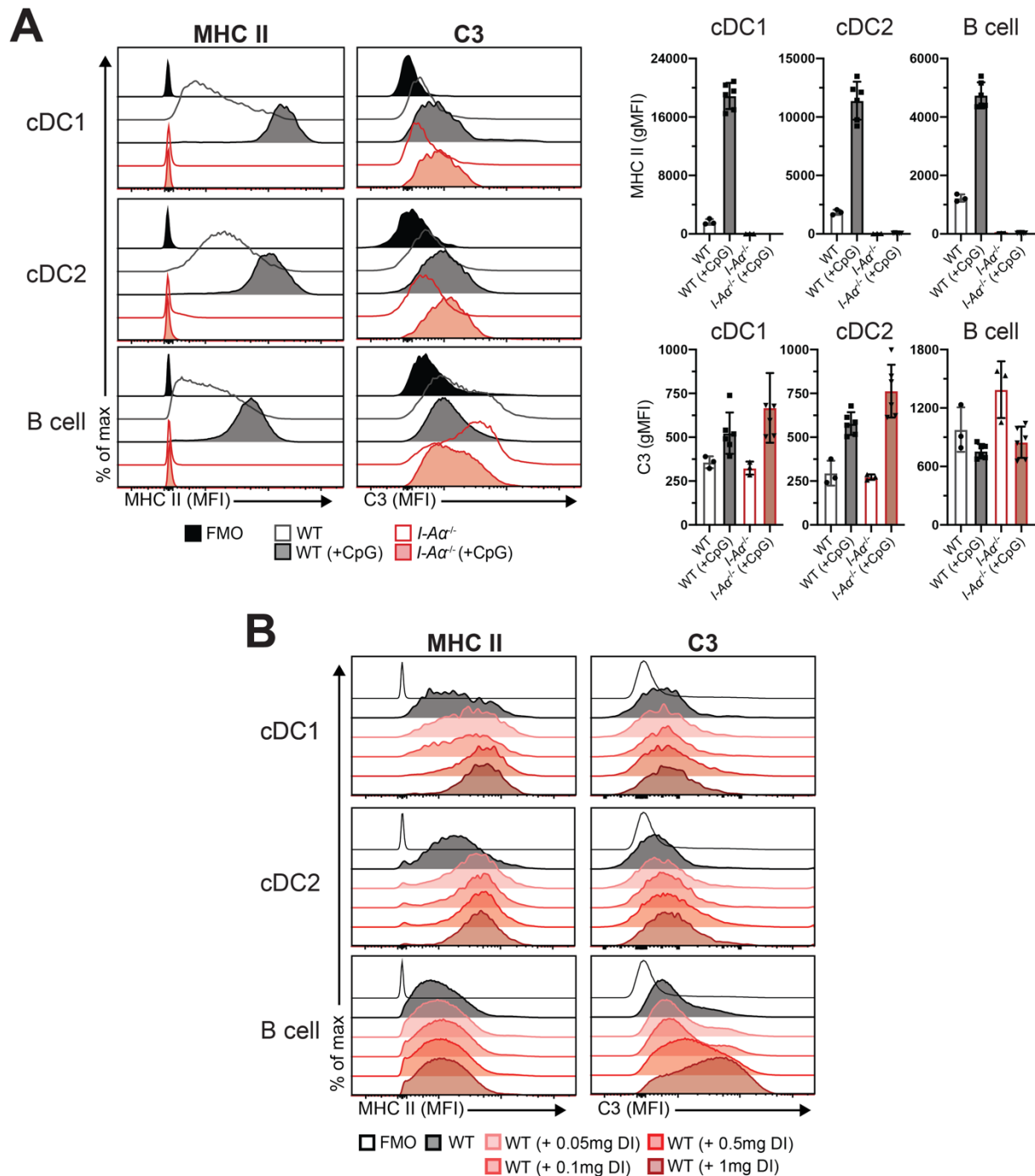


Figure 3.17 MHC II and C3 surface levels in cDCs and B cells upon *in vivo* activation. Expression levels of surface MHC II and C3 in splenic cDCs and B cells purified from (A) WT and *I-A α ^{-/-}* mice injected with 20 nmol CpG or (B) from WT mice injected with increasing amounts of delta inulin (DI) compared to the corresponding FMO control for which cells were incubated with streptavidin-APC only (minus biotin.11H9 anti-C3 mAb). Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, bars designate mean $\pm$ SD.

3.3 Discussion

This chapter describes the mechanisms for enriched C3 fixation at the surface of cDCs in MARCHF1-deficient mice, initially discovered in semi-quantitative proteomic analyses comparing PM fractions from WT and *Marchf1*^{-/-} cDCs.

3.3.1 A new role for MHC II as an acceptor for covalent association of C3

The intracellular RING-CH domain of MARCHF E3 Ub ligases contains a zinc finger motif that is critical for the ubiquitination of membrane proteins. For this it recruits E2 conjugating enzymes and directs the transfer of Ub molecules to the cytosolic tail of the substrate [517] [661]. Ubiquitination through MARCHF Ub ligases is known to be involved in the regulation of the intracellular transport of membrane proteins to and from the cell surface. Ubiquitination of cytosolic, luminal or even extracellular proteins through MARCHF Ub ligases has not been described in the literature and the membrane-bound immune receptors CD86 and MHC II are the only two known substrates of MARCHF1 in antigen presenting cells (APC) under physiological conditions. Hence, the outcome of our previously conducted proteomic screen, showing significantly enriched surface levels of complement C3 in MARCHF1-deficient cDCs [section 3.1.2], was unexpected as both proteins are thought to have distinct cellular localizations preventing direct ubiquitination of C3 through MARCHF1.

In initial experiments using flow cytometry and western blotting we clearly validated enriched C3 surface levels on *Marchf1*^{-/-} cDCs but also identified C3 deposition on the surface of WT cDCs. C3 is the central component in the complement system, predominantly produced and secreted by hepatocytes [565][566]. Its classical role as a serum effector molecule of innate immunity is traditionally thought to exclusively occur in the extracellular environment in order to opsonize pathogens or cell debris in the circulation, facilitating their clearance [584]. However, several recent studies have provided evidence for an intracellular repertoire of complement proteins, referred as the *complosome*, that provides not only immune defense functions but also modulates cellular homeostasis (reviewed in [626][627]). In particular the intracellular presence of C3 in various immune cells has been reported by several studies. First, various immune cells have been shown to locally produce complement proteins, depositing C3 on their cell surface in an autocrine manner [632][627][633], second, more recent studies have discovered the intracellular activation of complement C3 mediating T cell and macrophage functions [706][707][634] and lastly endocytosed C3 associated to Ag has

been shown to inhibit its lysosomal degradation in order to prolong Ag stability in DCs resulting in enhanced Ag presentation and T cell stimulation [576][575]. Furthermore, intracellular C3 has been described to have implications in cell signalling [624][625] and induction of gene transcription [708]. These studies clearly demonstrate the presence and functionality of C3 inside immune cells and we therefore considered the possibility that MARCF1-mediated ubiquitination is involved in regulating complosome activity. Direct ubiquitination of C3 has only been reported in hepatocytes under conditions of nutritional stress linked to its trafficking and secretion [709], but is not known to occur in immune cells and/or through MARCF1 Ub ligases. Furthermore, MARCF1 is known to only ubiquitinate membrane proteins. However, to address the potential that C3 deposition on cDCs is a consequence of impaired intracellular inhibition of complement activation caused by the lack of MARCF1 activity, we aimed to identify the source of C3 accumulated on cDCs and carried out bone marrow chimera experiments. In doing so we identified that the deposited C3 on the surface of cDCs originates from the extracellular environment, and if cells do not express MARCF1 the amount of C3 that accumulates on their surface is increased, indicating the deposition of C3 occurs most likely exclusively on the extracellular site of the plasma membrane and hence is not due to a direct intracellular effect but rather a secondary effect of MARCF1 deficiency.

In concordance with that we then have identified elevated surface expression of MHC II in DCs with impaired ubiquitination, and not the loss of MARCF1 per se, as the primary reason for enriched deposition of extracellular C3 on the cell surface of *Marchf1*^{-/-} cDCs and identified the covalent binding of C3 to surface MHC II molecules. We have also reported enriched surface expression of complement receptors CR1 and CR2 as well as the inhibitory complement regulators CD55 and Factor H in *Marchf1*^{-/-} cDCs. We assume that the differences in the expression of these complement regulators and receptors are a consequence and not a reason for increased C3 deposition, based on various reasons. First, virtually all the C3 molecules that could be detected on the surface of cDCs from WT or MARCF1-deficient mice bound exclusively to MHC II, as demonstrated by the complete loss of C3 surface expression in MHC II-deficient cDCs. Second, elevated surface levels of CD55 and Factor H might aim to decelerate complement activation, whereas CR1 and CR2 bind to C3 (bound to MHC II) “in cis” on the same membrane preventing their internalization.

Overall with this observation, we have reported the first evidence for a new role of MHC II,

namely functioning as an acceptor molecule for the covalent binding of constitutively activated C3, besides its only known function in presenting peptide antigens to CD4⁺ T cells. In a more general perspective, this describes an unknown instance of intersection between the innate and adaptive immune systems at a molecular level, with complement C3 as a central molecular component of innate immunity and MHC II as a central molecule of the adaptive immune system.

3.3.2 Differential binding of C3 to MHC II-expressing cells

Our results indicate that binding of C3 to MHC II does not occur uniformly across various MHC II-expressing cells (i.e. APCs) and also depends on the site within the organism as cells with the same ontogenetic origin but from different tissues/organs exhibited varying C3 surface levels. Splenic cDC1s exhibited the highest C3 surface levels, followed by cDC2s. A similar but reduced pattern was observed in cDCs from pooled lymph nodes and in thymic cDCs. Splenic macrophages, pDCs and B cells that lacked MARCF1 exhibited only little C3 surface levels. With the description of MHC II as a major acceptor for the covalent binding of C3, C3 deposition in these cells was, as expected, limited because MARCF1 deficiency in macrophages and pDCs results in only subtle elevation of surface MHC II, when compared to cDCs or B cells. However, MHC II surface levels are more drastically enriched in *Marchf1*^{-/-} B cells and cDCs, with equivalent amounts of MHC II molecules across both cell types so that drastically higher C3 surface levels in cDCs compared to B cells were surprising.

Cellular localization and migratory behavior

A potential explanation for the differential deposition of C3 across various APCs is that cells are differently exposed to activated C3 depending on their lifetime, localization and migratory behavior as well as cellular interactions in different organs and within the same lymphoid tissue. BrdU incorporation experiments, looking at the overall turnover rate of cDCs in spleen and lymph nodes, have demonstrated a shorter life span for splenic cDC1s compared to cDC2s and in general a longer life time for lymph node over splenic cDCs [268][188]. This means an overall longer life span of a cell and hence its potentially longer exposure time to extracellular C3 does not simply translate into enriched C3 surface deposition, as splenic cDC1s are short-lived but had the highest C3 surface levels. It rather suggests that the cell's localization and migratory behavior is more relevant to the exposure and acquisition of C3. Although complement activation has been reported in peripheral tissues, including the intestine, lung

and kidney [692][693][694][695], we observed no significant C3 surface levels in liver and lung cDCs during steady-state but high C3 deposition on cDCs in spleen and LN, indicating that the site of where C3 binds to surface MHC II of cDCs in mutant mice is restricted to lymphoid tissues. Considering C3 is highly abundant in the serum and the spleen is the main filter for blood-borne antigens, the constant blood flow and influx of serological C3 might result in a more extensive exposure of cells to C3 in the spleen and potentially explains why spleen cDCs exhibit higher C3 deposition when compared to non-splenic cDCs. The marginal zone (MZ) within the spleen could be of particular interest in this context, as afferent arterial vessels end in sinusoids at the MZ that surrounds the white pulp constantly delivering blood and replenish the tissue with 'fresh' C3 [710]. cDCs, especially cDC1s, have been shown to be highly abundant in the MZ [711][383][384] and also complement proteins have been reported to fulfill various functions in the MZ, including the clearance of Ag through complement C4 [712] and C3-dependent pathogens clearance through MZ macrophages [713].

C3 deposition also occurred on cDCs in lymph nodes, though to a lesser extent when compared to their splenic counterparts. The presence of C3 and cDCs in lymph nodes is well described in literature and both are known to be involved in the transport and presentation of Ag to B cells within and at B cell follicles [714][68], so that binding of C3 to surface MHC II within LN is plausible.

As our observations indicate that C3 binding to cDCs during steady-state is restricted to lymphoid tissues, cells that reside longer in these tissue might contribute to enriched C3 fixation and explain why cDCs from the spleen exhibit higher C3 deposition. Splenic cDCs are exclusively tissue-resident, while cDCs in the LN are known to be both migratory and resident. Currently no conclusive analysis has been undertaken to compare surface C3 levels between migratory and tissue-resident LN cDCs. This will require more detailed analysis of LN cDCs and the differentiation of cells in various draining LN (i.e. mesenteric vs. subcutaneous LN). Overall, imaging techniques, such as full organ histology with cross and longitudinal sections of spleen and lymph nodes, will be necessary to assess the architectural organization and cellular localization of cDCs, as well as the site of where C3 binding to MHC II on cDCs occurs.

Structural heterogeneity of MHC II α carbohydrates

The distinct localization and/or migratory behavior of cells does not ultimately explain the substantial differences in C3 deposition levels seen between splenic cDCs and B cells, as both

cell types are present in similar compartments of the spleen, such as in the MZ [715][52]. A further explanation is the differential affinity of MHC II in binding activated C3 that preferentially targets distinct structural components of MHC II, varying between different cells and/or tissues. We identified that the capacity of MHC II to bind C3 is not conferred by the polypeptide component of the molecule but by the MHC II α carbohydrate. This observation is in agreement with the general understanding that C3 displays only little protein-conformation specificity and with earlier reports describing preferences in binding of C3 to specific carbohydrates of certain proteins, such as of itself, complement C4 and immunoglobulins [675][676][677][678]. Examination of cells lacking MHC II surface expression indicated that virtually all the C3 molecules that could be detected on the surface of cDCs from WT and MARCF1-deficient mice were bound to MHC II α . This implies that the carbohydrate of MHC II α has some property that sets it apart from the other sugars found on other glycoproteins or, indeed, any carbohydrate-containing molecule exposed on the cell surface of cDCs for the binding of C3. Previous studies give support to this conclusion, demonstrating differences in the degree of sialylation and branching between different glycoproteins [716][717] and microheterogeneity in the MHC II carbohydrate within a particular and between different cell types [718][719]. The proportion of MHC II molecules containing such carbohydrate may vary between cells and tissues, providing an explanation for the different amounts of C3 we could detect on the surface of cDC1s, cDC2s and B cells, despite the fact all three cell types express similar amounts of MHC II molecules. Furthermore, the results of our immunoprecipitation/western blot experiments indicated that the proportion of MHC II molecules that was associated with C3 was small compared to the overall MHC II levels derived from lysates of cDCs. An explanation for this is that only a small proportion of MHC II molecules either has been present at the cell surface and/or carries the particular carbohydrate necessary to bind the thioester group of extracellularly activated C3. The particularity of the carbohydrate in MHC II α might also explain why *in vitro* incubation of cDCs with serum or the activation *in vivo* with CpG only leads to little C3 binding: in both scenarios there were too few surface MHC II molecules at the cell surface carrying the “correct” carbohydrate for the binding of extracellular C3. Detailed analysis of MHC II α carbohydrate heterogeneity, and characterization of the carbohydrate targeted by C3 will be required to confirm or falsify this hypothesis and to address if MARCF1-mediated ubiquitination affects MHC II glycosylation.

3.3.3 MARCF1-mediated internalization of MHC II as a route for C3 uptake

Marchf1^{-/-} and MHC II K225R^{KI/KI} mice allowed us to identify the covalent association of C3 and MHC II at the surface of cDCs. However, binding of C3 to surface MHC II was not limited to mutant cells with impaired or ceased MHC II ubiquitination but also identified on the surface of WT cDCs where MHC II levels are not artificially elevated. Notably, MARCF1-mediated ubiquitination of MHC II and its consequential internalization limited the number of MHC II-C3 complexes displayed on the surface of WT cDCs. This describes a potential route for the uptake of extracellular C3 into the cell. In this model, WT cDCs internalize C3 covalently associated to MHC II through MARCF1-mediated ubiquitination, whereas mutant cells with impaired MHC II ubiquitination lack internalization of C3 upon its covalent binding to MHC II. With emerging recognition of intracellular C3 as part of the complosome and immune modulator, various studies have demonstrated the internalization of C3 through complement receptors [720], receptor-mediated endocytosis [721][722] or when associated to Ag [576][575]. A recent study by Elvington *et al.* describes that various human immune cells can uptake serological C3(H₂O) [701]. In a process known as tick-over, small amounts of C3 continuously activate in the fluid phase by hydrolysis of the internal thioester bond, the initial step of the alternative pathway, which results in C3(H₂O) [587][701]. Although the authors suggest that loading of cells with extracellular C3 involves specific mechanisms [701], no particular machinery or surface molecule for the internalization has been reported. High resolution microscopy will be required to examine the intracellular trafficking of MHC II-C3 complexes in order to assess MARCF1-mediated ubiquitination of MHC II as a way for cDCs to internalize extracellular C3. In addition, functional analyses will aim to determine its role once inside the cell. Various hypotheses can be proposed: C3 bound to peptide loaded MHC II {1} is involved in MHC II turnover promoting its recycling back to the cell surface and/or reducing its lysosomal degradation upon internalization, the latter similar to the stabilizing function of C3 when associated to Ag [576][575], {2} the induced expression of certain genes, e.g. relevant for Ag processing and presentation, through intracellular C3 signalling or {3} the uptake of Ag or immune complexes that associate with C3 bound to MHC II.

3.3.4 Activation and cleavage of C3 bound to MHC II

Several observations made during our investigations of C3 fixation on MARCF1-deficient cDCs suggest that the underlying activation mechanism for the deposited C3 is the alternative pathway. First, we identified binding of C3 to MHC II in primary cDCs purified from mice that were kept in pathogen-free animal facilities. While complement activation through the classical or lectin pathway is known to be induced through pathogenic surface structures [586], activation through the alternative pathway occurs during steady state at low rate, even in the absence of pathogens [587][588]. Second, western blot and mass spectrometry analyses identified C3dg and C3d as the predominant complement C3 fragments bound to the carbohydrate of MHC II α in cDCs from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice. Complement activation leads to the cleavage of C3a from C3 to form active C3b, which attaches covalently to cell surfaces through a thioester bridge [723]. The proteolytic cleavage of activated C3b through the serine protease Factor I is assisted by various cofactors, including Factor H and DAF (CD55), and results in the generation of inactive C3dg and C3d. This is known to be critical for self-protection against spontaneously activated C3 through the alternative pathway, preventing the uncontrolled assembly of C3 convertases on host-cell surfaces [594][595]. While C3c is released into the fluid phase upon Factor I-mediated C3 cleavage, C3dg and C3d remain bound to the cell surface as both fragments retain the thioester of the initially activated C3b, relevant for the covalent binding to its target. Lastly, enriched Factor H and CD55 surface levels in *Marchf1*^{-/-} cDCs, as critical inhibitory regulators of the alternative pathway, support the hypothesis that the deposited C3 on the surface of cDCs derives from spontaneous tick-over activation. This suggests that the C3dg and C3d fragments, bound to MHC II on the surface of *Marchf1*^{-/-} cDCs, result from Factor I-mediated cleavage upon spontaneously activated C3 through the alternative pathway. The tick-over theory describes the spontaneous hydrolysis of the internal thioester bond of C3 causing the formation of C3(H₂O) in the fluid phase and is the starting point for the alternative pathway as C3(H₂O) functions as the initial C3 convertase for further complement activation [724]. However, it is thought that during steady state this complement activation only has minor implications as spontaneous C3 hydrolysis only occurs at a very low rate [587] and negative regulators prevent significant complement activation at the host-cell surface [594][595][588]. Our data suggests that constitutive C3 activation via the alternative pathway does not appear more

prominent *in vivo* because activated C3 molecules bind to MHC II and are quickly eliminated through the fast turn-over rate of the MHC II-C3 complexes during steady state, imposed by MARCF1-mediated ubiquitination.

Notably, cDCs from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice exhibited approximately 20x more C3 on their surface when compared to WT cDCs. The amount of C3 found on WT DCs was under the detection limit for mass spectrometry and WB analysis, which impeded us to decipher which C3 fragments bound to MHC II on the surface of WT cDCs. Consequently, we are not able to tell if and at what stage WT cDCs internalize C3, covalently bound to MHC II. Presumably this can be either directly after binding of activated C3b to MHC II or upon its successive cleavage through Factor I. However, the monoclonal antibody 3d29, specifically detecting C3dg and C3d [619], was most sensitive in detecting C3 on the surface of WT cDC1s by flow cytometry (**Figure 3.2C**), which indicates C3 gets fully cleaved on the surface of WT cDCs, similar to cDCs from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice, before being internalized.

We were not able to detect MHC II-dependent C3 deposition in WT mice following DC activation. Intravenous CpG injection into WT mice and incubation of DCs with serum and CpG *in vitro* indeed caused upregulation of surface MHC II, but C3 deposition was only subtle and independent of MHC II surface levels. A potential explanation for this might be that complement activation through CpG administration mainly triggers the classical pathway [703][704] and does not recapitulate the complement activation route that underpins binding of C3 to MHC II in mutant mice (i.e. *Marchf1*^{-/-} and MHC II K225R^{KI/KI}) during steady state. Instead, enhancing the alternative pathway through either inducing a mutant type of C3 that is unstable and prone to spontaneous hydrolysis or the inhibition/depletion of relevant regulators of the alternative pathway, such as Factor I and Factor H, will be necessary to confirm the alternative pathway and induce binding of C3 binding to MHC II in WT mice.

Mice deficient in MARCF1 expression or that express the MHC II K225R mutant molecule do not display obvious autoimmune or inflammatory defects, as might have been expected due to accumulation of C3 on cell surfaces. However, such disorders often do not arise spontaneously in laboratory mice bred in specific pathogen-free conditions, so it is possible that the mutant mice will display increased susceptibility to C3-mediated autoimmune or autoinflammatory reactions if subjected to the right challenge. Functional consequences of MHC II-C3dg complex accumulation are described in the following chapters.

The biological role of complement C3 and dendritic cells

Abstract

Binding of activated complement C3 to cell surfaces, such as of pathogens, apoptotic host or cancer cells, is a central part of the effector function in humoral immune responses of the innate immune system. Clearance of C3-opsonised cells is mediated through induced cell lysis or phagocytosis and is critical for the prevention of pathogenesis and the onset of autoimmunity. Beside its effector function in innate immunity, more recent studies have described implications for C3 in the adaptive immune system, including the regulation of antigen presentation in DCs and the promotion of T cell proliferation as well as B cell activation.

This chapter investigates the biological relevance of C3 deposition on cDCs in the spleen of mutant mice with impaired MHC II ubiquitination, causing enriched binding of extracellular C3 to surface MHC II (see Chapter 3). We demonstrate that covalent binding of C3 to MHC II α at the surface of cDCs has no impact on MHC II-restricted antigen presentation to naïve CD4 T cells but affects the cellular composition of immune cells in the spleen. Enumerations of splenocytes in various mutant mouse strains and mixed bone marrow chimeras identified a significant loss of splenic cDCs by 50-60%, associated with enriched C3 deposition, while cell numbers of other cell types were unaffected. Additionally, we discovered an unusual cell population that increases in scenarios of enriched C3 deposition on cDCs in several mutant mice and in mixed bone marrow chimera recipient mice. Detailed examination of these cells identified them as marginal zone (MZ) B cells, that acquire cDC-specific markers at the cell surface, including CD11c, CD8 and XCR1, without transcriptional expression for these proteins.

4 The biological role of complement C3 and dendritic cells

4.1 Introduction

4.1.1 C3 in adaptive immunity

The previous chapter describes the covalent binding of activated complement C3 to MHC II at the surface of lymphoid tissue cDCs. This molecular intersection between the innate and adaptive immune systems was unexpected as the complement system is traditionally considered as an effector arm of the innate immune system. Traditionally, opsonization of cells with complement C3 is known to mediate their clearance through various mechanisms, including phagocytosis by complement receptor CR1/2 and CR1g expressing APCs, predominantly by macrophages [571][572][573][574], immune shuttling and cell lysis, the latter through the induction of the terminal membrane attacking complex (MAC) [570] (reviewed in [584]). Complement C3 is known to opsonize free pathogens, including cell-free viruses [725] and a wide range of bacteria [726], virus-infected cells [727] and tumor or apoptotic host cells [728][729][730][575]. However, more recent studies have provided strong evidence that the complement system, in particular C3, contributes to adaptive immunity. For instance, it is well known that the two main anaphylatoxins of the complement cascade, C3a and C5a, enhance induced immune responses after their binding to the receptors C3aR and C5aR on T cells, supporting T cell proliferation and diminishing inhibitory regulation through Tregs [618][636][731]. For human T cells it has been shown that cell-bound iC3b enhances T cell proliferation through its binding to CR3 and CR4 on dendritic cells [579]. Furthermore, C3aR and C5aR signalling in APCs induces the release of cytokines and up-regulation of essential co-stimulatory molecules, shown for monocyte-derived DCs that bind C3 to increase the expression of MHC II, CD83 and CD86 [732][733]. The very last fragment of the C3 breakdown pathway C3dg has been described to be an important co-stimulatory factor for B cell activation. Thereby Ag-associated C3dg interacts with the complement receptor CR2 (CD21) on the surface of B cells that bind cognate Ag, which reduces the threshold for their activation [95][96]. Baudino *et al.* have described that C3 opsonization regulates endocytic transport of apoptotic cell structures resulting in enhanced T cell responses. Intracellular phagosome maturation and subsequent MHC II Ag presentation in DCs from C3-deficient mice resulted in an accelerated degradation of endocytosed antigens

and impaired antigen presentation and T cell proliferation when compared to DCs from C3-sufficient mice [575]. These studies clearly demonstrate various roles for C3 in Ag presentation and lymphocyte activation, essential steps in mounting adaptive immune responses.

This chapter investigates the impact of C3 binding to the surface of cDCs. It is widely known that spontaneous activation of C3 through the alternative pathway and the consequential deposition of C3 on host cells is inhibited through the action of various negative complement regulators [593][594][595][596]. However, the identification of enriched surface deposition of C3 on cDCs in mutant mice constitutively elevated surface MHC II, enabled us to investigate the role of C3 in DC biology.

4.2 Results

4.2.1 The effect of C3 binding to MHC II on antigen presentation

We have demonstrated that covalent association of extracellular C3 to surface MHC II complexes predominantly occurs in lymphoid tissue cDCs, enriched in mice with impaired MHC II ubiquitination. cDCs are the principle initiators of T cell responses via MHC-restricted Ag presentation. We therefore aimed to address if binding of C3 to surface MHC II affects presentation of soluble Ag to T cells. As C3 deposition was strongest on the surface of splenic cDC1s we compared Ag presentation of C3-coated and C3-deficient cDC1s. Although cDC1s are known to be specialized in MHC I-mediated cross-presentation [355][356][357][21][364][365][366], they are also capable of presenting antigenic peptides via MHC II to CD4⁺ T cells [380][381][382]. cDC1s were sorted to purity by flow cytometry, isolated from spleens of WT, *Marchf1*^{-/-} and *Marchf1*^{-/-} x *C3*^{-/-} mice (> 95% purity), pulsed with increasing doses of soluble ovalbumin (OVA) protein in the presence of TLR9 agonist, CpG, and finally co-cultured with OVA-specific OT-I or OT-II T cells. CD8⁺ OT-I and CD4⁺ OT-II T cells were purified from LN of transgenic mice (> 85% purity), expressing OVA-specific TCRs [for OT-I: MHC I-(2K^b)-OVA₂₅₇₋₂₆₁(SIINFEKL)-specific, for OT-II: MHC II(IA^b)-OVA₃₂₃₋₃₃₉(ISQAVHAAHAEINEAGR)-specific] [648][649][650]. OT-I and OT-II T cells were CellTrace Violet (CTV)-labelled before co-culturing with OVA-pulsed cDCs and Ag presentation was assessed based on numbers of dividing/proliferating T cells indicated by CTV-dilution (**Figure 4.1**).

WT cDC1s successfully stimulated OVA-specific OT-I and OT-II T cells in a dose-dependent manner with highest numbers of proliferating T cells in case of 1000 µg/ml OVA (100 µg in

total). *Marchf1*^{-/-} cDC1s displayed a reduction in the number of dividing OT-I and OT-II when pulsed with higher doses of OVA (333.3 and 1000 µg/ml), which is consistent with previous studies showing reduced Ag presentation in settings of impaired MHC II ubiquitination [535] [our data submitted for publication]. However, we did not observe any significant difference in numbers of dividing T cells between MARCHF1-deficient and *Marchf1*^{-/-} x *C3*^{-/-} cDC1s, indicating the presence of C3 had no impact on Ag presentation (**Figure 4.1**).

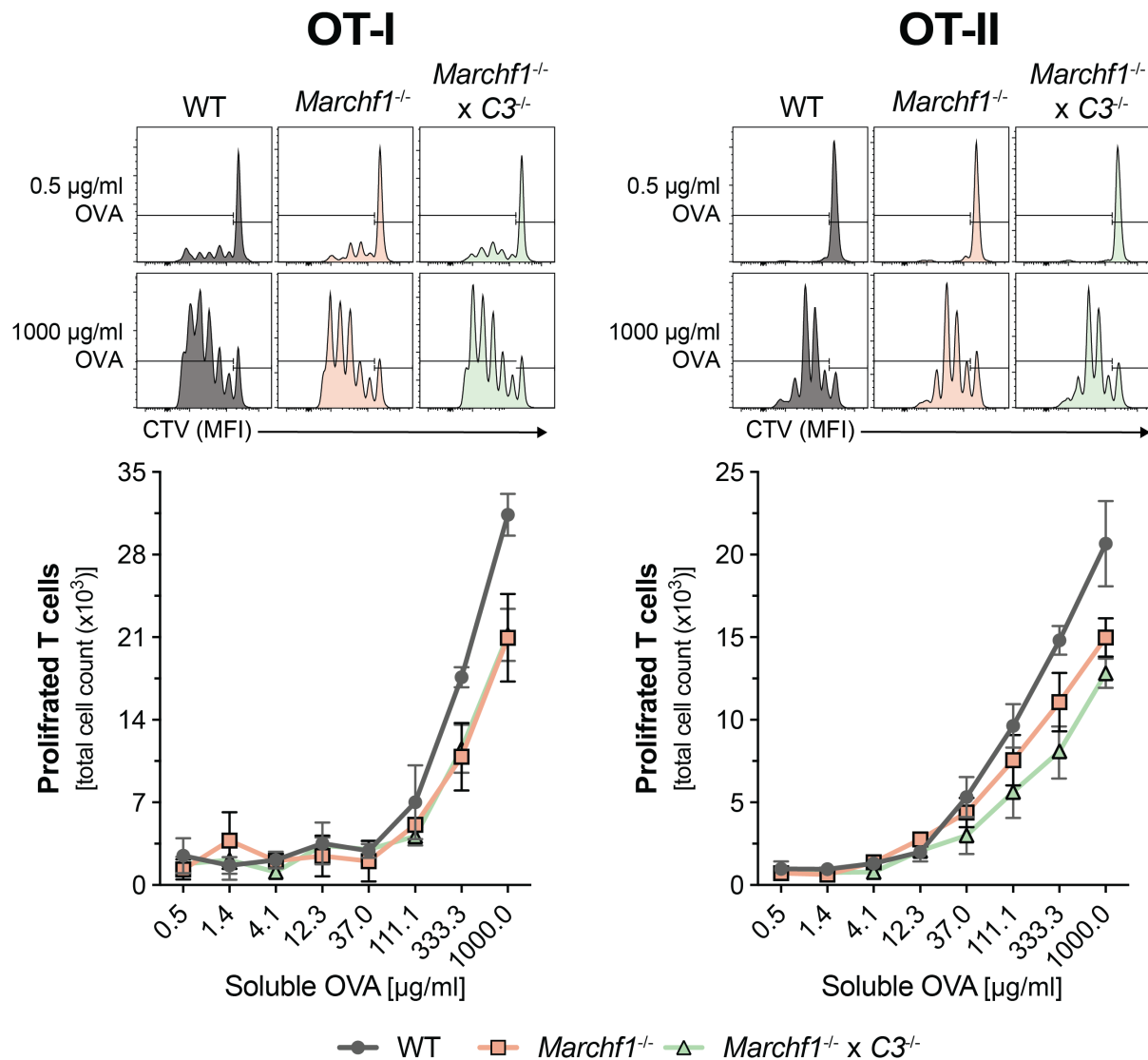


Figure 4.1 Antigen presentation capacity of C3-coated cDC1s. Purified CellTrace Violet (CTV)-labeled OT-I and OT-II T cells were incubated in triplicates for 72 h with cDC1s purified from spleens of either WT, *Marchf1*^{-/-} or *Marchf1*^{-/-} x *C3*^{-/-} mice upon CpG activation and pulsing with increasing doses of soluble OVA. Ag presentation capacity was assessed by flow cytometry analysis of CTV-dilution in order to determine numbers of proliferating/dividing OT-I/OT-II T cells. Each data point represents the measurements of a triplicate, displayed as mean and bars designating mean ± SD.

4.2.2 The impact of C3 deposition on cellular composition and dendritic cell homeostasis

C3 fixation on intact and healthy host cells is described to be shut down strictly through various negative complement regulators, including Factor I, Factor H and DAF (CD55) [593][594][595][596] and it has not been described in literature to directly regulate immune cell numbers. However, considering the high levels of C3 we have detected on the surface of cDCs, we aimed to determine cDC numbers and therefore undertook detailed analyses of the cellular composition in the spleen of MARCHF1-deficient mice.

Cellular composition in the spleen of *Marchf1*^{-/-} mice

In order to assess if MHC II-dependent C3 deposition affects numbers of cDCs, we examined the cellular composition in the spleen of mice with enriched C3 fixation (i.e. *Marchf1*^{-/-}, MHC II K225R^{KI/KI} and *Ubl3*^{-/-}). To gain an initial overview, we enumerated various major immune cells in the spleen of WT and *Marchf1*^{-/-} mice upon minimal purification: single-cell suspensions of splenocytes were generated through collagenase/DNase I (C/D) tissue digestion and subsequent selective lysis of erythrocytes using RCRB (NH₄Cl-based cell lysis). A combination of cell surface markers and gating strategies as well as microsphere count standards were used to determine cell numbers of various lymphocytes, granulocytes, macrophages and DCs via flow cytometry (**Figure 4.2**).

Spleen weight as well as total numbers of alive splenocytes were similar between WT and *Marchf1*^{-/-} mice, indicating no altered overall cellularity between both mouse strains (**Figure 4.2A**). Enumeration of spleen B cells, CD4⁺ and CD8⁺ T cells as well as macrophages, neutrophils, eosinophils and pDCs showed no altered numbers of these cells in the absence of MARCHF1 (**Figure 4.2B**). However, both cDC subsets were significantly reduced in case of MARCHF1 deficiency, with a reduction in numbers by ~60% for cDC1s and ~45% for cDC2s. Loss of cDCs in *Marchf1*^{-/-} mice was also significant based on the relative proportion of these cells in spleens (% of all alive cells) (**Figure 4.2C**). We discarded the possibility that *Marchf1*^{-/-} cDCs were lost in the gating strategy due to the lower surface levels of relevant identification markers (i.e. CD11c, CD11b, CD8 and XCR1), as all DC populations represented well-defined populations in the gating strategy to allow their clear identification ((**Figure 4.2D**)).

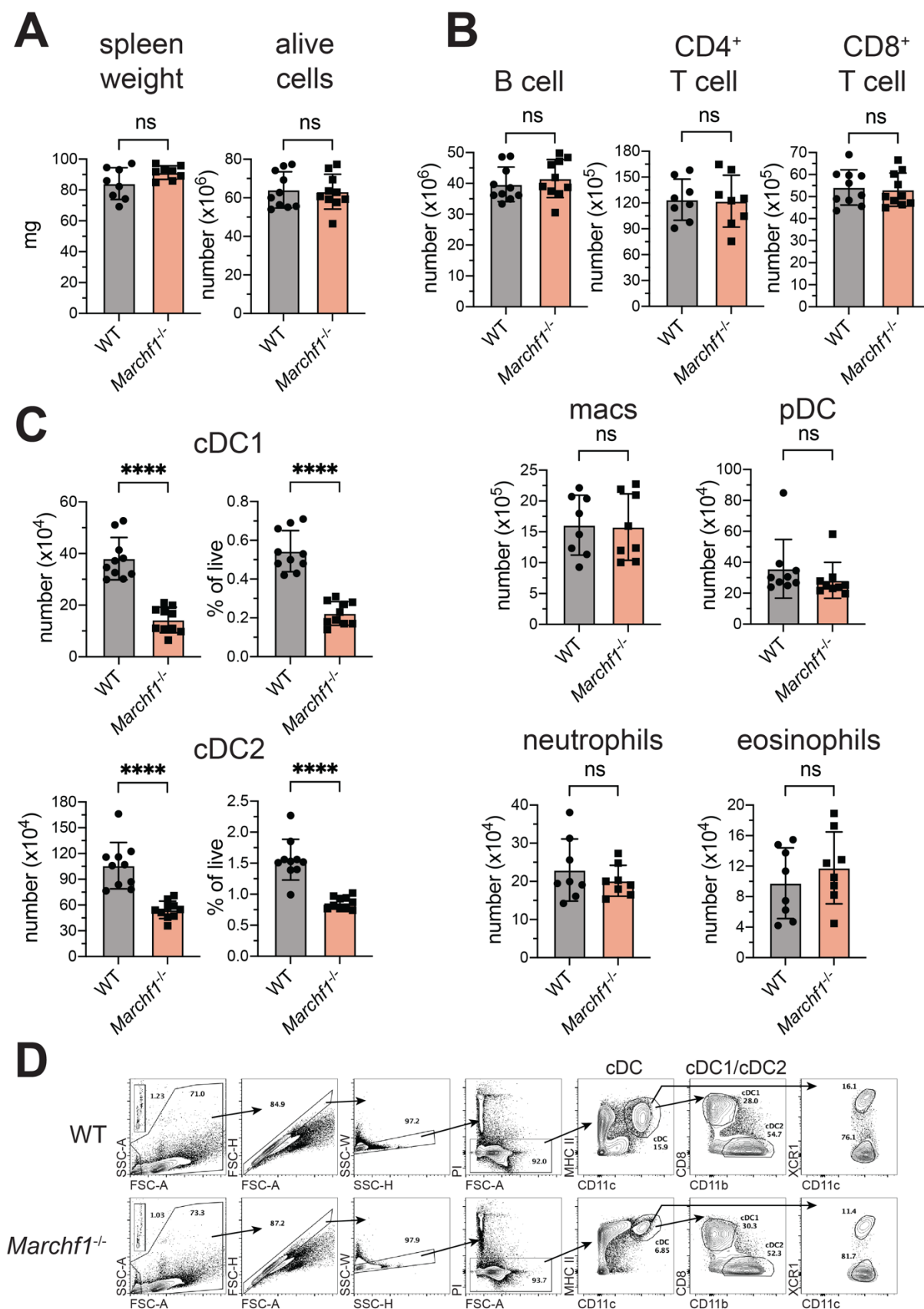


Figure 4.2 Enumeration of immune cells in the spleen of WT and *Marchf1*^{-/-} mice. Single-cell suspensions of total splenocytes from spleens of WT and *Marchf1*^{-/-} mice were generated through collagenase/DNase I (C/D) tissue digestion of finely cut spleens and subsequent selective lysis of red blood cells. Staining of cell surface markers allowed gating on cell population of interest and microsphere counting beads were used to determine numbers and/or frequencies. **(A)** Spleen weight and total count of alive cells (excluding cell doublets). **(B)** Total numbers of B cells, CD4⁺ and CD8⁺ T cells, macrophages (macs), pDCs, neutrophils and eosinophils. **(C)** Total numbers and frequencies of cDC1s and cDC2s. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, bars designate mean $\pm$ SD. Unpaired t test, two-tailed p value; ns: not significant, p value < 0.0001 (****). **(D)** Comparison of gating strategies for cDC1s and cDC2s from the low-density splenocyte suspension of WT and *Marchf1*^{-/-} mice, and CD11c versus XCR1 profiles of cDCs as an additional/alternative identification of cDC1

C3 deposition correlates with reduced cDCs

In order to verify the outcome of our previous analysis, that identified the specific loss of splenic cDCs in *Marchf1*^{-/-} mice, and to assess if this is due to enriched C3 deposition, we enumerated splenic cDCs from different mouse strains with varying levels of deposited C3 on their cDCs, namely *Marchf1*^{-/-}, MHC II K225R^{KI/KI} and *Ubl3*^{-/-} (all elevated/high C3) as well as mutant mice that were also deficient in C3. In contrast to our initial experiment, for which cDC numbers were determined from whole splenocytes suspensions (**Figure 4.2**), we purified cDCs in this experiment through either Nycodenz gradient centrifugation alone or gradient centrifugation + Ab-based immuno-depletion of non-cDCs [655](**Figure 4.3**).

Enumeration of cDCs from low-density splenocyte suspensions, achieved through Nycodenz gradient centrifugation, confirmed a ~50-60% reduction in numbers of both cDC subsets of MARCHF1-deficient mice (**Figure 4.3A**). cDCs from MHC II K225R^{KI/KI} mice, that exhibited equally high C3 surface levels when compared to *Marchf1*^{-/-} cDCs (Chapter 3, Figure 3.7 on page 103), were also reduced by ~50-60%. Interestingly, the loss of splenic cDCs in *Ubl3*^{-/-} mice was not as high as seen for *Marchf1*^{-/-} or MHC II K225R^{KI/KI} mice, with only ~20-25% reduction of cDC1 and no loss for cDC2 at all (**Figure 4.3A**), which correlated relatively to the observed levels of surface C3.

Purification of cDCs from WT spleens through Nycodenz gradient centrifugation and subsequent Ab-based immuno-depletion of non-cDCs achieved ~70-75% purity of cDCs, with a purity of ~15-20% for cDC1s and ~50-60% for cDC2s. Again, splenic cDCs from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice were reduced by ~50-60% in total cell numbers as well as frequencies when compared to WT mice (**Figure 4.3B**). Importantly, C3 deficiency in these mutant mice rescued cDC numbers and neither *Marchf1*^{-/-} x *C3*^{-/-} nor MHC II K225R^{KI/KI} x *C3*^{-/-}

mice exhibited a significant loss in cDCs (**Figure 4.3B**), appointing C3 as a determinant for the loss of splenic cDCs in these mice.

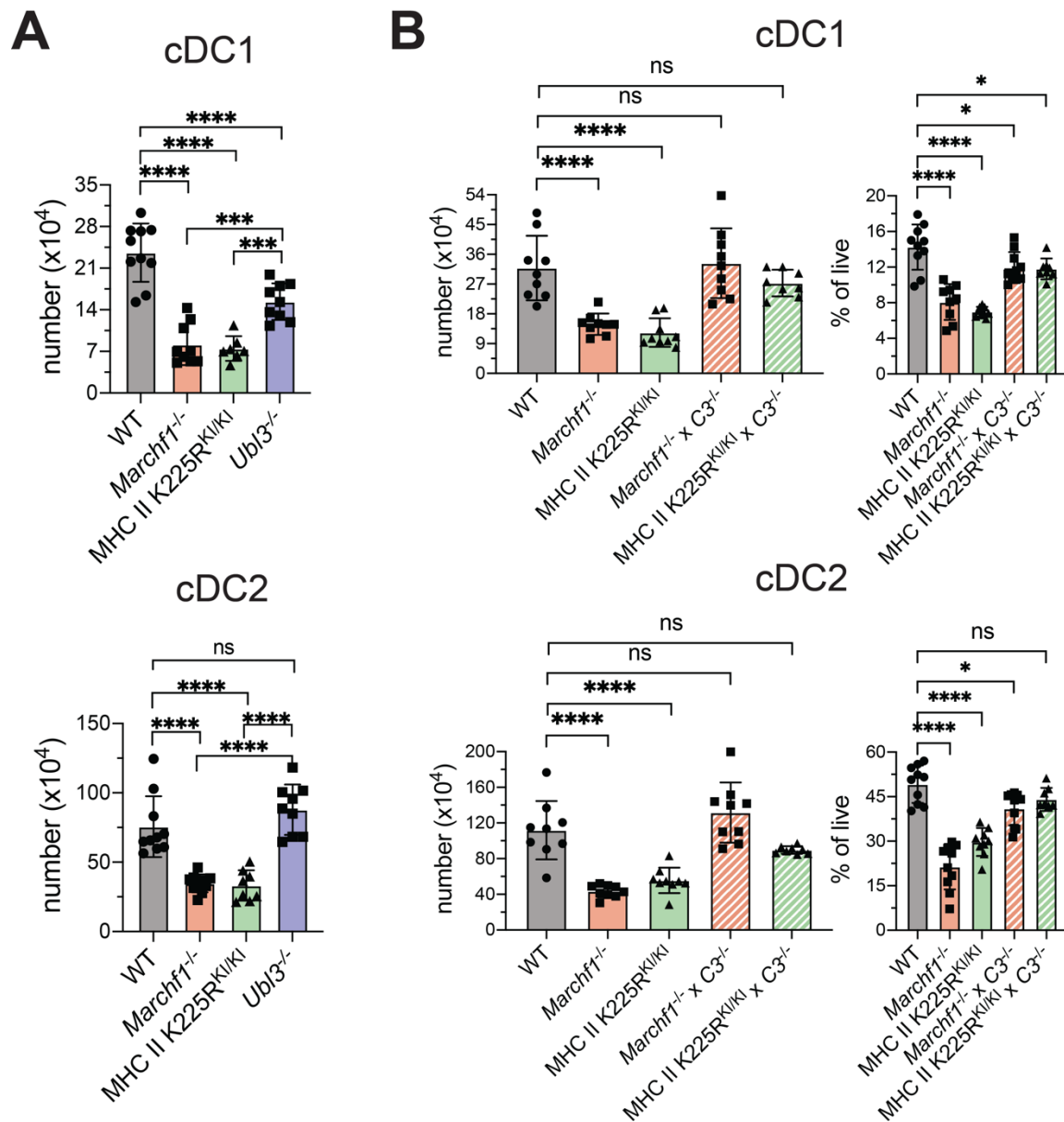


Figure 4.3 Enumeration of cDCs in the spleen of mice with varying C3 levels. Numbers and/or frequencies of splenic cDC1s and cDC2s from (A) WT, *Marchf1*^{-/-}, MHC II K225R^{KIKI} and *Ubi3*^{-/-} mice upon Nycodenz gradient centrifugation and (B) WT, *Marchf1*^{-/-}, MHC II K225R^{KIKI}, *Marchf1*^{-/-} x *C3*^{-/-} and MHC II K225R^{KIKI} x *C3*^{-/-} mice upon Nycodenz gradient centrifugation and subsequent Ab-based immuno-depletion of non-cDCs. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, bars designate mean ± SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.0002 (**), < 0.0001 (***).

In order to further verify the conclusion that C3 deposition is responsible for the loss of cDCs in *Marchf1*^{-/-} mice, we generated mixed BM chimeras where a 1:1 mix of WT and *Marchf1*^{-/-} BM was used to reconstitute WT or C3-deficient recipient mice. cDCs were purified from

spleens of recipient mice through gradient centrifugation and subsequent Ab-based immunodepletion of non-cDCs. We then determined frequencies of cDCs in their respective hematopoietic compartment (Ly5.1⁺ Ly5.2⁻ for WT and Ly5.1⁻ Ly5.2⁺ for *Marchf1*^{-/-}) as well as their total numbers (**Figure 4.4**). Confirming our previous observation, *Marchf1*^{-/-} cDCs were reduced in numbers and frequencies by ~50-60%, when compared to WT cDCs. Importantly, we did not observe any loss of *Marchf1*^{-/-} cDCs in *C3*^{-/-} recipient mice. Furthermore, numbers of *Marchf1*^{-/-} cDCs in C3-deficient recipient mice were enriched compared to WT mice (**Figure 4.4**), all indicating a role C3 in DC homeostasis in mice by reducing their numbers upon C3 deposition.

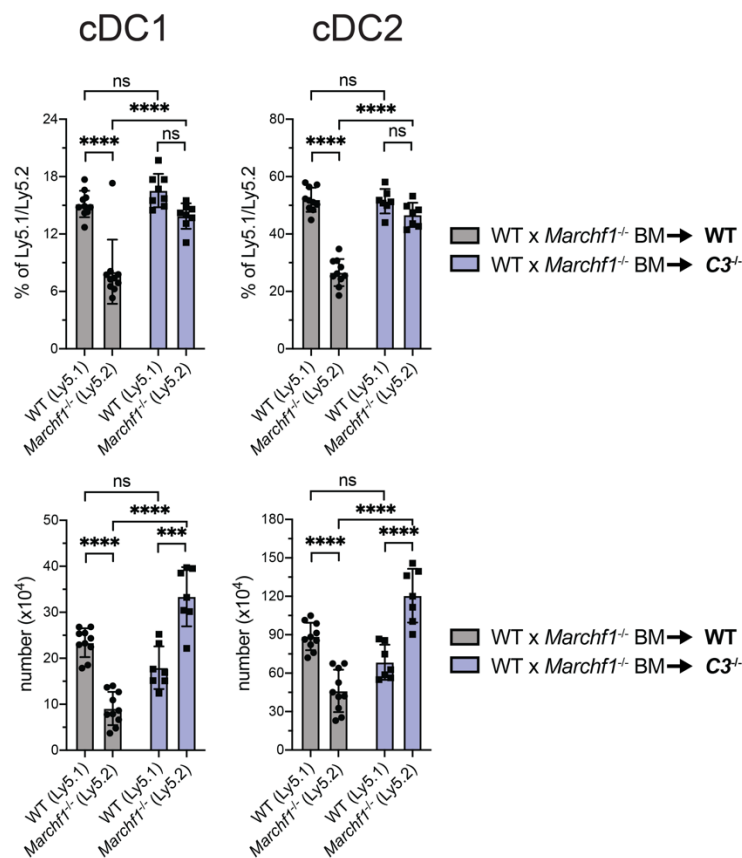


Figure 4.4 Enumeration of cDCs from mixed bone marrow chimera mice. Numbers and frequencies of WT and *Marchf1*^{-/-} cDC1s and cDC2s from mixed bone marrow (BM) chimera WT [grey] or *C3*^{-/-} [blue] recipient mice, both reconstituted with a 1:1 mix of WT (Ly5.1⁺ Ly5.2⁻) and *Marchf1*^{-/-} (Ly5.1⁻ Ly5.2⁺) BM. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, and bars designating mean ± SD. Two-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.0002 (**), < 0.0001 (****).

***C3*-dependent appearance of *CD11c*^{int} *CD24*^{high} *CD8*^{int} cells**

During our investigations on spleen cells in *Marchf1*^{-/-} mice we observed an unusual population of cells identified as *CD11c*^{int} *CD24*^{high} *CD8*^{int} (**Figure 4.5A**). This population was

barely detectable in WT low-density cell preparations but comprised approximately 20% of cells in their *Marchf1*^{-/-} counterparts and ~8% of all *Marchf1*^{-/-} splenocytes. The appearance of these cells in the spleen of MARCHF1-deficient mice was dependent on C3 expression as they were not detected in *Marchf1*^{-/-} x *C3*^{-/-} mice (**Figure 4.5B**).

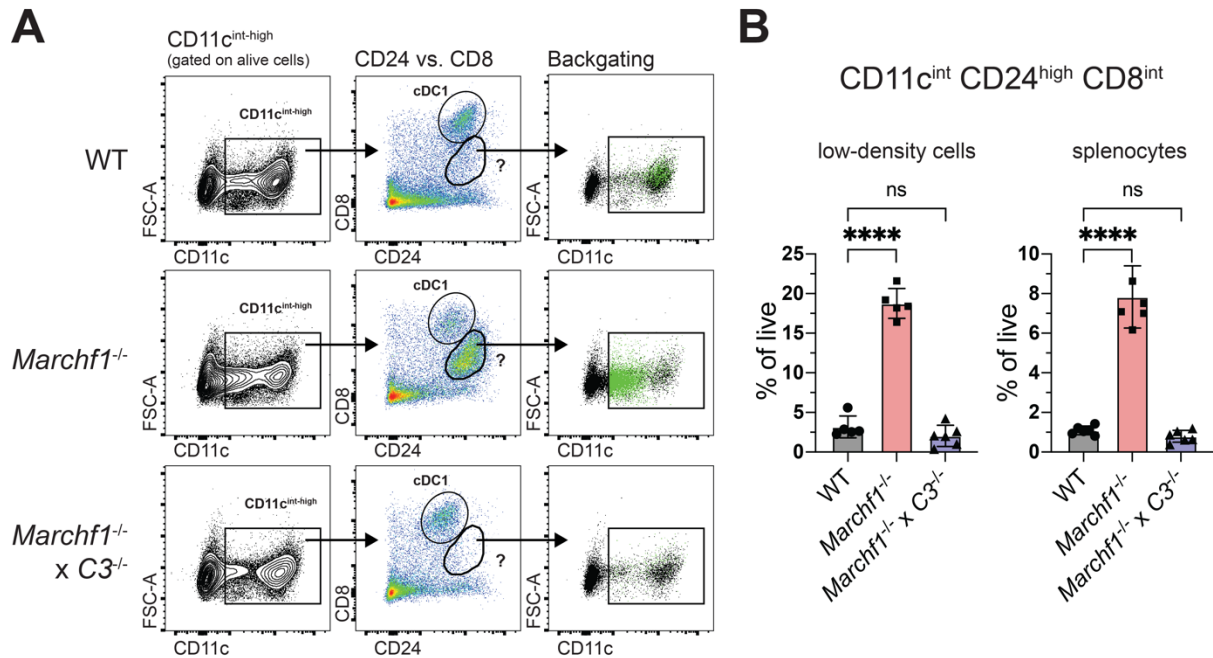


Figure 4.5 C3-dependent appearance of CD11c^{int} CD24^{high} CD8^{int} cells in *Marchf1*^{-/-} mice. (A) Representative gating strategies for the identification of CD11c^{int} CD24^{high} CD8^{int} cells from low-density splenocytes of WT, *Marchf1*^{-/-} and *Marchf1*^{-/-} x *C3*^{-/-} mice. Non-viable cells and cell doublets were eliminated [gates not shown] and cDC1s as well as CD11c^{int} CD24^{high} CD8^{int} cells were identified in CD24 versus CD8 profiles of CD11c^{int-high} cells. (B) Corresponding frequency of CD11c^{int} CD24^{high} CD8^{int} cells from whole spleen and low-density spleen cells. Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, and bars designating mean $\pm$ SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.0001 (****).

4.2.3 Characterization of CD11c^{int} CD24^{high} CD8^{int} cells

Surface marker phenotype

Considering CD11c^{int} CD24^{high} CD8^{int} cells were highly frequent in the spleen of *Marchf1*^{-/-} mice, we decided to further characterize these cells. All three surface CD markers (CD11c, CD24 and CD8) are expressed by a wide range of immune cells in the spleen, including B cells, T cells, macrophages, DCs and various granulocytes. We therefore expanded our investigation and looked at a wider set of surface markers, comparing expression levels in CD11c^{int} CD24^{high} CD8^{int} cells with the one in cells that are known to highly express the corresponding surface protein ('pos. control') (**Figure 4.6**).

Certain surface markers are expressed exclusively by cells of a distinct population, while others are expressed across various cell types. Specifically expressed surface protein complexes include TCR and CD3 (by T cells), Ig and CD19 (by B cells) as well as BST2 (Bone Marrow Stromal Cell Antigen 2) and SiglecH (by pDCs) and XCR1 (by cDC1s), whereas other surface markers are expressed across different cell types, including CD24 and CD62L (by lymphocytes), CD4 and CD8 (e.g. T cells and cDCs) as well as Clec12A (e.g. cDCs and pDCs) (**Figure 4.6A**).

Flow cytometry analyses revealed that CD11c^{int} CD24^{high} CD8^{int} cells highly express the B cell molecules B220, CD19, IgD and IgM, but not IgG1, at levels similar to B cells. They also showed moderate levels for surface CD62L, a marker for innate lymphocytes [734] but did not express pDC markers BST2 or SiglecH, T cell molecules CD3 and TCR, the NK cell marker NK1.1, nor the monocyte/granulocyte/neutrophil marker Ly6G (**Figure 4.6B**), indicating that CD11c^{int} CD24^{high} CD8^{int} cells were B cells. Indeed, CD11c expression has been described in a subset of B cells [170], but CD8 surface expression in B cells is highly unusual and only described in case of lymphoma [735][736]. Strikingly, our investigations revealed the expression of various surface cDC markers in these cells (**Figure 4.6B**). These included MHC II, CD86, CD53 and CD36, which are indeed known to be also expressed by B cells, but importantly, surface markers highly specific for cDC1s, such as XCR1, Flt3 and Clec9A were also expressed by CD11c^{int} CD24^{high} CD8^{int} cells, though all were expressed at lower levels than on *Marchf1*^{-/-} cDC1s. The cDC2 surface markers CD11b and CD4 were also expressed at intermediate levels by CD11c^{int} CD24^{high} CD8^{int} cells but no expression for ESAM was observed (**Figure 4.6B**).

We therefore concluded that the lack of MHC II ubiquitination and the consequential deposition of C3 on the surface of cDCs in the spleen of *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice, reduced cDC numbers but caused the accumulation of an unusual cell population with both cDC and B cell surface phenotype.

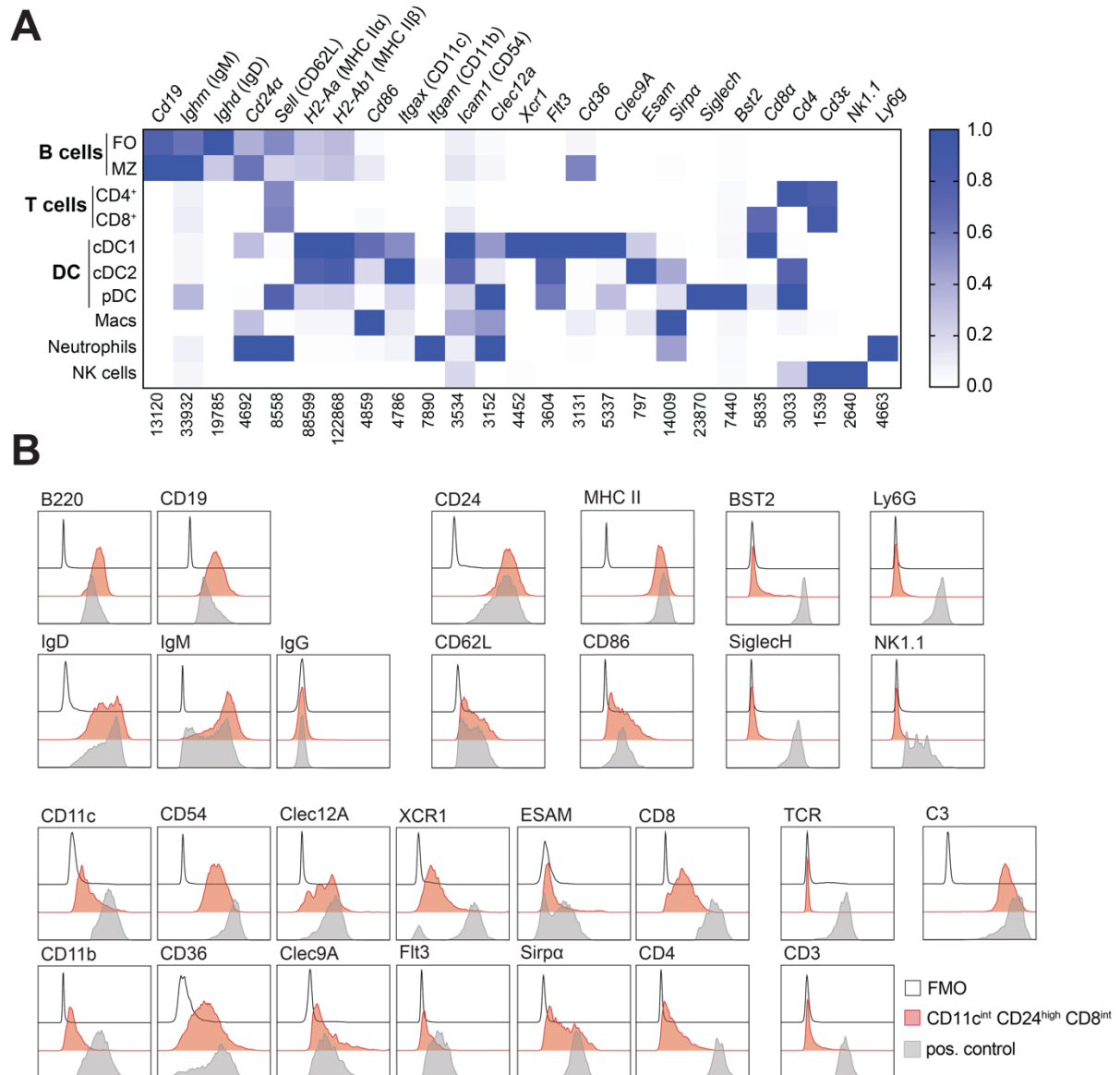


Figure 4.6 Surface marker phenotype of CD11c^{int} CD24^{high} CD8^{int} cells. (A) Relative mRNA levels of various surface markers in spleen follicular (FO) and marginal zone (MZ) B cells, naïve CD4⁺ and CD8⁺ T cells, pDCs and both cDC subsets (cDC1s and cDC2s) as well as macrophages (macs), neutrophils and NK cells based on ULI RNAseq. data from <http://www.immgen.org> [normalized to highest expression value of each gene (indicated in each column in the heatmap) across the different cell types). (B) Expression levels of various surface proteins in spleen CD11c^{int} CD24^{high} CD8^{int} cells of *Marchf1*^{-/-}/MHC II K225R^{KI/KI} mice compared to surface levels of cells that are known to express the corresponding marker (in grey pos. control: B220, CD19, IgM, IgD, CD24 and CD62L B cells, CD11c, CD86 and MHC II cDCs, BST2 and Siglech pDC, Ly6G neutrophils, Nk1.1 NK cells, XCR1 Clec9A, Clec12A, Flt3, CD54 and CD36 cDC1s, Sirpα, ESAM and CD11b cDC2s and CD4, CD8, CD3 and TCR T cells). Histograms are representative of at least two independent experiments for which cells were FcR-blocked before staining with flow cytometry Abs.

Antigen presentation

Considering CD11c^{int} CD24^{high} CD8^{int} cells exhibited intermediated to high levels of cDC-typical surface proteins, the main function of which is Ag presentation, we investigated their ability to present soluble Ag to T cells. For this cDC1s from WT and *Marchf1*^{-/-} as well as CD11c^{int} CD24^{high} CD8^{int} cells from *Marchf1*^{-/-} mice were sorted to purity by flow cytometry (> 95% purity), pulsed with soluble OVA protein and incubated with purified OVA-specific OT-I or OT-II T cells. OT-I and OT-II T cells were CellTrace Violet(CTV)-labelled and Ag presentation was assessed based on numbers of dividing/proliferating T cells indicated by CTV-dilution (**Figure 4.7**).

WT and *Marchf1*^{-/-} cDC1s successfully stimulated OVA-specific OT-I and OT-II T cells in a dose-dependent manner. As seen previously (Figure 4.1 and [535]), Ag presentation and induction of T cells through *Marchf1*^{-/-} cDC1s was reduced compared to WT cDCs, indicated by lower the number of dividing OT-I and OT-II cells. Surprisingly, CD11c^{int} CD24⁺ CD8^{int} cells completely lacked Ag presentation capacity (**Figure 4.7**), suggesting these cells were not functional DCs despite their expression of typical cDC surface markers.

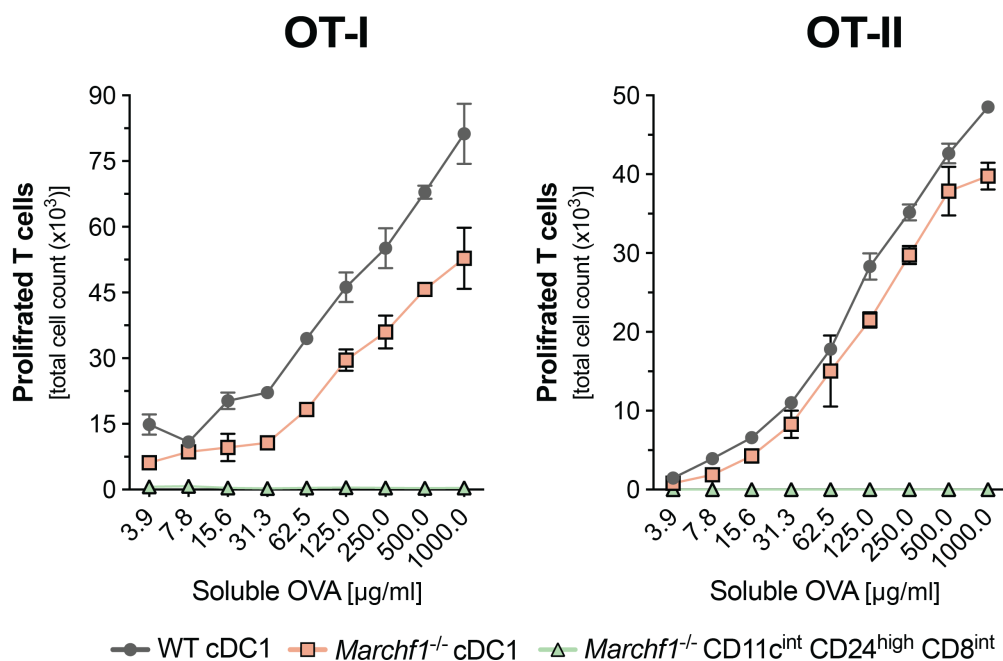


Figure 4.7 Antigen presentation capacity of CD11c^{int} CD24^{high} CD8^{int} cells. Purified CellTrace Violet (CTV)-labelled OT-I and OT-II T cells were incubated in triplicates for 72 h with either CD11c^{int} CD24^{high} CD8^{int} cells from *Marchf1*^{-/-} mice or cDC1s from WT and *Marchf1*^{-/-} mice upon CpG activation and pulsing with increasing doses of soluble OVA. Ag presentation capacity was assessed by flow cytometric analysis of CTV-dilution in order to determine numbers of proliferating and dividing OT-I/OT-II T cells. Each data point represents the measurements of a triplicate, displayed as mean and bars designating mean ± SD.

Transcriptome profiling

The identification of CD11c^{int} CD24^{high} CD8^{int} cells as '*B cell-cDC hybrid*' cells based on their surface marker signature, resembling both cDCs and B cells, led to the question if these cells were part of the B cell or cDC lineage, or if they were an entirely different population with both cDC and B cell properties. We therefore carried out transcriptome profiling, for which CD11c^{int} CD24⁺ CD8^{int} cells, B cells and cDC1s from *Marchf1*^{-/-} spleens, and B cells and cDC1s from WT spleens were sorted to purity by flow cytometry (> 95% purity). mRNA was then isolated from all five groups and whole transcriptome sequencing was carried out by Illumina sequencing (Illumina Hi Seq 2500) (**Figure 4.8**).

Transcriptomic profiling revealed over 6,500 genes differentially expressed between cDC1s, B cells and CD11c^{int} CD24^{high} CD8^{int} cells, with very few differences observed between WT and *Marchf1*^{-/-} cDC1s. Notably, the transcriptome profile of CD11c^{int} CD24⁺ CD8^{int} cells was very similar to those of WT or *Marchf1*^{-/-} B cells but very distinct from either WT or MARCHF1-deficient cDC1s (**Figure 4.8A**).

Representation of these RNAseq data in a multi-dimensional scaling plot using the 500 most differentially expressed genes resulted in a similar outcome: cDC1s from WT and *Marchf1*^{-/-} were clustered together in the transcriptomic space distinct from WT and *Marchf1*^{-/-} B cells, indicating very dissimilar transcriptome profiles between cDC1s and B cells. MARCHF1-deficient cDC1s showed only little differences in their gene expression signatures compared to their WT counterparts and CD11c^{int} CD24⁺ CD8^{int} were closely related to B cells but distinct from cDC1s (WT and *Marchf1*^{-/-}). However, some dissimilarities between CD11c^{int} CD24⁺ CD8^{int} cells and between WT and *Marchf1*^{-/-} B cells were observed in the transcriptomic space, indicating differences in the leading fold-change of the top 500 genes (**Figure 4.8B**).

Transcription levels of individual genes characteristic of B cells and cDC1s based on raw mRNA counts revealed that CD11c^{int} CD24⁺ CD8^{int} did not express the cDC1 genes *Cd8a*, *Itgax* (CD11c), *Xcr1*, *Flt3* or *Clec9a* but did express the B cell genes *Cd19*, *Ighd* and *Igm* (Ig heavy constant mu and delta) as well as *Cr2* and *Cd79a*. Interestingly, mRNA counts for *Ighm* and *Cr2* were higher but lower for *Ighd* in CD11c^{int} CD24⁺ CD8^{int} when compared to B cells (**Figure 4.8C**).

Overall, RNAseq profiling therefore suggested that CD11c^{int} CD24⁺ CD8^{int} cells in *Marchf1*^{-/-} mice belong to the B cell lineage without transcriptional expression of cDC surface markers, although proteins were present at their cell surface at significant levels.

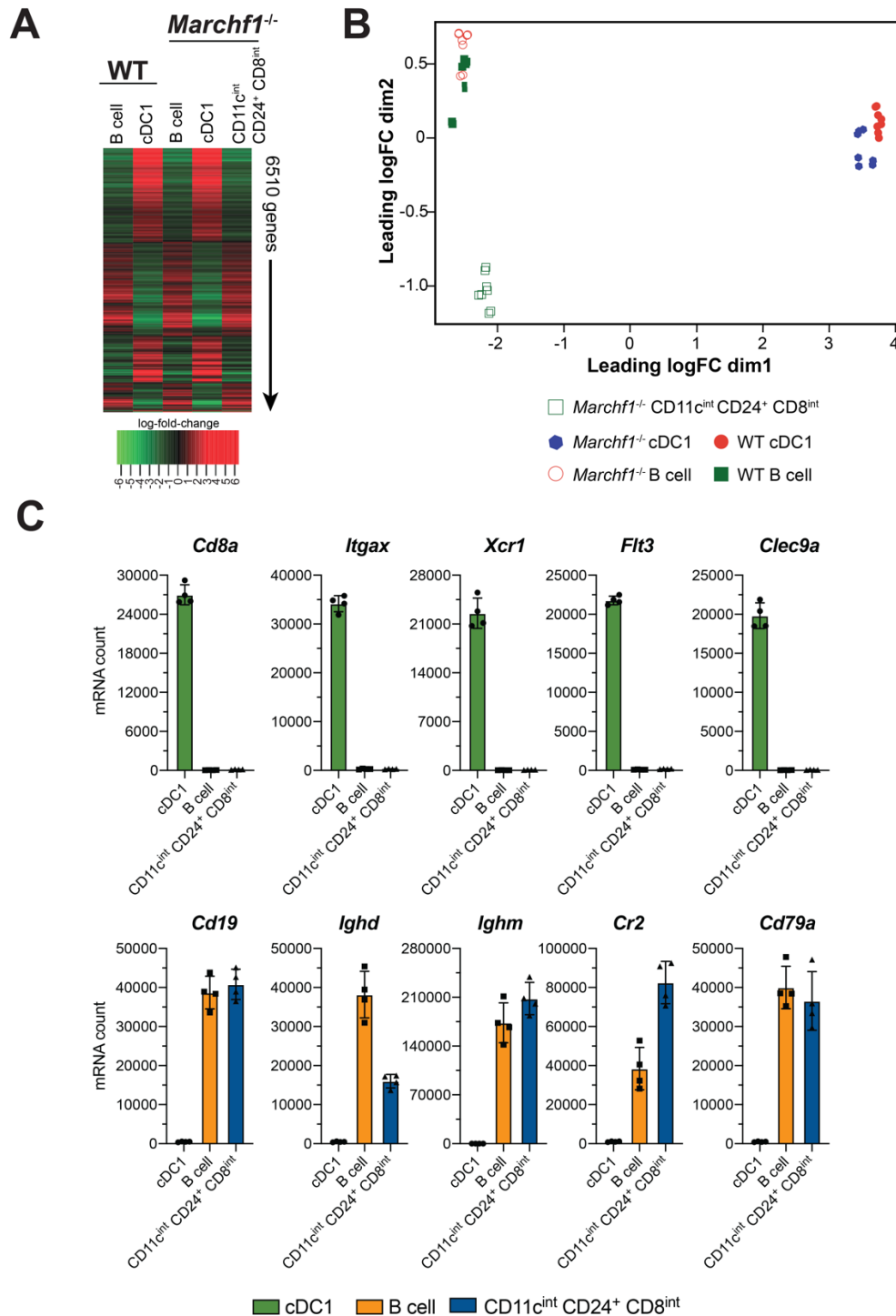


Figure 4.8 Transcriptome profiling of CD11c^{int} CD24⁺ CD8^{int} cells. RNAseq of sort-purified CD11c^{int} CD24⁺ CD8^{int} cells from *Marchf1*^{-/-} mice, as well as B cells and cDC1s from WT and *Marchf1*^{-/-} mice. **(A)** Heatmap of 6,500 differentially expressed genes across the five groups. **(B)** Multi-dimensional scaling plot of log-CPM values over dimensions 1 and 2, indicating similarity or dissimilarity between sample groups. Distances on the transcriptomic space correspond to the leading fold-change of the top 500 genes, differentially expressed. **(C)** Raw mRNA counts for genes characteristic of cDC surface markers [*Cd8a*, *Itgax* (CD11c), *Xcr1*, *Flt3* and *Clec9a*] and B cell surface markers (*Cd19*, *Ighm*, *Igha*, *Cr2* and *Cd79a*) in *Marchf1*^{-/-} cDC1s, B cells and CD11c^{int} CD24⁺ CD8^{int} cells.

C3-dependent surface expression of CD8 and CD11c on splenic B cells

Transcriptome profiling revealed that CD11c^{int} CD24⁺ CD8^{int} cells belong to the B cell lineage, displaying several cDC-specific surface proteins without transcriptional expression for these. The appearance of cDC markers on the surface of B cells was dependent on C3 expression as they appeared in MARCHF1-deficient but not in *Marchf1*^{-/-} x *C3*^{-/-} mice. To further verify that binding of C3 to MHC II on the surface of cDCs was responsible for the appearance of cDC markers on the surface of B cells, we {1} examined CD8 and CD11c levels in splenic B cells from mutant mice with varying levels of MHC II-dependent C3 deposition on their cDCs and {2} generated mixed BM chimeras where a 1:1 mix of WT and *Marchf1*^{-/-} BM was used to reconstitute WT or C3-deficient recipient mice (**Figure 4.9**).

Mutant strains with enriched C3 binding to MHC II on the surface of splenic cDCs, namely *Marchf1*^{-/-}, MHC II K225R^{KI/KI} and *Ubl3*^{-/-} mice, harbored approximately 10-20% of CD8⁺ and 6-12% of CD11c⁺ B cells (from Nycodenz gradient centrifugation preparations). CD11c and CD8 was barely detectable in B cells of neither WT nor mutant mice that also lacked C3 expression cells (i.e. *Marchf1*^{-/-} x *C3*^{-/-} and MHC II K225R^{KI/KI} x *C3*^{-/-}). Furthermore, MARCHF1-deficient mice that also lacked surface MHC II complexes (*Marchf1*^{-/-} x *IA-α*^{-/-}) also lacked CD8⁺ and CD11c⁺ B cells, confirming that both C3 and MHC II expression was essential in *Marchf1*^{-/-} mice for the appearance of cDC markers on B cells (**Figure 4.9A**).

Furthermore, mixed bone marrow chimera experiments confirmed this conclusion, as WT but not *C3*^{-/-} recipient mice, that were reconstituted with a 50:50 mice of WT and *Marchf1*^{-/-} BM, harbored CD8⁺ and CD11c⁺ B cells. Notably, the appearance of CD8 and CD11c was similar between WT (Ly5.1⁺ Ly5.2⁻) and *Marchf1*^{-/-} (Ly5.1⁻ Ly5.2⁺) B cells (**Figure 4.9B**).

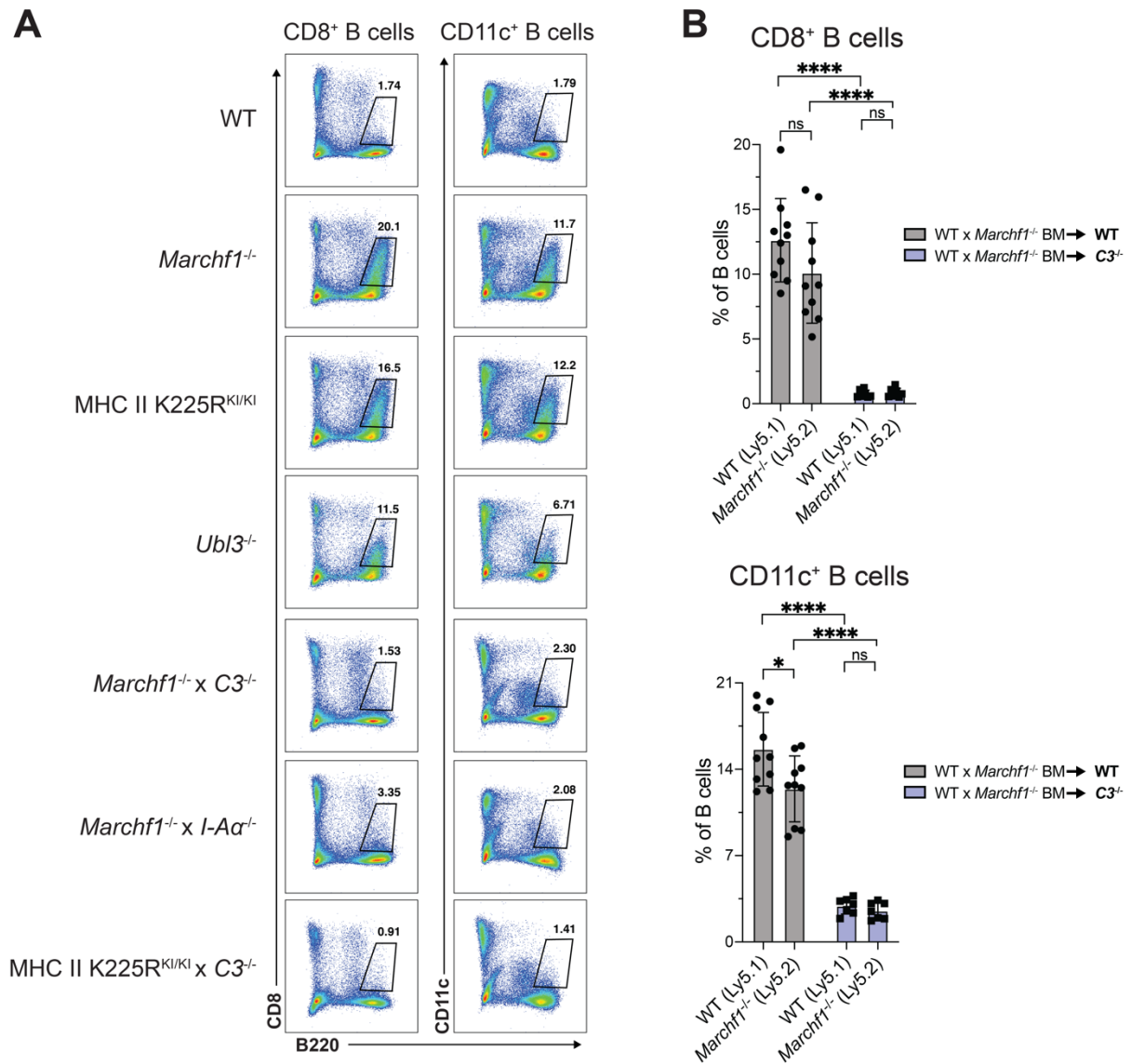


Figure 4.9 C3-dependent appearance of splenic CD8⁺ and CD11c⁺ B cells. (A) B220 vs. CD8 and B220 vs. CD11c plots of low-density splenocytes from WT, *Marchf1*^{-/-}, MHC II K225R^{KI/KI}, *Ubl3*^{-/-}, *Marchf1*^{-/-} x *C3*^{-/-}, *Marchf1*^{-/-} x *I-Aα*^{-/-} and MHC II K225R^{KI/KI} x *C3*^{-/-} mice. Non-viable cells and cell doublets were eliminated (gates not shown) and CD8⁺ and CD11c⁺ B cells were identified as B220^{high} and CD8⁺ or CD11c⁺, respectively. Gatings are representative of at least two independent experiments. **(B)** Numbers of WT and *Marchf1*^{-/-} CD8⁺ and CD11c⁺ B cells from mixed bone marrow (BM) chimera WT [grey] or *C3*^{-/-} [blue] recipient mice, both reconstituted with a 1:1 mix of WT (Ly5.1⁺ Ly5.2⁻) and *Marchf1*^{-/-} BM (Ly5.1⁻ Ly5.2⁺). Graphs display data pooled from a minimum of two independent experiments, with each symbol representing an individual mouse, and bars designating mean ± SD. Two-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.03 (*), < 0.0001 (****).

Gating for B cell subpopulation

Transcriptome profiling identified CD11c^{int} CD24⁺ CD8^{int} cells as B cells. We next aimed to identify if these cells belong to a specific B cell subpopulation. In brief, B cells develop in the bone marrow from where they migrate into the spleen to further develop from type 1

transitional (T1) to type 2 and type 3 transitional (T2 & T3) B cells, which then give rise to follicular (FO) or marginal zone (MZ) B cells in the primary follicles of the spleen (see section 1.2.1) [42]. Differential surface expression of IgM and IgD as well as CD21/CD35 and CD23 can be used to distinguish B cell subsets in the spleen (**Figure 4.10A**). Gating for transitional (T1-T3), FO and MZ B cells from whole splenocytes of WT and *Marchf1*^{-/-} mice did not reveal any major differences in the composition of B cell subsets between the two mouse strains (**Figure 4.10B**). Our RNAseq analyses indicated enriched expression of *Cr2*, *Ighm* but reduced levels of *Ighd* in CD11c^{int} CD24⁺ CD8^{int} cells when compared to bulk B cells (**Figure 4.8C**), indicating higher CR2 (CD21/CD35) and IgM but lower IgD expression, which in turn is characteristic for MZ B cells. Consistent with this, gating of CD11c^{int} CD24⁺ CD8^{int} cells for the different B cell subpopulations revealed a mature MZ B cell phenotype, displaying high levels of surface IgM and CD21/CD35 (**Figure 4.10B**). This suggests that MZ B cells in the spleen of mice with high C3 binding to MHC II at the surface of splenic cDCs, express cDC proteins at their surface.

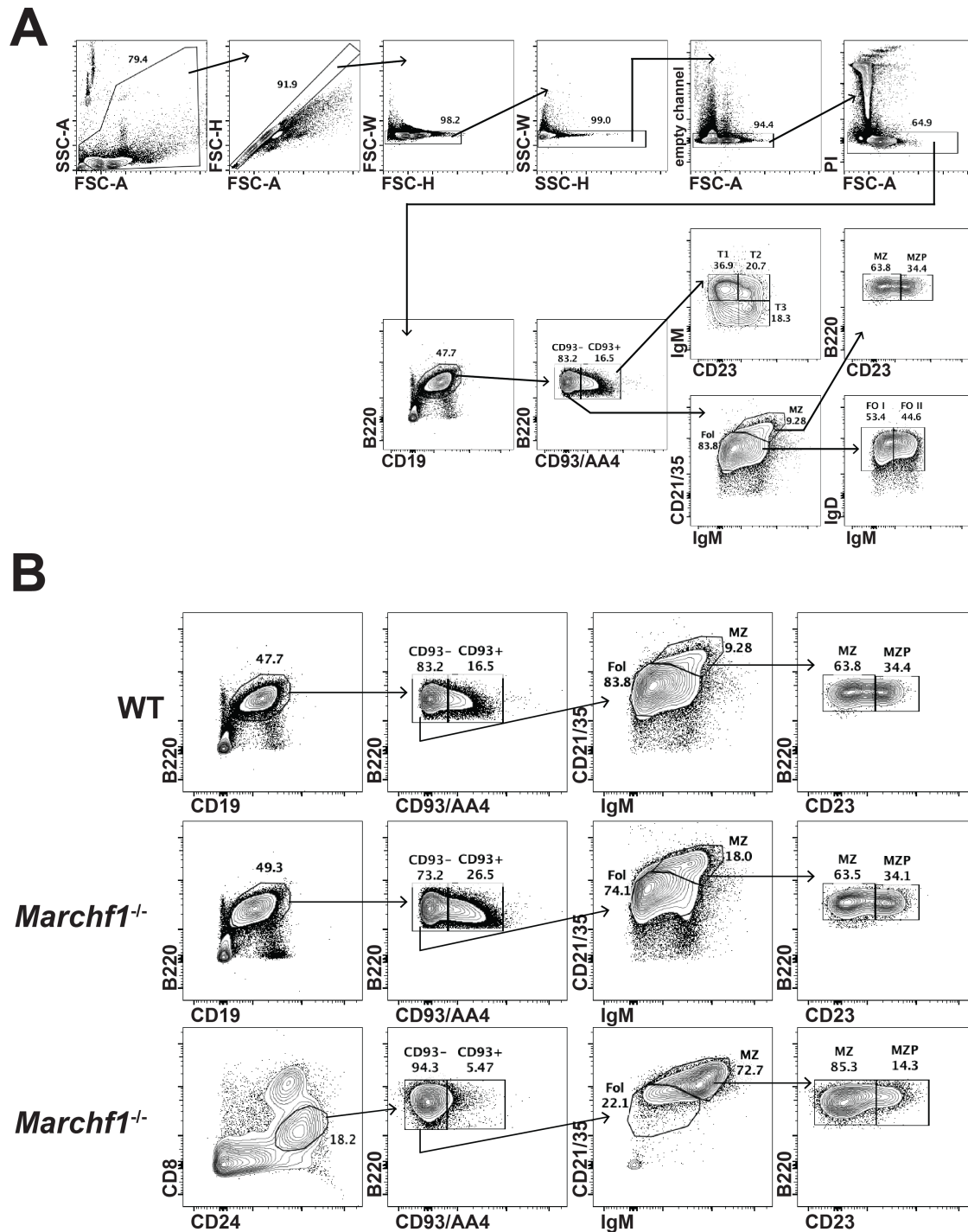


Figure 4.10 Gating of CD11c^{int} CD24⁺ CD8^{int} cells for B cell subpopulations. (A) Flow cytometry gating strategy for B cell subpopulations (T1-T3, FO and MZ) from whole splenocytes of WT mice (based on Allman *et al.* [737][738]). **(B)** Gating strategy for MZ B cells from whole splenocytes of WT and *Marchf1*^{-/-} mice (top and middle panel) and from splenic CD11c^{int} CD24⁺ CD8^{int} cells from *Marchf1*^{-/-} mice [bottom panel]. Non-viable cells and cell doublets were eliminated [gating as shown in (A)] and MZ B cells were identified as B220⁺, CD93⁻, IgM^{high}, CD21/35^{high} and CD23⁻, while progenitor MZ (MZP) B cells were identified as B220⁺, CD93⁻, IgM^{high}, CD21/35^{high} and CD23⁺.

4.3 Discussion

This chapter describes the biological impact of enriched C3 fixation on cDCs in mice with impaired MHC II ubiquitination. Covalent binding of C3 to MHC II at the surface of cDCs had no impact on MHC II-restricted Ag presentation but caused a significant loss of splenic cDCs, as well as the appearance of cDC markers at the surface of MZ B cells. RNAseq identified the lack of transcriptional expression for these surface markers in MZ B cells.

4.3.1 C3 binding to MHC II does not impact antigen presentation

It is known that Ag-associated complement C3 participates in the regulation of Ag presentation to B cells in lymph nodes, promoting GC formation through its co-ligation with the BCR and CD21 (+CD19+CD81) [639][637] as well as through retaining intact Ag on the surface of FDCs [739][94][727]. Its regulatory role in Ag presentation to T cells by cDCs has been reported by Villier *et al.* and Baudino *et al.* describing the inhibition of lysosomal degradation of Ag through C3 opsonization in order to prolong Ag stability in DCs and to enhance MHC-restricted Ag presentation [576][575]. To assess the direct effect of C3 binding to surface MHC II in cDCs on their ability to present Ag, we carried out *in vitro* Ag presentation assays with OVA-specific T cells (OT-I and OT-II). For this, purified primary cDCs were first incubated with soluble OVA protein and TLR9 agonist CpG, which led to DC activation as well as the uptake of OVA, its intracellular processing and finally the presentation of OVA-derived peptides in MHC molecules at the cell surface. OVA-pulsed cDCs were then incubated with OT-I and OT-II T cells to assess Ag presentation based on T cell proliferation. This experimental set up is a well-established assay to examine the capacity of cells to process and present Ag for MHC-restricted stimulation of T cells, demonstrated in numerous studies in the past [535][561][740][217][741].

As previously described [535], we observed impaired Ag presentation capacity in MARCHF1-deficient cDCs compared to WT cDCs, based on numbers of dividing OT-I and OT-II T cells. Reduced stimulation of CD4⁺ OT-II T cells by *Marchf1*^{-/-} cDCs, lacking MHC II ubiquitination, might be a consequence of decreased Ag processing and reduced loading of antigenic peptides into MHC II due to its impaired trafficking and turnover. Decreased Ag presentation to OT-I CD8⁺ T cells is likely due to reduced MHC I surface expression, which has been described as an indirect consequence of the lack of MHC II ubiquitination in cDCs [561]. However, we did not observe significant differences in the Ag presentation between

MARCHF1-deficient and *Marchf1*^{-/-} x *C3*^{-/-} cDC1s, indicating no direct effect of C3 binding to the surface of cDC1s, with regard to their Ag presentation capacity. Our biochemical analyses demonstrated that C3 deposition on the surface of cDC1s was due to its specific binding to MHC II α (see Chapter 3), so that no effect on MHC I-restricted presentation was expected. Additionally, we described that binding of C3 to MHC II α occurs specific to its carbohydrate. The carbohydrate of MHC II α is distant from its peptide binding cleft and the T cell coreceptor CD4 binds to the β chain of MHC II [742][743], so that binding of C3 to MHC II via its glycosylation chain likely does not affect its interactions with TCR or CD4, allowing effective signal transduction and T cell stimulation.

Ag presentation assays can also be carried out *in vivo*, in which proliferation of adoptively transferred OT-I and OT-II T cells is assessed upon immunization of mice with OVA antigen. However, we did not carry out *in vivo* Ag presentation assays as *Marchf1*^{-/-} mice harbored significantly less splenic cDCs, which was restored in *Marchf1*^{-/-} x *C3*^{-/-} mice, so that potential differences in Ag presentation could not be allocated to either altered cDC numbers or C3 binding to MHC II in this kind of experiment.

4.3.2 C3 deposition on cDCs causes their reduction in the spleen

The complement system is considered as an evolutionary ancient part of the innate immune system [567] that can discriminate between host and pathogens [583]. Cleavage of complement C3 is the central activation step in the system and can be induced through various mechanisms of the three known activation pathways. Two of these, namely the lectin and classical activation pathway, require recognition of pathogen-specific surface structures, such as PAMPS or cell surface bound antibodies [583][744][745], whereas the alternative pathway primarily uses reverse recognition: spontaneous hydrolysis of C3, known as *tick-over*, constitutively activates complement at low rate during steady-state and causes the binding of C3 to host-associated molecular pattern (HAMPs). These usually suppress further complement activation but in the absence of such host markers local complement amplification is induced [583]. Factor I and Factor H are the main complement suppressing regulators. Deficiency and mutations in Factor H are known to be associated with severe autoimmune diseases [746][747][748]. On the other hand, properdin is considered as a complement activating HAMP, promoting the clearance of tumor and apoptotic cells [749][750]. Other negative regulators suppressing amplification of spontaneously activated

C3 on host cell surfaces include DAF (CD55), MCP (CD46), CR1 and CD59 [596][583], while Factor I actively cleaves cell surface bound C3 [594][751].

We have identified MHC II in cDCs as a major acceptor molecule for spontaneously activated C3 through its covalent association at the cell surface. Enriched MHC II surface expression due to altered MHC II ubiquitination in cDCs led to enriched C3 surface deposition. We also identified enriched surface levels of negative complement regulators Factor H and CD55, indicating the alternative pathway as activation mechanism. In order to assess if impaired MHC II ubiquitination in cDCs and the consequential C3 deposition leads to their depletion, we determined cell numbers of various splenocytes in mutant mice and bone mixed chimera mice. First enumerations of major immune cells in the spleen revealed that cDCs were reduced by 50-60% in *Marchf1*^{-/-} mice while numbers of other immune cells, such as lymphocytes (T and B cells), macrophages, pDCs and granulocytes, were unaltered. Previously (Chapter 3), we have demonstrated that although *Marchf1*^{-/-} B cells and cDCs exhibit similarly elevated amounts of surface MHC II molecules, binding of C3 to MHC II is specific to cDCs. This provides the first indication that the loss of cDCs in *Marchf1*^{-/-} mice is due to C3 deposition and not due to the lack of MHC II ubiquitination. Further investigations confirmed this hypothesis: {1} cDC numbers were restored in *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice that also lacked C3 expression. {2} The degree of C3 deposition on splenic cDCs across various mutant mice correlated with the degree of their depletion. While *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice showed equally high deposition of C3 on their cDCs, causing a loss of cDCs by ~50-60% in both strains, C3 deposition in *Ubl3*^{-/-} mice was lower which translated into a less severe cDC depletion. Little C3 deposition on *Ubl3*^{-/-} cDC2s had no effect on their numbers in these mice, whereas *Ubl3*^{-/-} cDC1s exhibited some but lower C3 surface signal, when compared to cDC1s from MARCHF1-deficient or MHC II K225R^{KI/KI} mice, causing a reduction by only ~20%. Notably, C3 deposition in both *Marchf1*^{-/-} or MHC II K225R^{KI/KI} mice was approximately 4-5 times higher on splenic cDC1s than on cDC2s, which also led to a greater depletion of cDC1s than of cDC2s (~60% versus ~45%). Considering the amount of C3 on cDC1s was ~4-5 times higher than on cDC2s, a more severe difference in the reduction between the two cDC subsets might have been expected. However, C3 deposition and cell depletion do not necessarily correlate proportionally and cell depletion might be induced when a certain level/threshold of deposited C3 molecules has been reached at the cell surface, exceeded on both cDC subsets in these mice. {3} Finally, bone mixed chimera experiments further validated the

reduction of C3-opsonised cDCs, in which only *Marchf1*^{-/-} cDCs were reduced in WT but not in C3-deficient recipient mice, demonstrating that MHC II-dependent deposition of extracellular C3 on the surface of cDCs determines their depletion. Interestingly, numbers of cDCs in *C3*^{-/-} recipient mice were significantly enriched when compared to WT-recipient mice, further indicating a regulatory role for C3 in cDC homeostasis in WT mice, though this was only the case for *Marchf1*^{-/-} cDCs. It is unclear why this is the case, however enumeration of total cell numbers in mixed bone marrow chimera experiments may be misleading as reconstitution of BM might vary between both compartments (*here* WT versus *Marchf1*^{-/-} BM) within individual recipient mice and also between different recipient mice (*here* WT versus *C3*^{-/-} host), so that relative proportion of cDCs in their respective BM compartment (% of Ly5.1⁺/Ly5.2⁻ and Ly5.1⁻/Ly5.2⁺) is the more reliable readout.

Overall, these results indicate that MHC II ubiquitination in cDCs functions as a clearance mechanism for spontaneously activated C3 of the alternative pathway, preventing their depletion. Enumerations of cDCs in other tissues than the spleen will aim to answer the question if C3-dependent depletion is restricted to the splenic cDCs or if the degree of cDC loss corresponds to the level of C3 deposition across various lymphoid tissue cDCs in case this mechanism occurs systemically (i.e. comparison spleen, LN and thymus).

4.3.3 Surface expression of cDC markers in MZ B cells

The second major observation we made during our investigations on *Marchf1*^{-/-} splenocytes was the expression of cDC surface markers on MZ B cells. The appearance of these surface markers was independent of their transcriptional gene expression within the MZ B cells but strictly depended on C3 expression in mutant mice with impaired MHC II ubiquitination. We initially discovered this observation by the discovery of accumulated CD11c^{int} CD24^{high} CD8^{int} cells in the spleen of *Marchf1*^{-/-} mice. Examination of the surface marker phenotype of these cells, identified high expression levels of the B cell markers CD19, B220 and immunoglobulin (IgM and IgD), strongly indicating their B cell lineage. However, surprisingly we also detected intermediate levels of various cDC surface markers with a general trend for higher expression levels of cDC1 surface proteins (e.g. CD8, XCR1 and CD36) than of cDC2-specific markers (e.g. CD11b, CD4 and ESAM). Considering that the appearance of cDC surface markers on MZ B cells was dependent on the expression of C3 in *Marchf1*^{-/-} mice, higher C3 deposition on cDC1s, when compared to cDC2s, is a likely reason for enriched expression of cDC1 markers

on MZ B cells. Interestingly, CD11c^{int} CD24^{high} CD8^{int} cells also exhibited C3 on their cell surface to a similar level as *Marchf1*^{-/-} cDC1s. Considering CD11c^{int} CD24^{high} CD8^{int} cells were identified as MZ B cells, high C3 surface expression was surprising as we previously have demonstrated C3 surface levels on *Marchf1*^{-/-} B cells were low (see Chapter 3). However, this contradictory observation can be explained by the fact that previous gating strategies for B cells excluded any CD8⁺ cells (see Figure 2.1 in 'Materials and Methods' section on page 79), which means C3 expressing MZ B cells (CD11c^{int} CD24^{high} CD8^{int} cells) were also excluded in these analyses.

Surface expression of typical cDC surface markers in these cells did not convey Ag presentation capacity as indicated by our *in vitro* Ag presentation assay in which OVA-pulsed CD11c^{int} CD24^{high} CD8^{int} cells completely failed to induce OT-I and OT-II T cell proliferation. This was surprising as B cells are considered as professional antigen presenting cells [348] and various studies have demonstrated Ag presentation capacity by MZ B cells in *in vitro* and *in vivo* assays with the model Ag OVA [752] as well as through immunization with virus-derived nanoparticle Ag [753]. However, another study by Bennett *et al.* has demonstrated that Ag presentation through OVA-pulsed B cells is involved in T cell tolerance rather than their proliferation and expansion [754]. Overall, our Ag presentation assay has demonstrated that CD11c^{int} CD24⁺ CD8^{int} cells were not able to present Ag to T cells, a functional hallmark of cDCs, which demonstrated their lack of cDC lineage.

RNAseq of CD11c^{int} CD24^{high} CD8^{int} cells accomplished two major outcomes: {1} the definite identification of their B cell lineage and {2} the discovery of their lack of transcriptional expression for cDC-specific markers (i.e. genes for CD8, CD11c, XCR1, Flt3 and Clec9A). The heatmap provides an overview of more than 6500 differentially expressed genes and clearly identified a similar expression profile between CD11c^{int} CD24^{high} CD8^{int} cells and WT or *Marchf1*^{-/-} B cells, distinct from cDC1s of either WT or MARCHF1-deficient mice. Representation of RNAseq results in a multi-dimensional scaling plot confirmed this outcome, but also identified some differences in the transcriptome profiles between CD11c^{int} CD24^{high} CD8^{int} cells and *Marchf1*^{-/-} and WT B cells, which were not detectable in the heatmap. These differences are most likely not due to the lack of MARCHF1 expression, as MARCHF1 deficiency is expected to have only little impact on the overall gene expression, indicated by tight clustering of WT and *Marchf1*^{-/-} cDC1s in the multi-dimensional scaling plot as well as low numbers of differentially expressed proteins identified in our proteomic analyses (see section 3.1.2). A more plausible explanation for this is a discrepancy between the tested

groups: while the group of WT B cells contained the entire B cell population (B220⁺ CD19⁺), cell sorting through flow cytometry of *Marchf1*^{-/-} B cells separated CD11c^{int} CD24^{high} CD8^{int} cells from the bulk B cells population. As CD11c^{int} CD24^{high} CD8^{int} cells were later identified as MZ B cells, this means MZ B cells were excluded from the group of *Marchf1*^{-/-} B cells, explaining the observed differences in the transcriptome profiles between WT and *Marchf1*^{-/-} B cells. Subsequent flow cytometry analysis identified that CD11c^{int} CD24^{high} CD8^{int} cells were indeed MZ B cells. Although cDC surface proteins were clearly detected on the surface of *Marchf1*^{-/-} MZ B cells, no transcriptional expression was detected based on raw mRNA counts of the corresponding genes. This implies that MZ B cells in MARCHF1-deficient mice acquire cDC surface proteins from other cells (i.e. from C3-coated cDCs). A potential explanation is either the incorporation of extracellular vesicles (EV) that are shed from the surface of cDCs, carrying cDC markers [755], or the direct extraction of surface molecules from the cDC surface, a phenomenon known as ‘trocytosis’ [756]. Trocytosis, sometimes also referred to as ‘trans-endocytosis’ [411] or ‘cellular cross-dressing’ [757], has been described for T and B cells in various studies, demonstrating the selective acquisition of surface proteins from APCs [758][759][760][761].

Further examination of CD8 and CD11c surface levels in splenic B cells across various mutant mice and in mixed bone marrow chimera mice confirmed that the appearance of cDC markers on B cells strictly correlated with C3 binding to MHC II on the surface of cDCs. While splenic B cells in mutant mice with constitutively high MHC II-C3 surface complexes (i.e. *Marchf1*^{-/-}, MHC II K225R^{KI/KI} and *Ubl3*^{-/-}) exhibited CD11c and CD8 surface expression on B cells, neither WT nor mutant mice that also lacked C3 or MHC II expression showed any CD11c⁺ or CD8⁺ B cells. Interestingly, similar to the loss of cDCs, the degree of C3 deposition seems to correlate with the level of CD8 and CD11c on B cells and/or the number of CD11c⁺ or CD8⁺ B cells, as *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice harbored more CD11c⁺ or CD8⁺ B cells than *Ubl3*^{-/-} mice. Of further note, CD8 expression was in general higher than CD11c, indicating preferential acquisition of certain surface markers, which has been demonstrated for the acquisition of surface proteins via trocytosis by *Daubeuf* et al. [758]. This hypothesis is further supported by the fact that certain cDC surface markers are higher on CD11c^{int} CD24^{high} CD8^{int} cells than others as seen during their surface phenotype examination (Figure 4.6). Lastly mixed bone marrow chimera experiments confirmed that the appearance of cDC markers on B cells was dependent on C3 expression and elevated MHC II surface level, as only

WT but not C3-deficient recipient mice harbored CD11c⁺ and CD8⁺ B cells. Notably, surface expression of CD11c and CD8 was similar between WT and MARCHF1-deficient B cells, indicating that acquisition of cDC proteins was not due to MARCHF1 deficiency within B cells but due to C3 deposition on *Marchf1*^{-/-} cDCs. The role of C3 deposition in the acquisition of cDC markers by B cells from cDCs via trogocytosis is examined in Chapter 5.

The role of complement C3 in the
trogoncytosis between
dendritic cells and B cells

Abstract

Trogocytosis, also known as trans-endocytosis, is the intercellular transfer of plasma membrane fractions between different immune cells upon their cellular conjugation. Mostly, trogocytosis is described for lymphocytes that extract surface proteins embedded in membrane fractions upon the recognition of their cognate Ag. Importantly, the extraction of membrane proteins via trogocytosis is considered as a coordinated cellular process between living cells and although its exact molecular mechanisms remain elusive, it has been described in the context of cell communication, Ag acquisition, cell killing as well as immune regulation.

This chapter describes the C3-mediated trogocytosis of cDCs by MZ B cells *in vitro* and *in vivo*, in which B cells acquire membrane fractions as well as surface proteins and peptide-loaded MHC II molecules from cDCs. Acquisition of cDC surface molecules via trogocytosis was significantly enriched when cDCs were coated with C3, in an MHC II-dependent manner, and furthermore depended on the expression of complement receptor CR2 in B cells. With this, we report the first evidence for new role of the C3-CR2 ligand-receptor pair, namely promoting trogocytosis between cDCs and B cells, in which C3, covalently bound to MHC II at the surface of cDCs, is the driving mediator. The engagement of specific receptors in this process as well as the fact that some membrane proteins were more efficiently acquired by trogocytic B cells than others suggests that C3-mediated trogocytosis is a dynamic and coordinated cellular process carried out by MZ B cells. Future experiments will investigate the biological consequences of C3-mediated trogocytosis with particular regard to its potential to deplete cDCs as well as functional benefits gained by MZ B cells through the acquisition of cDC surface molecules. These results explain the accumulation of trogocytic MZ B cells in mutant mice with altered MHC II ubiquitination and its consequential C3 deposition on cDCs, as described in the previous chapters.

5 The role of complement C3 in the trogocytosis between dendritic cells and B cells

5.1 Introduction

The previous two chapters describe the covalent binding of activated C3 to MHC II at the cell surface of cDCs and their consequential depletion as well as the appearance of cDC markers at the surface of MZ B cells. RNAseq identified the lack of transcriptional expression of these surface markers in MZ B cells, which suggests their intercellular transfer from C3-coated cDCs. It is well known that membrane-bound proteins can be exchanged between cells through various mechanisms, including the transfer of extracellular vesicles (EV), through membrane nano-tubulus and direct cell-cell contact [762], the latter often referred to as ‘trogocytosis’. We therefore investigated the role of C3 in the process of trogocytosis between cDCs and B cells.

5.1.1 Trogocytosis

Trogocytosis describes the transfer of membrane fractions and membrane-associated proteins from one cell to another upon their close contact by which recipient cells inherit foreign molecules [756][763].

The first evidence for the intercellular transfer of plasma membrane molecules between immune cells emerged in the early 1970s, although not named as trogocytosis, in which various studies, by Grey *et al.* in addition to Hudson *et al.*, identified and characterized the expression of B cell-derived immunoglobulins (Ig) on T lymphocytes [764][765]. Later Sprent *et al.* identified the basis for this was the specific capture of Ig upon TCR binding to its cognate Ag [766]. Furthermore, MHC molecules have been identified on T cells and other lymphocytes [767][768], acquired through similar mechanisms of intercellular membrane exchange in a TCR-signalling-dependent manner [769][770].

In the mid 2000s, more and more publications on the intercellular exchange of membrane proteins between APCs and lymphocytes have emerged, in which Hudrisier *et al.* have been a leading group in the field [659][758][759][771][772][773]. Although the term trogocytosis has been used most commonly, various other studies used other vocabulary, such as trans-endocytosis, cellular cross-dressing or even cannibalistic gnawing/nibbling, while basically all

describing the same phenomenon [411][757][763][774]. Despite the evidence for bidirectional membrane transfer [775], trogocytosis is considered to be unidirectional [760][761], in which the receptor-carrying cell acquires membrane fractions from the donating cell. Trogocytosis is a much more common and frequent phenomenon than previously thought [776] with versatile implications in cell communication [776], Ag acquisition [142][777], cell killing [778][779][780], as well as immune regulation [781][782][783]. Apart from its roles in the immune system, trogocytosis also has been described to participate in the development of the nervous system and embryonic development [774][784]. Although its exact molecular mechanisms remain elusive, it is thought to be a mixture of phagocytosis and endocytosis as it depends on actin ring contraction as well as small GTPases and endocytic proteins [763][776]. However, future studies will be required to decipher if trogocytosis has clathrin-mediated or caveolin-mediated endocytic mechanisms.

Most studies on trogocytosis report that B and T lymphocytes strip off various molecules from the plasma membrane of APCs upon their conjugation, often after the formation of an immunological synapse [756][759][761][785][786][781]. However, more recent studies have identified that trogocytosis is also carried out by other immune cells, including basophils [777], macrophages [787], neutrophils [778][779] and NK cells [788][789]. Furthermore, thymic DCs can acquire Ag from medullary thymic epithelial cells (mTEC) through trogocytosis of pMHC I and pMHC II, which is central in immune tolerance [432]. Importantly, trogocytosis is considered as a coordinated cellular process, mediated through receptor signaling [756], and occurs only between living cells, so that acquisition of foreign molecules through trogocytosis is not a result of phagocytosis of apoptotic cells [769][790]. Various surface molecules can be swapped between cells through trogocytosis, including Ag, ligands, MHC, adhesion and co-stimulatory molecules which can affect the phenotype and function of both the donating, as well as the receiving cell [756][776]. Furthermore, the multi-intercellular transfer of membrane proteins has been described in which cells pass on acquired molecules to further cells, a phenomenon described as '*serial trogocytosis*' [791].

Considering most studies on trogocytosis describe membrane exchange between APCs and lymphocytes, researchers have used this phenomenon in the past to discover Ag and/or reactive T cells in order to understand and manipulate immune responses to tumors [786][760]. For example, Li *et al.* have developed a cell-based selection platform for TCR

ligand discovery that exploits the membrane transfer through trogocytosis, in which reactive T cells can be identified on the basis of transferred membrane to target cells that present cognate peptide-MHC molecules [760].

Beside its implication in immune regulation and Ag exchange, trogocytosis has been described to be involved in the prevention of the autoimmune disorder hemolytic anemia through the ablation of red blood cells [792]. However, the opposite effect can occur when antibodies, that bind to self-antigens in order to prevent autoreactivity, are removed through trogocytosis [793]. Another example of 'harmful' trogocytosis is '*oncologic trogocytosis*', which occurs between ovarian epithelial cancer cells and stromal cells, in which cancer cells can acquire drug resistance protein and thus chemoresistance [776][794].

5.2 Results

The presence of cDC-specific membrane proteins at the cell surface of MZ B cells in the absence of gene transcription can be explained by the intercellular exchanges of membrane fractions as a result of trogocytosis. We therefore examined trogocytosis between B cells and cDCs from mutant mice where cDCs were opsonized with C3 (i.e. *Marchf1*^{-/-} and MHC II K225R^{KI/KI}) by various methods.

5.2.1 *In vitro* trogocytosis of C3-opsonized cDCs

Daubeuf *et al.* have established a method to investigate trogocytosis *in vitro*, in which incubation of Ag-loaded APCs with T or B cells leads to trogocytosis, demonstrated with OVA-pulsed APCs and OT-I T cells [659]. According to this protocol, the most practical way to monitor trogocytosis is by detecting the acquisition of labels incorporated into the target cell membrane through for example, fluorescent lipids or surface biotinylation. Although the protocol of Daubeuf *et al.* has been optimized to detect, quantify and characterize T and B cells that respond to cellular antigens, the authors also suggest the assay is applicable to all types of lymphocytes, and any cell type that carries out trogocytosis as a result of the specific engagement of their cell-surface receptors [659]. We therefore examined trogocytosis between B cells and C3-coated cDCs *in vitro* in the setting of this established protocol.

Trogocytosis of PKH26-stained cDCs

Using the *in vitro* trogocytosis assay, established by Daubeuf *et al.* [659], we first aimed to address if trogocytosis occurs between B cells and cDCs and, if so, whether C3-coated cDCs

from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice were more efficiently trogocytosed by B cells than their WT counterparts. This would provide an explanation why mutant mice with C3-coated cDCs harbored a large number of trogocytic MZ B cells, identified by their expression of cDC surface markers.

To do this, we purified splenic cDCs and B cells from WT and *Marchf1*^{-/-} mice (WT & *Marchf1*^{-/-} B cells >98% purity, WT cDCs >80% purity and *Marchf1*^{-/-} cDCs >60% purity), stained either cDCs or B cells with PKH26, a highly fluorescent lipophilic dye used for membrane staining [795][796], and then co-cultured them for 2 hours. Trogocytosis was assessed by acquisition of PKH26 dye by unlabeled cells upon co-culturing with PKH26-stained cells through flow cytometry (**Figure 5.1**).

Incubation of PKH26-stained cDCs with B cells led to acquisition of PKH26, indicated by the obtained PKH26 (PE) signal on B220⁺ CD19⁺ cells, while B cells incubated on their own remained, as expected, negative for surface PKH26. Capturing of PKH26-labelled membrane fractions by B cells was approximately doubled when incubated with *Marchf1*^{-/-} cDCs, compared to WT cDCs, based on both % of PKH26⁺ B cells (~70% versus ~35%) as well as gMFI values of PKH26 (PE) in B cells (~450 versus ~850). This suggested that *Marchf1*^{-/-} cDCs were more susceptible for trogocytosis than WT cDCs. Interestingly, acquisition of membrane fractions from cDCs was slightly higher by WT than in *Marchf1*^{-/-} B cells in scenarios where cDCs were MARCHF1-deficient (**Figure 5.1A**).

Transfer of PKH26-labelled membrane from cDCs to B cells was unidirectional, as no or only little PKH26 was transferred from PKH26-labelled B cells to cDCs, regardless of the expression of MARCHF1 by either cell type, which indicated that trogocytosis is a coordinated cellular process (**Figure 5.1B**).

To assess if increased trogocytosis of MARCHF1-deficient cDCs was indeed due to high C3 surface levels in cDCs, we repeated the *in vitro* trogocytosis assay with cDCs from WT, *Marchf1*^{-/-}, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x *C3*^{-/-} mice. Again, incubation of WT B cells with PKH26-stained *Marchf1*^{-/-} cDCs resulted in enriched (approximately doubled) trogocytosis compared to their incubation with labelled WT cDCs, based on acquired PKH26 signal. Incubation of B cells with cDCs from MHC II K225R^{KI/KI} mice led to a similarly enriched transfer of PKH26-dyed membrane fractions, while cDCs from MHC II K225R^{KI/KI} x *C3*^{-/-} mice showed no enriched susceptibility for trogocytosis (**Figure 5.1C**). Importantly, PKH26 surface levels were comparable between all four group of cDCs before co-culturing (**Figure 5.1D**),

which excluded the possibility that enriched acquisition of PKH26 was due to higher surface staining.

These results suggest that MHC II-dependent C3 deposition on cDCs promotes trogocytosis of cDCs by B cells, in which the latter acquire membrane proteins from cDCs through the unidirectional transfer of membrane fractions.

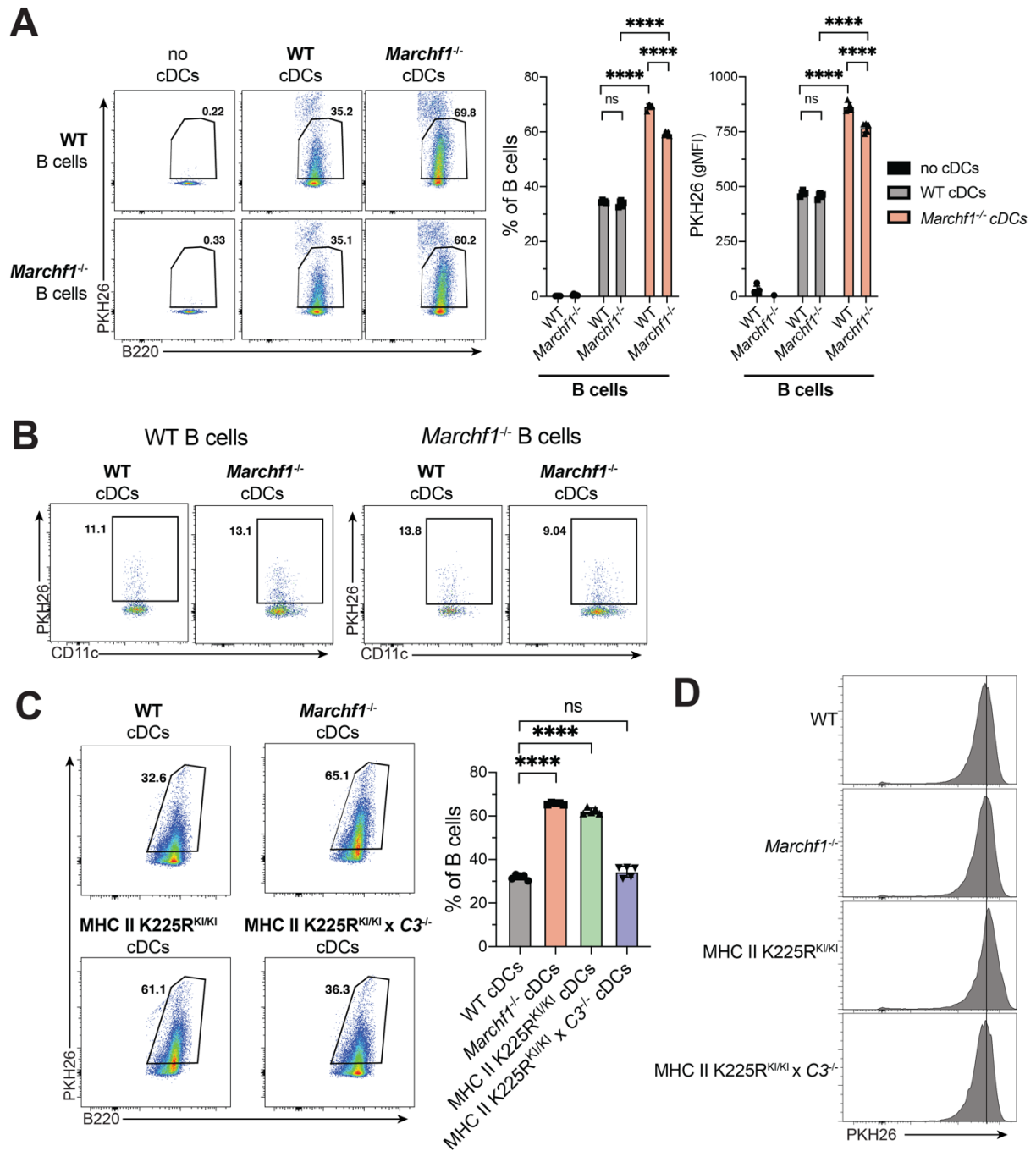


Figure 5.1 *In vitro* trogocytosis assay between WT and *Marchf1*^{-/-} cDCs and B cells upon PKH26-staining. Flow cytometry analysis of co-cultured PKH26-stained cDCs and B cells *in vitro*. (A) B220 vs. PKH26 plots of WT and *Marchf1*^{-/-} B cells upon co-culturing with PKH26-stained WT or *Marchf1*^{-/-} cDCs. (B) CD11c vs. PKH26 plots of WT and *Marchf1*^{-/-} cDCs upon co-culturing with PKH26-stained WT or *Marchf1*^{-/-} B cells. (C) B220 vs. PKH26 plots of WT B cells upon co-culturing with PKH26-stained WT, *Marchf1*^{-/-}, MHC II K225R^{KI/KI} or MHC II K225R^{KI/KI} x C3^{-/-} cDCs. (D) PKH26 surface levels of WT, *Marchf1*^{-/-}, MHC II K225R^{KI/KI} or MHC II K225R^{KI/KI} x C3^{-/-} cDCs before co-culturing in (C). Each data point in bar plots of (A) and (C) represents the measurements of a pentaplicate, displayed as mean and bars designating mean ± SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.0001 (****).

Trogocytosis of cDCs membrane proteins

In vitro trogocytosis assays with PKH26-stained cells suggested the intercellular exchange of membrane fractions from cDCs to B cells, enriched when cDCs were coated with C3. Next, we aimed to analyze the transfer of membrane proteins in this setting.

For this, we first co-cultured splenic B cells purified from WT Ly5.1⁺ Ly5.2⁻ mice with cDCs from MHC II K225R^{KI/KI} or MHC II K225R^{KI/KI} x C3^{-/-} mice that were Ly5.1⁻ Ly5.2⁺ in a time course of five hours, excluding any surface staining (i.e. PKH26). We then examined the acquisition of Ly5.2 (CD45.2) by B cells over time via flow cytometry (**Figure 5.2A**).

Confirming the PKH26 transfer data, B cells captured membrane fractions from Ly5.1⁻ Ly5.2⁺ cDCs and hence also acquired CD45.2 (Ly5.2) shortly after co-culturing (30min). Again trogocytosis was higher (~2-3 times) when B cells were incubated with C3-coated cDCs (i.e. MHC II K225R^{KI/KI}) when compared to either WT or C3-deficient cDCs (i.e. MHC II K225R^{KI/KI} x C3^{-/-}) with highest levels of captured CD45.2 at the 2h timepoint of co-culturing, while B cells incubated on their own remained, as expected, negative for Ly5.2 (**Figure 5.2B**).

Next, we assessed trogocytosis of cDC-specific surface markers for which we co-cultured splenic B cells with *Marchf1*^{-/-} cDCs for 2h and then examined acquisition of CD8, CD11c, XCR1 and CD11b by B cells. B cells expressed CD8, CD11c and XCR1 after incubation with cDCs, much more so if the cDCs were *Marchf1*^{-/-} than if they were WT (**Figure 5.2B**), even though the surface expression of these three molecules was equivalent or even slightly reduced in *Marchf1*^{-/-} versus WT cDCs (i.e. CD8 and XCR1) (**Figure 5.2C**). Interestingly, some membrane proteins were more efficiently acquired by trogocytic B cells (i.e. CD8) than others (i.e. XCR1), and CD11b was transferred almost equally well from WT and *Marchf1*^{-/-} cDCs, with only slightly enriched transfer from *Marchf1*^{-/-} cDCs (**Figure 5.2B**).

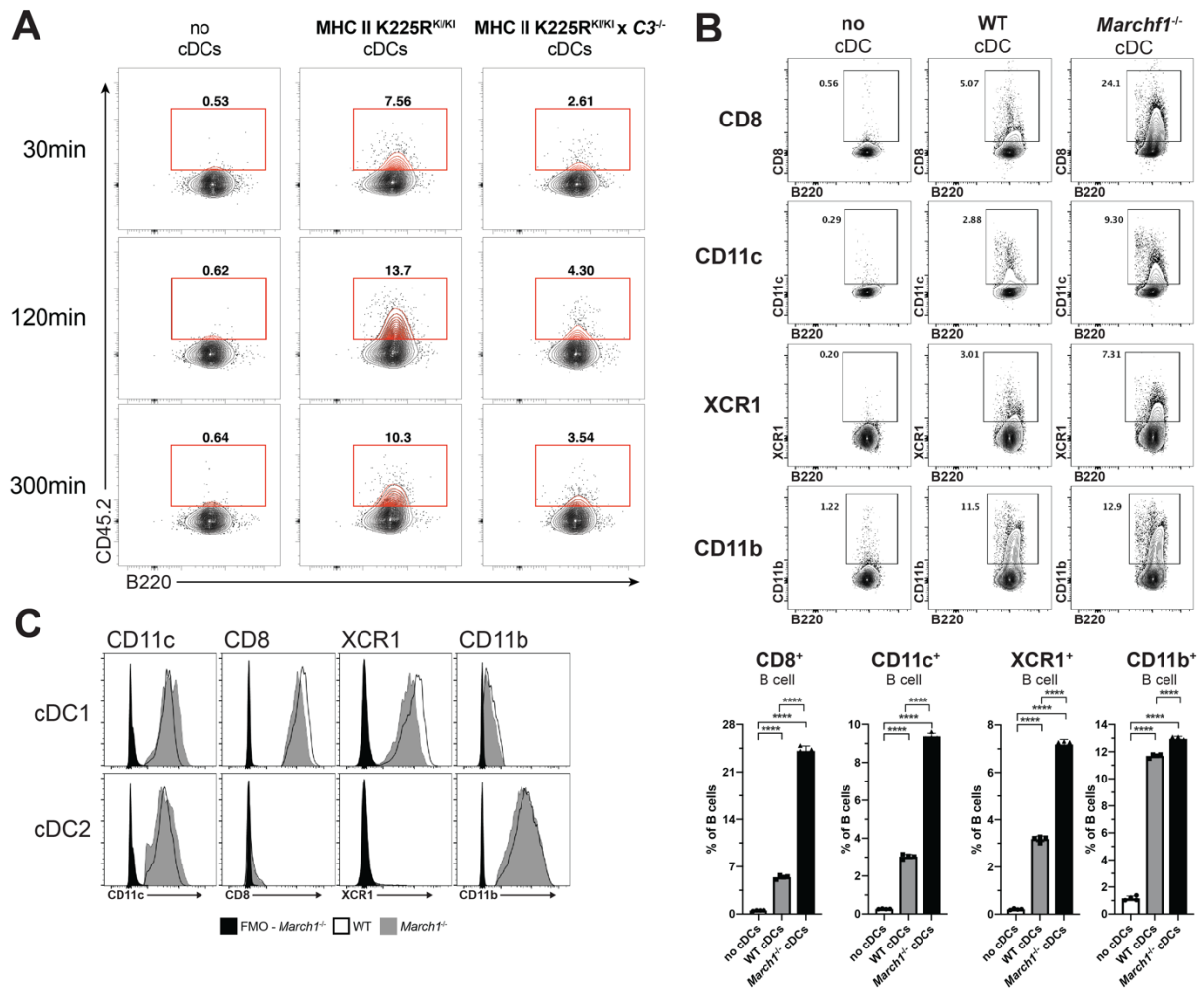


Figure 5.2 Acquisition of cDC surface proteins by B cell in *in vitro* trogocytosis assay. Examination of surface proteins in B cells captured from cDCs upon co-culturing by flow cytometry. (A) CD45.2 vs. B220 plots of Ly5.1⁺ Ly5.2⁻ B cells co-cultured with cDCs from MHC II K225R^{KI/KI} or MHC II K225R^{KI/KI} x C3^{-/-} mice (both Ly5.1⁺ Ly5.2⁺) for 30, 120 and 300 min. (B) Surface levels of CD8, CD11c, XCR1 and CD11b in B cells incubated for 2h on their own or with WT and *Marchf1*^{-/-} cDCs. Each data point represents the measurements of a quadruplicate, displayed as mean and bars designating mean $\pm$ SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.0001 (****). (C) Representative histograms showing surface expression of CD11c, CD8, XCR1 and CD11b in cDC1s and cDC2s from WT and *Marchf1*^{-/-} mice, compared to the corresponding FMO control.

C3-mediated trogocytosis of cDCs requires CR2 expression

Next, we aimed to address the question if trogocytosis of C3-coated cDCs requires a receptor on the surface of B cells, relevant for the enriched membrane transfer. Our previous biochemical analyses revealed that the dominant C3 fragments, covalently bound to MHC II on the surface of cDCs, were C3dg and C3d (see Chapter 3). Complement receptor 2 (CR2) is known to bind C3d/C3dg and is highly expressed by B cells, in particular MZ B cells [797]. In order to address if CR2 is the corresponding receptor for trogocytosis of C3-coated cDCs, we

repeated the *in vitro* trogocytosis assay with B cells that were deficient in CR2 and assessed their acquisition of CD8 and CD11c in this setting (**Figure 5.3**).

As observed previously, WT B cells, that expressed CR2 ($Cr2^{+/+}$), acquired the surface proteins CD8 and CD11c from cDCs, to significantly higher levels if cDCs were coated with C3 (i.e. MHC II K225R^{KI/KI}) than if they were WT or deficient in C3 (i.e. MHC II K225R^{KI/KI} $\times$ $C3^{-/-}$). Notably, CR2-deficient B cells ($Cr2^{-/-}$) trogocytosed cDC membrane from WT and MHC II K225R^{KI/KI} cDCs similarly, although the latter were coated with C3 (**Figure 5.3**), which suggested that CR2 expression was necessary for enriched trogocytosis of C3-opsonized cDCs.

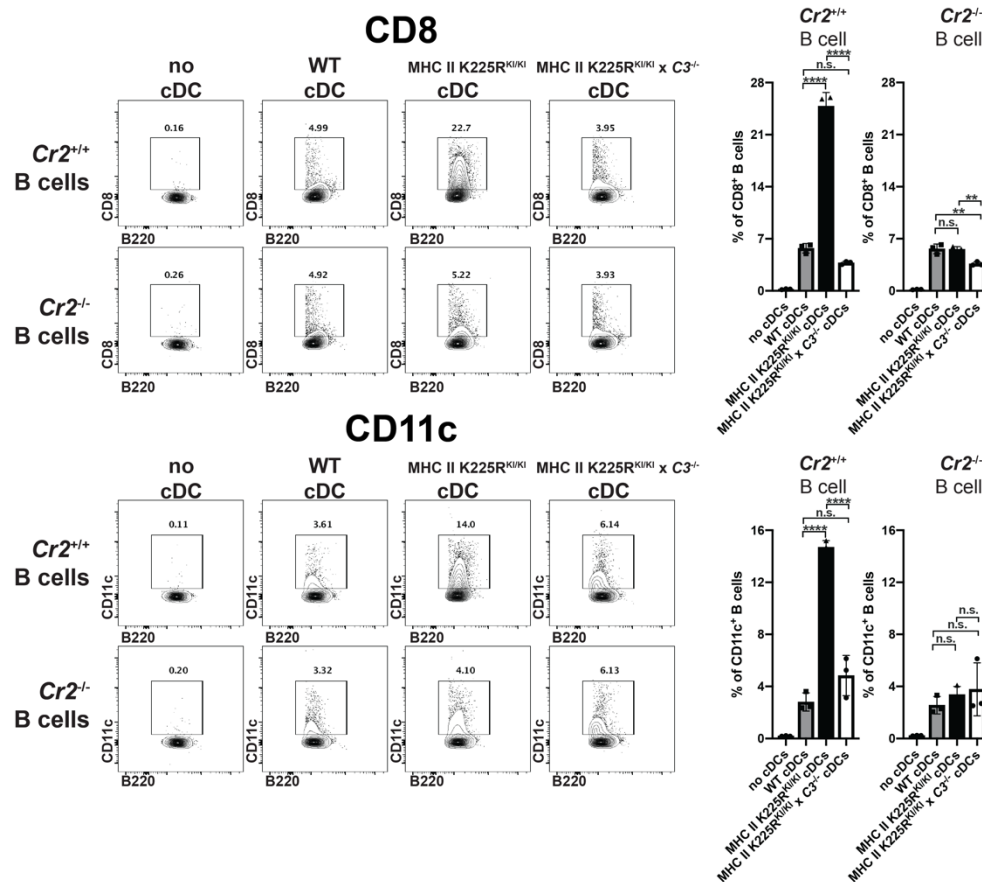


Figure 5.3 *In vitro* trogocytosis assay between cDCs and CR2-deficient B cells. Examination of surface levels of CD11c and CD8 in WT and $Cr2^{-/-}$ B cells upon co-culturing with cDCs from WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} $\times$ $C3^{-/-}$ mice. Each data point represents the measurements of a quadruplicate, displayed as mean and bars designating mean $\pm$ SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.002 (**), < 0.0001 (****).

5.2.2 *In vivo* trogocytosis of C3-opsonized cDCs

Co-culturing experiments of B cells and cDCs *in vitro* have demonstrated the directed intercellular transfer of surface molecules from cDCs to B cells, with increased trogocytosis efficiency in scenarios of enriched C3 deposition on cDCs. The accumulation of trogocytic B cells, identified by their expression of cDC-specific surface proteins, in the spleen of mutant mice with enriched C3 deposition on cDCs, indicates that C3-mediated trogocytosis between cDCs and B cells occurs *in vivo*. However, we aimed to further verify *in vivo* trogocytosis through various methods.

In vivo trogocytosis of Ab-targeted XCR1

To examine trogocytosis between C3-coated cDCs and B cells *in vivo*, we intravenously injected WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} mice with a fluorescence(FITC)-conjugated anti-XCR1 mAb (isotype: IgG2b) in order to specifically target and label the surface of cDC1 *in vivo*. We then purified light-density splenocytes via Nycodenz gradient centrifugation and examined trogocytosis based on acquired FITC(XCR1) signal in splenic B cells over time (**Figure 5.4**).

Labelling of cDC1s *in vivo* through intravenous injection of FITC-conjugated anti-XCR1 mAbs stained cDC1s in all three mutant mice over a time period of 20h with a small reduction in proportion of stained cDC1s [% of CD8⁺ FITC(XCR1)⁺ cells] at the 20h timepoint, while injection of a FITC-conjugated IgG2b isotype control did not label cDC1s *in vivo* (**Figure 5.4A**). WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} mice that had been intravenously injected with an FITC-conjugated anti-XCR1 mAb but not with an FITC-conjugated isotype control harbored splenic B cells that were positive for FITC(XCR1) over a time period of 20h with highest signal obtained at the 2h timepoint. Acquisition of FITC-stained XCR1 molecules was approximately 2-3 times higher in B cells from MHC II K225R^{KI/KI} mice when compared to B cells of WT or MHC II K225R^{KI/KI} x C3^{-/-} mice at all three time points, indicating C3-mediated trogocytosis between B cells and cDC1s *in vivo* (**Figure 5.4B**).

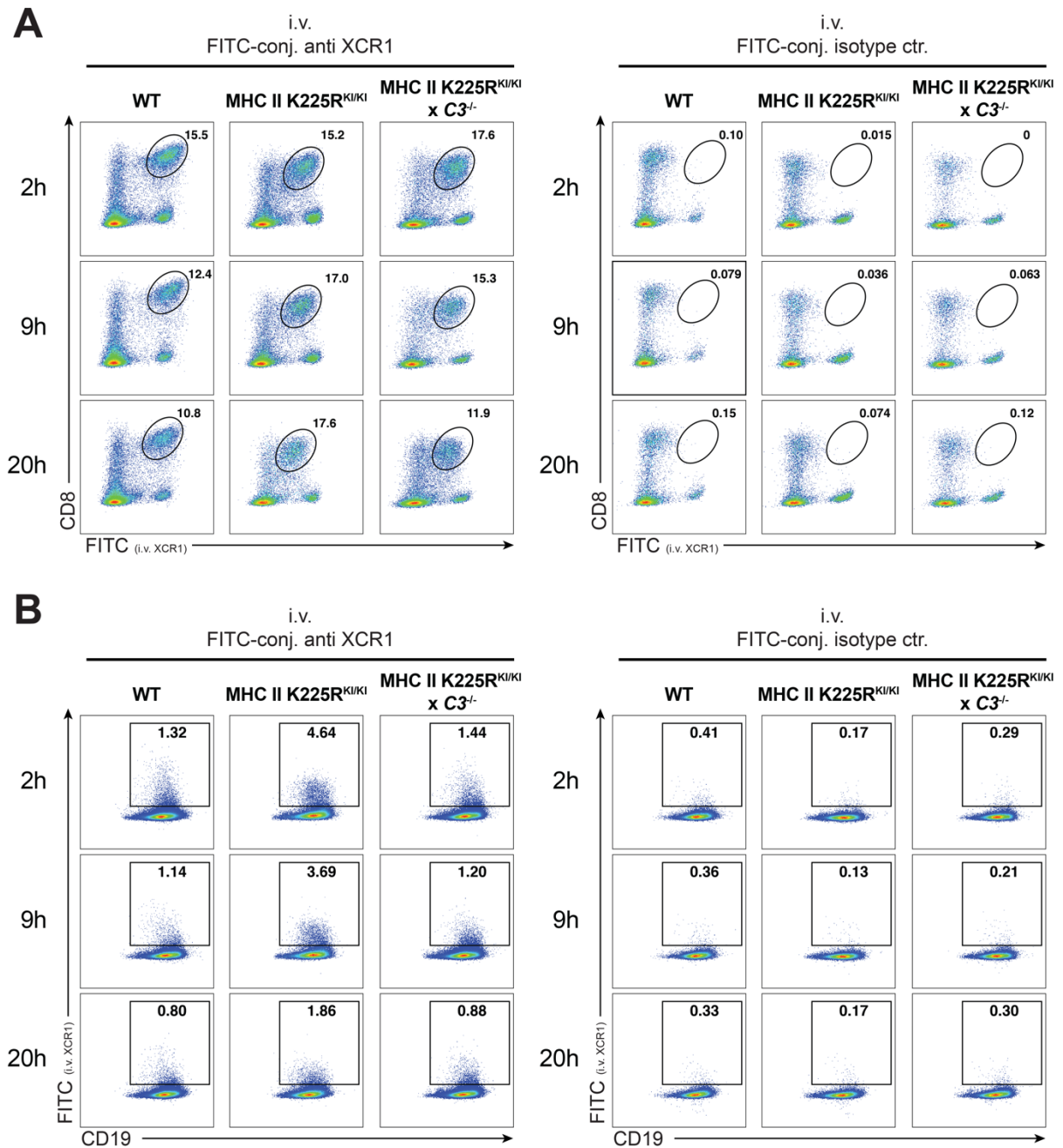


Figure 5.4 *In vivo* trogocytosis assay between cDC1s and B cells upon intravenous injection of FITC-anti XCR1 mAb. (A) FITC (empty channel) vs. CD8 plots of cDCs (CD11c⁺ MHC II⁺) purified from spleens of WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} mice that have been injected with an FITC-conjugated anti-XCR1 mAb [left panel] or FITC-conjugated isotype control [right panel]. (B) CD19 vs. FITC (empty channel) plots of B cells (CD19⁺ B220⁺) purified from spleens of WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} mice that have been injected with an FITC-conjugated anti-XCR1 mAb [left panel] or FITC-conjugated isotype control [right panel].

In vivo trogocytosis of pMHC II (I-Ea₅₂₋₆₈)

A major biological implication of trogocytosis is the intercellular transfer of Ag from APCs to other cells, which can affect phenotype and function of both the donating APC as well as the

receiving cell, as shown for T and B lymphocytes and non-lymphoid immune cells in multiple studies [142][777][690][781][782][783]. We therefore investigated the acquisition of peptide-loaded MHC II (pMHC II) molecules by B cells from C3-coated cDC1s through trogocytosis *in vivo*. For this we used the self-antigen I-Ea and the YAc detection mAb that specially detects the I-Ea₅₂₋₆₈ peptide presented at the cell surface in MHC II (I-Ab) [798]. Presentation of self-protein I-Ea has been shown to occur frequently *in vivo* with implications in T cell selection [798] and its detection through the YAc Ab has been successfully used to track Ag trafficking and presentation by flow cytometry [58].

To assess trogocytosis of I-Ea peptide-loaded MHC II molecules, we intravenously injected WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} mice with an anti-Clec9A mAb (rat IgG2a κ) that was conjugated to the I-Ea₄₆₋₇₄ antigenic peptide (Clec9A-OVA_{FGD}-YAc), in order to deliver the self-Ag to cDC1s *in vivo*. Control mice were injected with a rat IgG2a κ isotype control that also carried the I-Ea₄₆₋₇₄ antigenic peptide. We then examined presentation of I-Ea peptide loaded in MHC II [pMHC II (I-Ea₅₂₋₆₈)] on splenic cDCs as well as B cells from immunized mice by flow cytometry through a two-step staining using the biotinylated YAc mAb + streptavidin-PE. As a negative control [fluorescence-minus-one (FMO) control] cells were incubated with streptavidin-PE only, excluding the biotinylated YAc mAb (**Figure 5.5**).

Surface expression of I-Ea₅₂₋₆₈-loaded MHC II was highest on cDC1s and not or only little detected in cDC2s or total B cells from either WT, MHC II K225R^{KI/KI} or MHC II K225R^{KI/KI} x C3^{-/-} mice, indicating specific Ag delivery and presentation of I-Ea self-antigen by cDC1s upon Clec9A-targeted immunization. pMHC II (I-Ea₅₂₋₆₈) surface expression was approximately 5-6x higher in cDC1s with impaired MHC II ubiquitination (i.e. MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-}) when compared to WT cells. Interestingly, MZ B cells of MHC II K225R^{KI/KI} mice exhibited significant surface levels of pMHC II (I-Ea₅₂₋₆₈) when compared to WT or C3-deficient MZ B cells (i.e. MHC II K225R^{KI/KI} x C3^{-/-}) (**Figure 5.5**), which suggests that C3 deposition on cDC1s mediates acquisition of Ag by MZ B cells upon Clec9A-targeted Ag delivery.

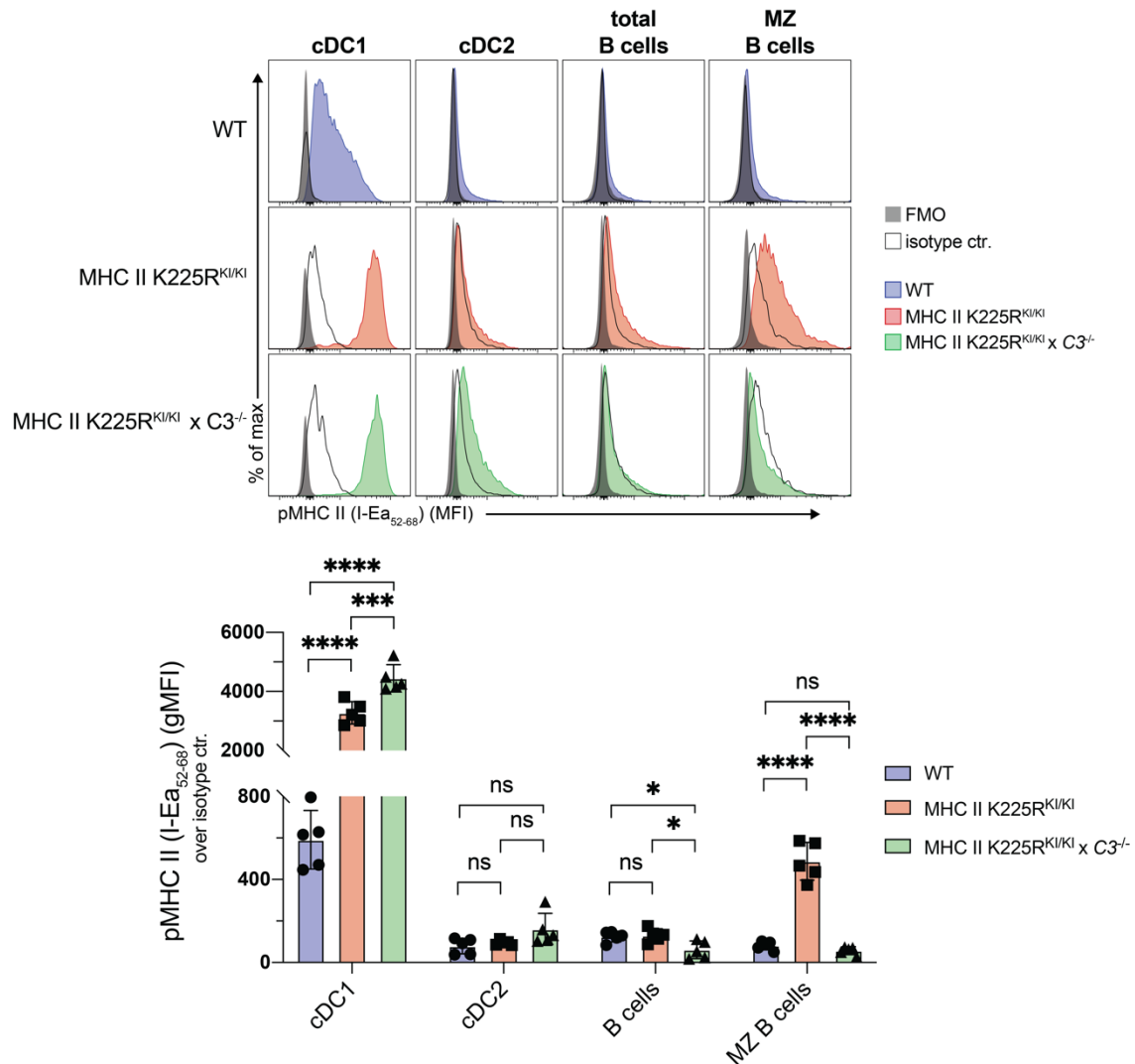


Figure 5.5 In vivo trogocytosis of pMHC II (I-Ea₅₂₋₆₈) between cDC1s and B cells. Surface expression levels of I-Ea peptide bound MHC II (I-Ab) [pMHC II (I-Ea₅₂₋₆₈)] in splenic cDC1s, cDC2s, total B cells and MZ B cells purified from mice that have been immunized with anti-Clec9A Ab conjugated with I-Ea Ag (Clec9A-OVA_{FGD}-YAe) or I-Ea antigen-conjugated isotype control, compared to the corresponding fluorescence-minus-one (FMO) control for which cells were incubated with streptavidin-PE only [minus biotin. YAe mAb]. Each data point represents an individual mouse, displayed as mean and bars designating mean $\pm$ SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.03 (*), < 0.0002 (**), < 0.0001 (****).

5.2.3 Antigen presentation by C3-opsonized cDC1s to B cells

Activation of B cells requires the recognition of native, intact Ag through their BCR. Although B cells can bind free Ag, in secondary lymphoid tissues Ag is often presented to B cells when tethered to membranous structures [68][77][78], such as the surface of SCS macrophages and FDCs in LN and spleen. Furthermore, it is known that cDCs can also present intact Ag to B cells inducing B cell responses (reviewed in [68]). Various studies, by Caminschi *et al.*, Lahoud *et*

al. as well as Kato *et al.*, have reported that Ag delivery to the surface of cDC1 via Clec9A-specific mAb induces T_{FH} and GC responses with isotype-switching and affinity maturation even in the absence of adjuvants [11][83][146][147][148]. These studies demonstrate the rapid B cell activation through the recognition of native Ag presented on the surface of cDC1s upon Clec9A-targeted immunization, in which B cells effectively acquire help from cognate $CD4^+$ T cells at the T/B border within the spleen and lymph nodes [11][147][148].

We previously have shown that binding of C3 to MHC II at the surface of cDC1s has no impact on their capability to present Ag to $CD4^+$ T cells, based on numbers of proliferating OT-II T cells (see Chapter 4). However, considering C3-coated cDC1s are highly trogocytosed by MZ B cells, which indicates their close interaction in the spleen, we examined the effect of C3 opsonization on Ag presentation between cDC1s and B cells. For this we used B cells from transnuclear OB1.*Rag1*^{-/-} (OB1) mice, that exclusively harbored IgG1⁺ B cells, specific to the linear epitope sequence DKLPFGGDSI which is contained in a surface-exposed loop of OVA [652][651]. Previous studies have shown that cDC1-targeted immunization of mice through the injection of anti-Clec9A mAbs carrying the OVA peptide DKLPFGGDSI (anti-Clec9A-OVA_{FGD}) led to activation of OVA-specific OB1 B cells *in vivo* [11]. To assess if C3 deposition on cDCs and their consequential trogocytosis affects B cells activation upon Clec9A-targeted immunization, we adoptively transferred OB1 cells into WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} mice recipient mice one day prior to immunization with 0.5 µg anti-Clec9A-OVA_{FGD}. Activation of splenic OB1 cells was then analyzed 1; 6 and 24 hours after immunization by flow cytometry based on CD86 and MHC II surface expression (**Figure 5.6**).

Purified OB1 B cells from unimmunized WT mice showed intermediate surface levels of MHC II and low CD86 surface expression. Immunization with anti-Clec9A-OVA_{FGD} led to an increase in both MHC II and CD86 surface levels of adoptively transferred OB1 B cells in all three recipient mice (i.e. WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-}) with highest levels at the 24h timepoint after immunization, indicating successful activation of B cells through specific Ag delivery to the surface of cDC1s. Surprisingly, OB1 B cells were more activated in WT than in MHC II K225R^{KI/KI} or MHC II K225R^{KI/KI} x C3^{-/-} recipient mice, as both exhibited significantly reduced upregulation of MHC II and CD86 (**Figure 5.6**).

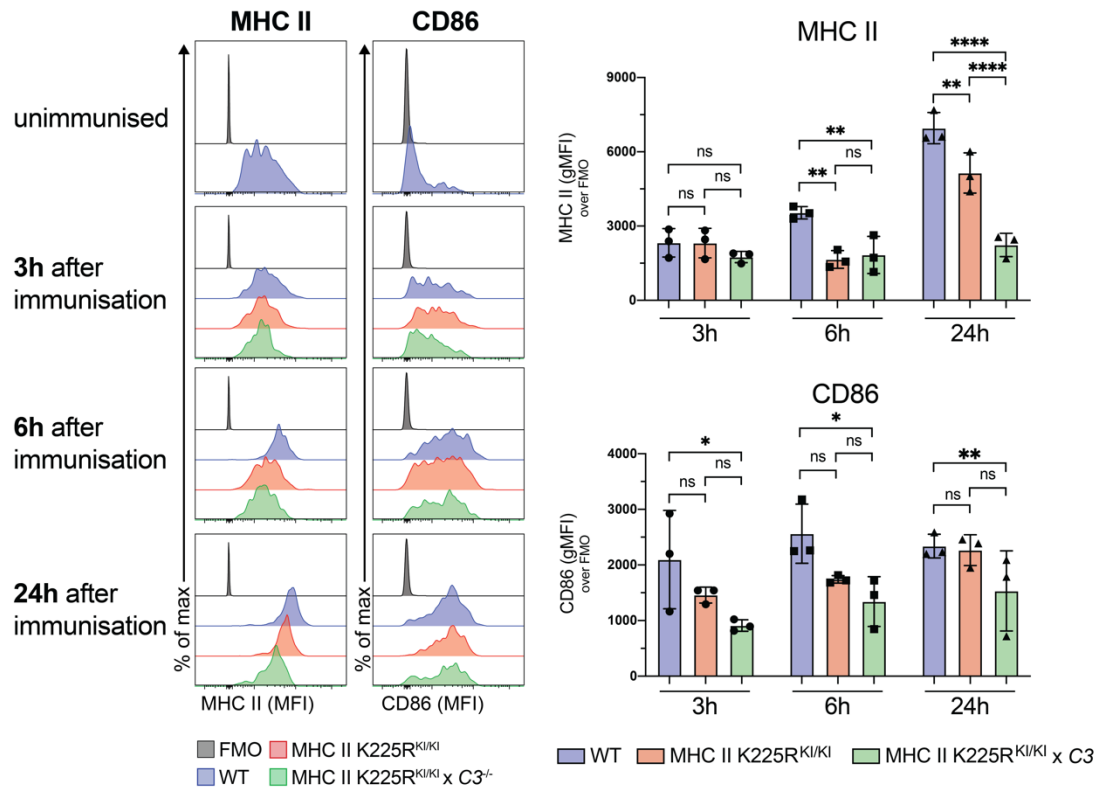


Figure 5.6 *In vivo* OB1 B cell activation upon cDC1-targeted immunization with anti-Clec9A-OVA_{FGD}. Surface expression levels of MHC II and CD86 in adoptively transferred OB1 B cells purified from the spleens of WT, MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} post immunization (3h, 6h and 24h) with anti-Clec9A-OVA_{FGD}. Each data point represents an individual mouse, displayed as mean and bars designating mean $\pm$ SD. One-way ANOVA followed by Bonferroni's multiple comparisons test; ns: not significant, p value < 0.03 (*), < 0.002 (**), < 0.0001 (****).

5.3 Discussion

This chapter describes C3 as a mediator for trogocytosis between cDCs and B cells, by which B cells acquire membrane fractions as well as surface proteins and peptide-loaded MHC II molecules from cDCs in a receptor-dependent manner. It is well known that membrane-bound proteins can be exchanged between cells through trogocytosis upon their direct cell-cell contact and DCs have been identified to transfer Ag to B cells in order to induce B cell activation [66][142]. Although in this case Ag is just presented on the surface to B cells and not actively acquired by them, other cells, like basophils [777] and Tregs [781], have been shown to strip off peptide-loaded MHC II molecules embedded in the membrane of cDCs, in order to acquire Ag presentation capacity themselves or to suppress Ag presentation in cDCs, respectively. Our *in vitro* and *in vivo* models have identified the unidirectional transfer of membrane and membrane associated proteins from the cell surface of C3-coated cDCs to B cells. Importantly, this transfer required the engagement of complement receptor CR2

which indicates that C3-mediated trogocytosis is a coordinated cellular process. These observations provide an explanation for the accumulation of trogocytic MZ B cells and the significant loss of cDCs in mutant mice with altered MHC II ubiquitination and its consequential C3 deposition on cDCs, as described in Chapter 3 and 4.

5.3.1 *In vitro* trogocytosis of C3-coated cDCs

Using an established *in vitro* trogocytosis assay [659], we have identified that MHC II-dependent C3 deposition on cDCs promotes their trogocytosis by B cells, in which the latter strip off membrane proteins from the surface of cDCs.

Initial experiments identified the intercellular transfer of PKH26-stained membrane from cDCs into unlabeled target B cells, a common method to identify and monitor trogocytosis, enriched when cDCs were coated with C3. Importantly, C3-dependent trogocytosis was unidirectional as it occurred only from cDCs to B cells but not the other way around. This is in concordance with previous studies [760][761] and supports the understanding that trogocytosis is a coordinated cellular process that is receptor-dependent [756][769][770] and in which the receptor-carrying cell is the receiving party in this process. Various studies have demonstrated that these receptors are the TCR and BCR for T and B cells, necessary for their trogocytic activity as binding to the cognate Ag enabled required signaling as well as their conjugation to the donating APC [659][756][759][761][781][785][786]. Here, we demonstrate a receptor that has not been previously described to participate in trogocytosis, namely CR2 on the surface of B cells that specifically binds to C3dg and/or C3d, which is covalently bound to MHC II at the surface of cDCs. Interaction of C3dg/C3d and CR2 is known to be an important co-stimulatory signalling axis in the activation of B cells [95][96], but its role in promoting trogocytosis between cDCs and B cells has not been described in the literature. Conclusively, our results show that the receptor-carrying cell, namely CR2-expressing B cells, is the receiving cell in the intercellular membrane transfer between cDCs and B cells.

We first identified trogocytosis of membrane proteins from C3-coated cDCs based on the acquisition of CD45.2 in *Ly5.2^{-/-}* B cells through their co-culturing *in vitro*. Notably, we were able to observe acquisition of CD45.2 even after a short time period upon their incubation (30 min), which wasn't surprising as trogocytosis has been shown to occur very rapidly, even within minutes [770]. This in turn argues against an exosome-based mechanism behind the intercellular exchange of membrane proteins between B cells and C3-coated cDCs, as the

formation of extracellular vesicles by DCs is known to take several hours [799][800].

We were able to rigorously discard the explanation for enriched trogocytosis based on differences in the expression levels of trogocytosed surface molecules between donating WT and C3-coated cDCs from *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice, as surface expression of cDC markers was equivalent or even slightly reduced in *Marchf1*^{-/-} versus WT cDCs. However, we interestingly observed that some cDC surface molecules were more efficiently acquired by trogocytic B cells (i.e. CD8) than others (i.e. XCR1). Differences in the efficiency of trogocytosis of certain surface molecules have been described before by Daubeuf *et al.* with preferential acquisition of distinct surface markers by trogocytic cells [758]. A potential explanation in the setting of C3-mediated trogocytosis is that certain proteins may lay in closer proximity to the MHC II-C3 complex within the microdomains of the plasma membrane of cDCs. As C3-mediated trogocytosis of cDCs is ligand(C3)-receptor(CR2)-dependent, it is plausible to assume that MHC II-C3 complexes are primarily targeted by trogocytic B cells and membrane proteins in close proximity of these complexes are more likely to be co-transferred than surface markers that are more distant. Furthermore, our *in vitro* trogocytosis assay also reveals indication that cDC1s are more efficiently trogocytosis in a C3-dependent manner than cDC2s. CD11b was transferred almost equally well from WT and *Marchf1*^{-/-} cDCs, with only slightly enriched transfer from *Marchf1*^{-/-} cDCs, which indicates that trogocytosis of CD11b is less dependent on C3 deposition. This is in concordance with our previous observation, showing a general trend for higher expression levels of cDC1 surface proteins (e.g. CD8, XCR1 and CD36) than of cDC2-specific markers (e.g. CD11b, CD4 and ESAM) on trogocytic B cells, which in turn indicates that C3-mediated trogocytosis is more cDC1-specific. As CD11b expression is high in cDC2s but low in cDC1s and we previously have described higher deposition of C3 on cDC1s than on cDC2s (see Chapter 3), higher C3 deposition on cDC1s than on cDC2s, is a likely reason for enriched trogocytosis of cDC1s by MZ B cells *in vitro*. This is further supported by the fact that trogocytosis only occurs when an extremely high concentration of MHC I-restricted antigenic peptide is added externally to the target cells, in case of trogocytosis between T cells and APCs [756][769]. Hence, extremely high C3 surface levels on cDC1s compared to cDC2s might be an explanation for preferred trogocytosis of cDC1s. However, *in vitro* trogocytosis assays with sorted cDC1s vs. cDC2s will be required to confirm or falsify this hypothesis.

Further future co-culturing experiments with cDCs and B cells will enable further examination

of C3-mediated trogocytosis. First, a repeat of the *in vitro* trogocytosis assay with sort-purified B cells will be required in order to compare the trogocytic activity of the three main subsets of splenic B cells (i.e. FO, T1-T3 and MZ B cells). Presumably, MZ B cells will have the highest trogocytic activity as they have the highest expression of surface CR2 and we previously have identified trogocytic B cells as MZ B cells *in vivo*. Second, microscopy and in particular live cell imaging using time-lapse microscopy will help to examine the dynamics of C3-dependent trogocytosis in more detail and will furthermore address if the intercellular membrane transfer indeed depends on close cell-cell interactions between C3-coated cDCs and B cells or if it is due to the exchange of extracellular vesicles. Furthermore, imaging will be necessary to identify the route and fate of trogocytosed membrane/proteins in the receiving cell. Since we hypothesize that C3-mediated trogocytosis is a coordinated cellular process based on the requirement of the CR2-C3 interaction, it will be necessary to assess if CR2-signalling is needed in the trogocytic B cell, which can be addressed by using mutant forms of CR2 lacking signalling function. Lastly, it would be interesting to see whether trogocytosis of C3-coated cDCs *in vitro* causes induces their cell deaths, which provides an explanation for their reduced numbers in mutant mice with impaired MHC II ubiquitination (i.e. *Marchf1*^{-/-} and MHC II K225R^{KI/KI}). Cell killing through trogocytosis, often referred to as ‘*trogoptosis*’, has been demonstrated for NK cells [788], neutrophils [778][779], as well as for the parasite *hystolytica* [780].

5.3.2 *In vivo* trogocytosis of C3-coated cDCs

Confirming the results measuring trogocytosis of C3-coated cDCs by B cells *in vitro*, our *in vivo* approaches achieved a similar outcome by showing enriched acquisition of cDC-specific surface proteins in the scenario of enriched C3 deposition. The accumulation of trogocytic B cells, identified by their expression of cDC-specific surface proteins, in the spleen of mutant mice with enriched C3 deposition on cDCs (described in Chapter 2), indicates that C3-mediated trogocytosis between cDCs and B cells occurs *in vivo*. However, this was further confirmed, first by XCR1-labelled surface proteins as well as by I-Ea peptide loaded MHC II molecules displayed at the surface of splenic (MZ) B cells *in vivo* upon cDC1-targeted immunization through the intravenous injections of anti-XCR1 FITC-conjugated mAbs and anti-Clec9A-OVA_{FGD} (YAc) mAbs. In both cases acquisition of these molecules by B cells was due to enriched C3 deposition on cDCs, as no expression was seen in either WT or C3-deficient

B cells (MHC II K225R^{KI/KI} x C3^{-/-}). However, we cannot exclude the possibility that cDC1-targeted immunization with either anti-XCR1 FITC-conjugated mAb or anti-Clec9A-OVA_{FGD} (YAe) mAbs specifically targeted cDC1s in MHC II K225R^{KI/KI} mice as we have previously demonstrated that MZ B cells exhibited intermediate surface levels of XCR1 and Clec9A in these mice. Presumably, expression of these cDC1 surface molecules in MZ B cells was due to their intercellular transfer via trogocytosis of cDC1s *in vivo*, which means that injection of anti-XCR1 and anti-Clec9A mAbs might directly target the surface of MZ B cells in these mice and they then generate MHC II-IEa complexes themselves.

Functional analyses will be required to assess the biological consequences of C3-mediated trogocytosis between cDCs and B cells. First, we will aim to address if trogocytosis in mutant mice with enriched C3 deposition is the reason for the significant loss of splenic cDCs in MHC II K225R^{KI/KI} mice. Trogocytosis-mediated cell killing of tumor cells has been shown to be carried out by neutrophils [779][778] and host cells can be killed by the parasite *hystolytica* through trogocytosis [780][801]. Killing of host immune cells through trogocytosis by other host immune has not been described in literature. However, it is plausible that extensive trogocytosis of C3-coated cDCs causes a lack of cell membrane integrity and induces cell death, which is the mechanism for trogoptosis of host cells in case of *hystolytica* [780][801]. If so, a potential downstream effect of increased DC death through trogocytosis might be the activation of other cells in a DAMP-mediated manner. We are currently generating MHC II K225R^{KI/KI} mice that are also deficient in CR2 expression (MHC II K225R^{KI/KI} x Cr2^{-/-}). Similar to the experiments comparing cell numbers between MHC II K225R^{KI/KI} vs. MHC II K225R^{KI/KI} x C3^{-/-} (Chapter 4), we will enumerate trogocytic B cells as well as cDCs from the spleen of MHC II K225R^{KI/KI} x Cr2^{-/-} mice, in order to address if CR2 expression is necessary for *in vivo* trogocytosis and whether this is the cause of cDC ablation.

A second major aim is to functionally characterize trogocytic B cells that have inherited cDC markers with particular regard to their acquisition of Ag from cDCs. The results of our anti-Clec9A-OVA immunization experiments in order to examine the role of C3 deposition in the activation of OB1 B cells were non-conclusive as their activation were significantly lower in both MHC II K225R^{KI/KI} and MHC II K225R^{KI/KI} x C3^{-/-} recipient mice. If reduced numbers of cDCs would be the reason for impaired activation of OB1 B cells in MHC II K225R^{KI/KI} upon anti-Clec9A-OVA immunization, their activation in immunized MHC II K225R^{KI/KI} x C3^{-/-} recipient should have been restored as cDC numbers are normal in these mice. This might indicate that

C3 deposition on cDCs takes part in the activation of B cell upon Clec9A-targeted immunization, which requires future experiments with WT versus $C3^{-/-}$ mice.

Overall, this system might not have been the appropriate one to investigate if B cells can utilize Ag acquired from cDCs through trogocytosis, as OB1 B cells are known to depend on help from cognate $CD4^{+}$ T cells at the T/B border within the spleen [83][146][147][148]. Considering trogocytosis of cDCs is carried out by MZ B cells, which are known to induce T cell-independent immune responses we will carry out experiments that investigate more specifically the activation of MZ B cells.

Final discussion

6 Final discussion

This thesis has examined the mechanisms of MARCHF1-mediated C3 deposition on the surface of conventional dendritic cells (cDCs), initially discovered in a proteomic screen to identify new MARCHF1 protein substrates. The data presented in this thesis describe two hitherto unknown instances of intersection between the innate and adaptive immune system, one at the molecular and the other at the cellular level. The main outcome and implications, as well as emerging hypotheses and future directions from the work in this thesis are discussed below.

6.1 Summary of main findings

Effective immunity requires the orchestrated cooperation of multiple molecular and cellular components of both the innate and adaptive arms of the immune system. The complexity of intercellular interactions engaged in immune responses is matched at the molecular level by the involvement of components of both the innate (e.g. complement, TLRs, inflammasomes, IL-1) and adaptive (e.g. TCR, BCR, immunoregulatory cytokines) immune system.

The first part of this thesis describes a new molecular cooperation between the innate and adaptive immune systems by demonstrating the covalent association of complement component 3 (C3) and major histocompatibility complex class II (MHC II), two central components of the immune system. Biochemical characterization revealed that the binding of C3 to MHC II was highly specific and involved the cleaved C3 fragments, C3dg and C3d, suggesting a new undescribed role for MHC II surface molecules by functioning as an acceptor for the covalent association of spontaneously activated C3 (**Figure 6.1A**).

The second part of this thesis describes the second new intersection between the innate and adaptive immune system, in this case at the cellular level. cDCs are themselves capable of linking innate with adaptive immunity through their major role in capturing Ag and stimulating naïve T cells to initiate adaptive immune responses. Here, we have uncovered a novel interaction between splenic cDCs and MZ B cells, innate B lymphocytes and producers of natural Abs [107][109][110][111][112]. Their close cell-cell interaction triggered the unidirectional transfer of plasma membrane proteins from cDCs to MZ B cells, a trogocytic event that was dependent on recognition of MHC II-C3 complexes on the cell surface of cDCs by CR2, expressed on MZ B cells (**Figure 6.1A and 6.1B**). The question if this causes a loss of

cell membrane integrity in cDCs and cell death, providing an explanation for their reduced numbers in scenarios of high C3 deposition, will require further investigations.

6.2 The covalent association of complement C3 and MHC II

The role of MHC II as the central molecule in the presentation of antigenic peptides to CD4⁺ T cells has been characterized over decades of research. However, apart from its main function in Ag presentation, it has been described to have further implications, such as in signal transduction. Thereby, MHC II-mediated signaling from the cell surface as well as from intracellular compartments has been shown to induce apoptosis in B cells and pDCs [802][803][804], as well as cytokine production in regulatory T cells and BM-derived DCs [805][806]. Similarly, an emerging concept describes the role of complement C3 as a modulator for various adaptive immune responses [577][626][627]. Beyond its traditional role as an opsonizing reagent in humoral immune responses of the innate immune system, C3 has been shown to regulate Ag presentation in DCs [575][576], as well as to stimulate of B and T cell responses [578][579]. These observations demonstrate that both molecules, MHC II and complement C3, have a more versatile role in our immune system that go beyond their traditionally described ones.

In this study, we now present data that further support this hypothesis by describing the hitherto unknown covalent binding of C3 to MHC II at the surface of cDCs. To our knowledge this finding represents the first evidence of a new role for MHC II molecules at the cell surface, in which it functions as an acceptor for the covalent association of spontaneously activated complement C3. C3 activation via the tick-over mechanism in the alternative pathway is thought to occur at a very low rate and to play little or no role in the immune system [587][588][724]. Our results challenge these two notions as we show that the reason why constitutive C3 activation does not appear more prominent *in vivo* is because activated C3 molecules bind to MHC II and are quickly eliminated by virtue of the fast turn-over rate of the MHC II-C3 complexes, imposed by MARCHF1-mediated ubiquitination. Mice deficient in MARCHF1 expression or that express the MHC II K225R mutant molecule were the key to identify the role for MHC II as a C3 acceptor due to its prolonged time at the cell surface to efficiently target and bind slowly activated C3 molecules of the alternative pathway at a detectable level. However, binding of C3 to MHC II was also identified at the cell surface of WT cDCs, where MHC II levels were not artificially elevated, though to a much lesser extent

due to Ub-mediated internalization of MHC II. This potentially describes a novel route for the uptake of extracellular C3 into the cell, in which C3 covalently associated to MHC II is internalized through MARCHF1-mediated ubiquitination. It will be necessary to address if and how internalized MHC II-C3 complexes are trafficked inside the cell and to see if directly degraded in the lysosomal compartment, secreted as extracellular vesicles or if they reside in certain intracellular compartments to fulfil intracellular functions as part of the complosome [626][627].

The hypothesis that C3 is degraded in the lysosomal compartment together with MHC II upon Ub-mediated trafficking implies a potential route for the disposal of activated C3. Considering the activation of the complement system can generate remarkable amounts of active complement C3 molecules, clearance of activated C3 post pathogen encounter is critical to prevent the onset of unspecific self-reactivity. While complement receptor-mediated phagocytosis by various cells, such as macrophages and erythrocytes, is known to be a key mechanism in this [571][572][573][574], Ub-mediated trafficking and lysosomal degradation of MHC II, hijacked by C3, is another potential route for the disposal of excessive C3. It has to be noted that our *in vivo* activation experiments failed to recapitulate MHC II-dependent C3 deposition on cDCs in WT mice upon intravenous injection of CpG. Therefore, future *in vivo* activation experiments with additional adjuvants and/or various infection models will be required to see if binding of activated C3 to surface MHC II is involved in its clearance in case of classical/lectin pathway activation or if it is limited to the alternative pathway. However, based on the presented data in this thesis, C3 activation via tick-over appears to represent less of an *accidental* event and more of an *adaptive* property of the complement system with functional consequences (based on the consequential C3-mediated trogocytosis, discussed in section 6.3).

Surprisingly, *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice do not display obvious autoimmune or inflammatory defects, as might have been expected due to the accumulation of C3 on cell surfaces, although *Marchf1*^{-/-} mice are known to exhibit higher inflammatory cytokine levels [520]. However, such disorders often do not arise spontaneously in laboratory mice bred under specific pathogen-free conditions, so it is possible that these mutant mice would display increased susceptibility to C3-mediated autoimmune or autoinflammatory reactions if subjected to the right challenge.

Furthermore, the capacity of MHC II to specifically bind spontaneously activated C3 is not

conferred by the polypeptide component of the molecule but by the carbohydrate in its α chain, which is in agreement with previous studies describing binding preferences of C3 to specific carbohydrates [675][676][677][678]. Virtually all the C3 molecules that could be detected on the surface of WT, *Marchf1*^{-/-} and MHC II K225R^{KI/KI} mice were bound to MHC II, which implies that the MHC II α carbohydrate has some property that sets it apart from the other sugars at the cell surface and makes it prone to be attacked by the C3 thioester group. Potential particularities of the carbohydrate include a certain degree/type of sialylation and branching, which has been shown to vary between different glycoproteins within a particular cell population and between cell types [716][717][718][719]. Moreover, the proportion of MHC II molecules containing such carbohydrate may vary between cells, providing an explanation for the different amounts of C3 we could detect on the surface of cDC1s, cDC2s and B cells, despite the fact all three cell types express equivalent amounts of MHC II molecules. Detailed analysis of MHC II α carbohydrate heterogeneity, and characterization of the carbohydrate targeted by C3 will be required to confirm or falsify this hypothesis.

A schematic illustration summarising the molecular events of the binding of complement C3 to MHC II and its subsequent processing at the cell surface of dendritic cells is given in **Figure 6.1A** (see page 190).

6.3 A novel axis of communication between cDCs and MZ B cells

The direct interaction between cDCs and B cells has been reported in numerous studies, describing cDCs to induce T cell-independent B cell immune responses in lymph nodes and the spleen and in the context of Ag presentation to B cells upon Clec9A-targeted immunization [11][68][71][83][142][146][147][148]. Here, we now report a novel cellular intersection between the innate and adaptive immune system by describing the trogocytosis between cDCs and MZ B cells as an unexpected consequence of C3 deposition on the DC surface through its recognition by CR2.

The study by Balázs *et al.* is one of the most cited publication describing the interaction between DCs and MZ B cells causing their activation [111]. However, this study has various limitations and fundamental differences to our observation made on the interaction of MZ B cells and cDCs: {1} DCs in this case were circulating blood DCs, whereas we here describe the interaction between MZ B cells and spleen-resident cDCs, known as major contributors in the induction of immune responses against blood-borne Ag [396][450]. {2} Second, we describe

cDC-MZ B cell interactions during steady state as opposed to pathogen-triggered upon capturing of bacterial Ag. {3} Lastly, the stimulation of B cells described in the study by Balázs *et al.* is based of soluble mediators, instead of direct cell-cell interaction like we describe, leading to C3-mediated trogocytosis of cDC membrane fractions.

The phenomenon of trogocytosis is poorly understood, with indications for mechanisms of both phagocytosis and endocytosis [763][776], but our results provide some interesting insights. First, it can be strongly, if not exclusively said that it depends on a single receptor-ligand interaction on the surface of the acceptor and donor cells, as was proven here by the strong dependence of C3 on the surface of cDCs, and CR2 on the MZ B cells. It is unclear if the role of this interaction is passive e.g to increase cell-cell adhesion, or active e.g to initiate a cellular mechanisms of membrane transfer, triggered by engagement of CR2 on MZ B cells, or MHC on cDCs. The second conclusion of our results is that trogocytic uptake does not affect only the interacting proteins but also portions of membrane sufficiently large to enable bystander transfer of other membrane proteins (e.g. CD8 and XCR1). Thirdly, we have also shown that trogocytosis is not bidirectional, at least in the case reported here, as we did not observe transfer of B cell membrane to cDCs.

Trogocytosis may result in the transfer of cytoplasmic content in addition to plasma membrane from cDCs to MZ B cells, but we did not address this possibility. Limited understanding of the mechanisms of trogocytosis prevents us from providing a more clear explanations for these observations. Indeed, we cannot discard the possibility that membrane transfer does not occur directly via apposition of the two membranes involved, and is instead the result of MZ B cell uptake of microvesicles such as extracellular vesicles released by cDCs [799][800]. However, trogocytic uptake of cDC membrane may enable MZ B cells to acquire intact Ag captured by cDC receptors, akin to the acquisition of Ag deposited on marginal zone macrophages by follicular B cells. MZ B cells are considered as semi-innate type of B cells and major producers of natural Abs, which recognize molecular patterns shared by multiple species of bacteria and other pathogens. Such Abs play a major protective role against infection early in life, before adaptive humoral B cell responses have had time to develop [113][114]. Yet, MZ B cell responses are poorly understood. They are considered to be largely autonomous, independent of T cells and other cell types, as no good evidence has yet been found for intercellular interaction during development of such immune responses [107][109][110][111][112].

Other functional outcomes of transfer mediated by cDC surface proteins may be possible, but these will require future examination. The consequences of transfer are clearer for cDCs, as an increase in this process in MARCHF1-deficient or MHC II K225R^{KI/KI} mice caused decreases in cDC numbers. A simple explanation for this is that while cDCs can tolerate a certain degree of trogocytic sequestration of plasma membrane, they cannot repair the damage that must accompany the removal of substantial portions of membrane in the mutant mice, causing cell death. Through this mechanism, MZ B cells emerge as regulators of DC development, raising also the possibility that MHC II ubiquitination has been conserved through evolution not only to promote antigen presentation but also to regulate C3 accumulation and DC homeostasis. MZ B cell trogocytosis of cDC membrane was observed under normal conditions but had to be finely regulated because it contributed to cDC homeostasis and its exacerbation caused reduction in DC numbers. We showed that the regulatory mechanism that prevented this deleterious outcome was MARCHF1 ubiquitination of MHC II, which limited the amount of C3 displayed on the cell surface of cDCs. A corollary of these findings is that MHC II ubiquitination and C3 deposition regulate cDC homeostasis. However, this hypothesis has to be investigated in the close future, for which we are currently generating MHC II K225R^{KI/KI} mice that are also deficient in CR2 expression (i.e. MHC II K225R^{KI/KI} x *Cr2*^{-/-}), in order to examine if CR2 expression is necessary for *in vivo* trogocytosis and this in turn is responsible for the cDC ablation in these mice.

A schematic illustration summarizing DC trogocytosis by B cells in the spleen as a consequence of enriched C3 deposition on the cell surface of dendritic cells is given in **Figure6.1A** and **Figure6.1B**.

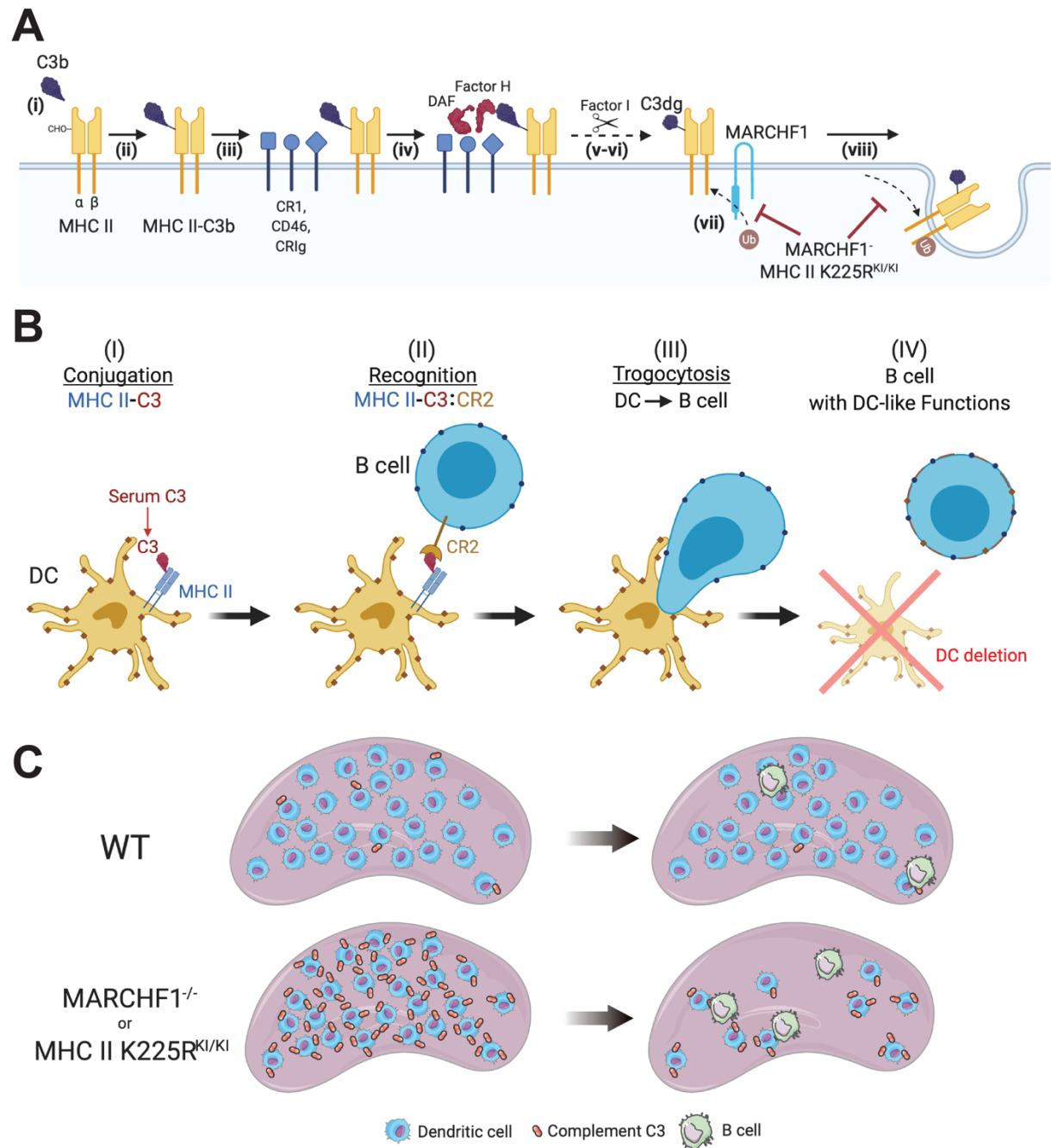


Figure 6.1 A schematic illustration summarizing the molecular binding of complement C3 to MHC II and its subsequent processing at the surface of dendritic cells and their consequential trogocytosis by B cells in the spleen. (A) Representation of C3 binding to a plasma membrane MHC II of a healthy host cell and its inactivation to prevent cellular damage. (i) C3 constitutively converts to C3b (tick-over), which binds to the carbohydrate of membrane MHC II causing (iii) binding of complement regulating proteins, such as CR1, CD46/MCP and CR1g, in cis and (iv) recruitment of serum DAF and Factor H which (v) recruit Factor I to (vi) degrade C3b into C3dg. In WT DC the formed MHC II-C3 complexes are cleared from the cell surface by endocytosis via MARCHF1 ubiquitination. The lack of MHC II ubiquitination interrupts this process at step (vii), causing accumulation of MHC II-C3dg on the DC surface of *Marchf1*^{-/-} and MHC II K225RKI/KI mice. **(B)** Trogocytic transfer of DC membrane to B cells mediated by CR2 recognition of MHC II-C3 complexes. Constitutively activated C3 binds to MHC II on DC. B cell Complement Receptor 2 recognises C3dg/C3d, triggering trogocytic transfer of DC plasma membrane to B cells. The process leads to the acquisition of new functions by MZBC using DC receptors and to the elimination of the trogocytosed DC. **(C)** Trogocytosis of C3-coated DC by B cells occurs subtle in the spleen of WT mice but is significantly enhanced in the spleen of mice with enhanced C3 deposition due to impaired MHC II (i.e. *Marchf1*^{-/-} and MHC II K225RKI/KI), causing a depletion of cDCs as well as increased numbers of B cells presenting DC-typical surface proteins.

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