



UNIVERSITY *of*
TASMANIA

MENZIES
Institute for Medical Research



The Role of the CC-Chemokine Receptor 6, CCR6, in B cell differentiation during the humoral immune response

by

Jennifer Louise Bannan

Menzies Institute for Medical Research, at the University of Tasmania

Bachelor of Science (Hons)

Mj. Biochemistry and Molecular Biology

Submitted in fulfilment of the requirements for the

Doctor of Philosophy, Medicine

University of Tasmania

November, 2014

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Signed: _____

Date: 07 / 04 / 15

Authority of Access

This thesis is not to be made available for loan or copying for two years following the date this statement was signed. Following that time, the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Signed: _____

Date: 07 / 04 / 15

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Signed: _____

Date: 07 / 04 / 15

Abstract

The T cell-dependent humoral immune response consists of a series of discrete stages that culminate in the differentiation of effector B cells, capable of secreting antibodies. These cellular events occur in specific microenvironments within secondary lymphoid organs. The positioning of B cells during the humoral response allows them to receive the appropriate cellular and genetic cues necessary for their subsequent differentiation. Consequently, the precise positioning of B cells is essential for the generation of an effective humoral response.

The chemokine receptors, a family of G-protein coupled receptors, are responsible for directing the migration and organisation of lymphocytes in the immune system. It has been established that the chemokine receptors, CXCR5, CCR7, CXCR4 and EBI2, work together to co-ordinate the movement of B cells in the humoral response, by integrating stimuli from various chemokine ligands in the surrounding environment. Whilst a number of chemokine receptors on B cells have been functionally defined, others are not well understood. One such chemokine receptor, CCR6, has an expression pattern consistent with a role in B cell differentiation, however, the contribution of CCR6 to the humoral response remains undefined.

This thesis focused on addressing the role of CCR6 in the humoral response. I hypothesised that CCR6 expression is necessary for efficient B cell differentiation during T cell-dependent humoral immune responses. Specifically, this thesis has examined 1) the expression level of CCR6 during B cell activation, 2) the consequences of B cell differentiation in the absence of CCR6, 3) the potential mechanisms that underlie the contribution of CCR6 to B cell differentiation and finally, 4) the relevance of CCR6 in B cell-mediated autoimmunity.

Initially, CCR6 expression was assessed on B cells after activation with T cell-dependent antigens. Flow cytometry analysis demonstrated that antigen-specific B cells significantly upregulate CCR6 expression upon activation *in vitro*. This finding was also confirmed *in vivo*. Furthermore, cell sorting and immunofluorescence revealed that CCR6 is expressed during T-B cell interactions *in vivo*. Consequently, this work established that CCR6 is upregulated on activated B cells.

Considering the distinct expression of CCR6 during B cell differentiation, particularly activation, the impact of CCR6 on the humoral response was evaluated. B cell differentiation was quantified in WT and CCR6^{-/-} mice that were challenged with the immunogen, NP-KLH, over a 6-week period.

Flow cytometry and immunofluorescence analysis revealed that the CCR6^{-/-} mice generated more germinal centre B cells at 3 and 5 days after antigen challenge, compared to WT mice. This correlated with a significant reduction in the frequency of naïve B cells at days 3, 5 and 14, as well as a significant increase in the memory B cell population at days 3, 5, and 10, after challenge in the CCR6^{-/-} mice compared to the WT mice. In addition, when extrafollicular B cells were analysed in MD4 and MD4.CCR6^{-/-} mice immunised with HEL-SRBC, the MD4.CCR6^{-/-} mice were found to have significantly fewer extrafollicular B cells 6 days after challenge, compared to the MD4 mice. Even though B cell differentiation was found to be dysregulated in the absence of CCR6, this dysregulation was not permanent as it only occurred early in the response, indicating a role for CCR6 in the initiation of the humoral response.

Investigation into the potential mechanisms underlying the B cell dysregulation observed in the CCR6^{-/-} mice resulted in several findings. Immunofluorescence of splenic germinal centre follicles showed no gross structural abnormalities in the CCR6^{-/-} mice. In addition, qRT-PCR indicated that somatic hypermutation and differentiation in CCR6^{-/-} germinal centre B cells was not affected. However, CCR6^{-/-} germinal centre B cells contained significantly more Bcl-6 mRNA than WT, potentially accounting for the increased germinal centre response. Furthermore, this increase in Bcl-6 mRNA was attributed to a significant increase in T follicular helper cell-secreted IL-21. Also, flow cytometry analysis demonstrated that the T follicular helper cell and follicular dendritic cell populations of CCR6^{-/-} mice were reduced compared to WT mice, indicating that germinal centre affinity selection is impaired in the absence of CCR6. Despite the increased germinal centre response observed in the CCR6^{-/-} mice, antibody quantification by ELISA demonstrated that the production of antigen-specific IgM and IgG in CCR6^{-/-} mice was equivalent to that in WT mice. Additionally, an adoptive transfer model demonstrated that the loss of CCR6 on leukocytes other than B cells is responsible for the B cell dysregulation identified in the CCR6^{-/-} mice. Taken together, the reduced extrafollicular response and increased germinal centre response likely counterbalance each other in the CCR6^{-/-} mice. Hence, the loss of CCR6 does not have an overall detrimental effect on the humoral response.

Finally, the clinical relevance of CCR6 was examined in a mouse model of systemic autoimmunity and in the human B cell-mediated systemic autoimmune disease, Systemic Lupus Erythematosus (SLE). Flow cytometry analysis showed that the FasL^{gld} mice, which develop a spontaneous generalised systemic autoimmune disease, have a significantly lower frequency of CCR6⁺ B cells than WT mice. However, CCR6 expression on B cells was significantly higher in the FasL^{gld} mice compared to the WT mice. This finding was examined in a preliminary study of SLE. Participants

diagnosed with SLE had a significantly higher expression of CCR6 on B cells, compared to healthy controls. The increased CCR6 expression observed in autoimmunity highlights the potential for chemokine receptors to be used as biomarkers and therapeutic targets of disease.

Overall, this thesis presents several novel findings. It is the first to demonstrate the upregulation of CCR6 on activated B cells, as well as the first to define the T cell-dependent humoral response in CCR6^{-/-} mice and to examine the potential of chemokine receptors to be used in clinical medicine. As a result of this work, I propose that CCR6 aids the organisation of activated B cells during the T cell-dependent humoral response. This thesis provides a significant contribution to our understanding of the development of efficient humoral responses and insight into how responses can be manipulated in disease.

Acknowledgements

The completion of my PhD has been a long and challenging journey and I am elated that I have finished. However, I could not have succeeded without the support of many people. I hope that my acknowledgements do justice to the support that I have received and the full extent of my gratitude.

First, I would like to thank my primary supervisor, Professor Heinrich Körner, for his support. Thank you for the opportunity to work on this project. Thank you for strengthening my character and my resolve. The process of completing my PhD has taught me life-long skills.

Thank you to my co-supervisor, Dr. David Gell, for your support. Your independent perspective was refreshing and much appreciated.

I would also like to thank Kathy Buttigieg and Jocelyn Darby for their complete belief in me. I have learnt so much from both of you – personally and professionally. Thank you for your brilliant technical skills in the laboratory, your excellent immunology expertise and your seemingly never-ending general knowledge. You have taught me to be practical and to let go. You were reassuring, encouraging and confident in my ability, and for this I am very thankful. Your friendship and laughter have been invaluable. I could not have finished without you. Thank you.

Furthermore, I am very grateful to the Menzies Institute for Medical Research, at the University of Tasmania, for their professional support, which has allowed me to present and discuss this research to an international audience, and successfully complete my PhD. I would also like to thank the Menzies Graduate Research Coordinators: I have really appreciated your support. To my fellow students at Menzies, thank you for making my experience enjoyable.

Finally, I would like to sincerely thank all of my friends and family for their encouragement and understanding. To my parents, John and Louise, you have taught me to be inquisitive about the world and encouraged my pursuit in science. Thank you for your endless love and support, and your unwavering belief in me. I could not have completed my PhD without you. Thank you.

Table of Contents

Declaration of Originality	ii
Authority of Access	iii
Statement of Ethical Conduct	iv
Abstract	v
Acknowledgements	viii
List of Figures	xi
List of Tables	xii
List of Abbreviations	xiii
Chapter 1: Introduction	1
1.1. <i>The Immune System</i>	2
1.2. <i>Lymphocytes and Lymphoid Organs</i>	4
1.3. <i>B Lymphocytes</i>	6
1.3.1. Antibodies	6
1.3.2. B cell development	7
1.4. <i>The Humoral Immune Response</i>	10
1.4.1. Stage 1: B cell activation	12
1.4.2. Stage 2: T-B cell interactions	13
1.4.3. Stage 3: Precursor B cell proliferation	14
1.4.4. Stage 4: The expansion of Extrafollicular Foci	14
1.4.5. Stage 5: The Germinal Centre reaction	16
1.5. <i>Lymphocyte Migration</i>	19
1.5.1. Chemokines and Chemokine Receptors	19
1.5.2. Chemokine Receptors in Humoral Immunity	21
1.6. <i>The CC-chemokine receptor 6</i>	23
1.6.1. The identification of CCR6	23
1.6.2. The ligand for CCR6	23
1.6.3. The function of CCR6	24
1.7. <i>Autoimmunity</i>	28
1.7.1. CCR6 and Autoimmunity	28
1.7.2. CCR6 and SLE	31
1.8. <i>Scope of Study</i>	32
Chapter 2: Materials and Methods	34
2.1. <i>Animals</i>	35
2.2. <i>Participants</i>	35
2.3. <i>Standard Solutions</i>	36
2.4. <i>Antigens and Immunisations</i>	37
2.5. <i>Flow Cytometry and Cell Sorting</i>	37
2.6. <i>Immunofluorescence</i>	40
2.7. <i>Magnetic Cell Separation</i>	41
2.8. <i>Tissue culture</i>	41
2.9. <i>Adoptive Transfers</i>	41
2.10. <i>Quantitative Real-Time Polymerase Chain Reaction</i>	41
2.11. <i>Preparation for Next Generation Sequencing</i>	42
2.12. <i>Serum Isolation</i>	44
2.13. <i>Enzyme-linked immunosorbent assay</i>	44
2.14. <i>Statistical Analysis</i>	45
Chapter 3: CCR6 expression on activated B cells during the humoral response	46

3.1. CCR6 expression is upregulated upon T cell-dependent B cell activation in vitro	47
3.2. CCR6 is expressed by T cell-dependent activated B cells in vivo	51
3.3. Concluding Remarks	56
Chapter 4: The cellular kinetics of the humoral response in the absence of CCR6	58
4.1. CCR6 counterbalances excessive splenic Germinal Centre formation	59
4.2. CCR6 regulates efficient effector B cell differentiation	63
4.3. CCR6 contributes to humoral responses in multiple secondary lymphoid organs	66
4.4 CCR6 expression is linked to the formation of Extrafollicular Foci	68
4.5. CCR6 influences Marginal Zone B cells during T cell-dependent humoral responses	70
4.6. Concluding Remarks	72
Chapter 5: The mechanisms underlying CCR6 deficient B cell differentiation	74
5.1. CCR6 contributes to the Germinal Centre reaction	75
5.2. The effects of CCR6 are not intrinsic to B cells	89
5.3. CCR6 deficiency results in an altered chemokine receptor expression	92
5.4. Concluding Remarks	96
Chapter 6: The expression profile of CCR6 in Systemic Lupus Erythematosus	99
6.1. CCR6 expression is dysregulated in FasL ^{gld} mice	100
6.2. Prominent CCR6 expression identifies SLE	106
6.3. Concluding Remarks	111
Chapter 7: Discussion and Conclusion	114
7.1. CCR6 is upregulated upon follicular B cell activation	115
7.2. CCR6 contributes to efficient B cell differentiation	117
7.3. CCR6 is associated with SLE	121
7.4. Conclusion	123
Chapter 8: References	125

List of Figures

<u>Figure 1.1.1.</u> The mammalian immune response	3
<u>Figure 1.2.1.</u> The structure of secondary lymphoid organs	5
<u>Figure 1.4.1.</u> B cell differentiation in the T cell-dependent humoral immune response	11
<u>Figure 1.4.4.1.</u> The mechanisms of the Extrafollicular response	15
<u>Figure 1.4.5.1.</u> The mechanisms of the Germinal Centre reaction	17
<u>Figure 1.5.1.1.</u> The structure of chemokine receptors	20
<u>Figure 1.5.2.1.</u> The chemokine receptor-directed positioning of B cells during the T cell-dependent humoral response	22
<u>Figure 1.6.3.1.</u> The expression profile of CCR6 on B cells during the T cell-dependent humoral response	28
<u>Figure 3.1.1.</u> Optimisation of CCR6 detection on B cells	48
<u>Figure 3.1.2.</u> CCR6 expression during T cell-dependent B cell activation in vitro	50
<u>Fig. 3.1.3.</u> In vitro T cell-dependent B cell activation in the absence of CCR6	51
<u>Figure 3.2.1.</u> CCR6 is highly expressed by CD38 ⁺ GL7 ⁺ precursor B cells in vivo	53
<u>Figure 3.2.2.</u> CCR6 is expressed by T-B cell conjugates in vivo	55
<u>Fig. 3.2.3.</u> Quantification of T-B cell conjugates in vivo	56
<u>Figure 4.1.1.</u> The T cell-dependent Germinal kinetics in the absence of CCR6	60
<u>Figure 4.1.2.</u> The frequency of splenic Germinal Centre follicles during the T cell-dependent humoral response in the absence of CCR6	62
<u>Figure 4.2.1.</u> Effector B cell differentiation during the T cell-dependent humoral immune response in the absence of CCR6	65
<u>Figure 4.2.2.</u> Characterisation of the CD38 ^{hi} GL7 ⁻ B cell population in the T cell-dependent humoral response	66
<u>Figure 4.3.1.</u> The T cell-dependent humoral response of lymph node B cells	68
<u>Figure 4.4.1.</u> CCR6 deficiency impairs the Extrafollicular response	69
<u>Figure 4.5.1.</u> The contribution of CCR6 to Marginal Zone B cell differentiation in the T cell-dependent humoral response	71
<u>Figure 5.1.1.</u> The structure of Germinal Centre follicles in the CCR6 deficient humoral response	76
<u>Figure 5.1.2.</u> The molecular characteristics of CCR6 deficient Germinal Centre B cells	78
<u>Fig. 5.1.3.</u> The molecular characteristics of CCR6 deficient T Follicular Helper cells	81
<u>Fig. 5.1.4.</u> The T Follicular Helper cell kinetics of the T cell-dependent humoral response in the absence of CCR6	83
<u>Fig. 5.1.5.</u> The Follicular Dendritic cell kinetics of the CCR6 deficient T cell-dependent humoral response	84
<u>Figure 5.1.6.</u> T cell-dependent antibody production in the absence of CCR6	85
<u>Figure 5.1.7.</u> Preparation of antigen-specific Germinal Centre B cells for Immunoglobulin Sequencing	87
<u>Figure 5.1.8.</u> Optimisation of the sample preparation protocol for antigen-specific Immunoglobulin Sequencing	88
<u>Figure 5.2.1.</u> The contribution of CCR6 deficient leukocytes to Germinal Centre formation in the T cell-dependent humoral response	91
<u>Figure 5.3.1.</u> The chemokine receptor expression profile of CCR6 deficient B cells in the T cell-dependent humoral response	93
<u>Figure 5.3.2.</u> The influence of CXCR5 and CCR7 on T cell-dependent Germinal Centre B cell formation	95
<u>Figure 6.1.1.</u> The CCR6 ⁺ B cell compartment in murine systemic autoimmunity	101
<u>Figure 6.1.2.</u> The characteristics of CCR6 expression during B cell differentiation in murine systemic autoimmunity	103
<u>Figure 6.1.3.</u> The expression of CCR6 on T Follicular Helper cells in murine systemic autoimmunity	105
<u>Figure 6.2.1.</u> The expression of CCR6 in the circulating B cell population during Systemic Lupus Erythematosus	107
<u>Figure 6.2.2.</u> The expression of CCR6 on the circulating T Follicular Helper cell population in Systemic Lupus Erythematosus	109

List of Tables

<u>Table 1.3.2.1.</u> Stages of B cell development in the bone marrow	8
<u>Table 1.3.2.2.</u> Follicular B cell subsets in the spleen	9
<u>Table 1.5.1.1.</u> Chemokine Receptors	21
<u>Table 1.7.1.1.</u> CCR6 autoimmune disease associations	30
<u>Table 2.1.1.</u> Strains of Mus musculus used	35
<u>Table 2.2.1.</u> Characteristics of Participants	36
<u>Table 2.3.1.</u> Frequently used Solutions	36
<u>Table 2.5.1.</u> Antibodies used to detect murine B cells	38
<u>Table 2.5.2.</u> Antibodies used to detect chemokine receptors in murine samples	38
<u>Table 2.5.3.</u> Antibodies used to detect murine T cells	38
<u>Table 2.5.4.</u> Secondary antibodies used for flow cytometry in murine samples	39
<u>Table 2.5.5.</u> Antibodies used to detect human B cells by flow cytometry	39
<u>Table 2.5.6.</u> Antibodies used to detect human T cells by flow cytometry	39
<u>Table 2.6.1.</u> Primary antibodies used for immunofluorescence	40
<u>Table 2.6.2.</u> Secondary antibodies used for immunofluorescence	40
<u>Table 2.10.1.</u> Primers used for quantitative Real-time Polymerase Chain Reaction	42
<u>Table 2.11.1.</u> Next Generation MID Sequences	44
<u>Table 2.13.1.</u> Antibodies used for Enzyme-linked Immunosorbent Assay	45
<u>Table 6.2.1.</u> Statistical power of Systemic Lupus Erythematosus study	111

List of Abbreviations

AID: Activation-induced Cytidine Deaminase	IRF4: Interferon regulatory factor 4
ANOVA: Analysis of Variance	ITAMs: Immunoreceptor Tyrosine-based Activation Motifs
AP-1: Activator Protein -1	J: Joining
Bcl-6: B-cell lymphoma 6	JAK: Janus Kinase
BCR: B cell Receptor	KLH: Keyhole Limpet Hemocyanin
Blimp-1: B lymphocyte maturation protein-1	LFA-1: Lymphocyte function-associated antigen 1
Blmk: B-cell linker protein	Lpr: Lymphoproliferation
BSA: Bovine Serum Albumin	LPS: Lipopolysaccharide
CD: Cluster of Differentiation	m: murine
cDNA: complementary DNA	MAPK: Mitogen-Activated Protein Kinases
CSR: Class Switch Recombination	MHC: Major Histocompatibility Complex
CT: Cycle Threshold	MRL: Murphy Roths Large
D: Diversity	mRNA: Messenger Ribonucleic Acid
DMEM: Dulbecco's Modified Eagle Medium	MS: Multiple Sclerosis
DNA: Deoxyribonucleic Acid	MZ: Marginal Zone
DNP: 2,4-Dinitrophenyl	NFAT: Nuclear Factor of Activated T cells
DTT: Dithiothreitol	NFκB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
EBF1: Early B cell factor-1	NK: Nature Killer
EBI2: Epstein-Barr virus-induced G-protein coupled receptor 2	NP: 4-Hydroxy-3-nitrophenylacetyl
EDCI: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	PBMCs: Peripheral Blood Mononuclear Cells
EDTA: Ethylenediaminetetraacetic acid	PBS: Phosphate Buffered Saline
EF: Extrafollicular Foci	PCR: Polymerase Chain Reaction
eGFP: enhanced Green Fluorescent Protein	PPs: Peyer's Patches
ELISA: Enzyme-linked Immunosorbent Assay	qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction
FACS: Fluorescent Activated Cell Sorting	RBCs: Red Blood Cells
FCS: Fetal Calf Serum	RA: Rheumatoid Arthritis
FDCs: Follicular Dendritic Cells	Rag: Recombination Activating Gene
FoxP3: Forkhead box P3	rRNA: ribosomal Ribonucleic Acid
GALT: Gut-Associated Lymphoid Tissue	SAP: SLAM Associated Proteins
GC: Germinal Centre	S.c. Subcutaneous
<i>Gld</i> : Generalised Lymphoproliferation Disease	SEM: Standard Error of the Mean
h: human	SHM: Somatic Hypermutation
HEL: Hen Egg Lysozyme	SLAM: Signalling Lymphocyte Activation Molecule
HEVs: High Endothelial Venules	SLE: Systemic Lupus Erythematosus
HRP: Horseradish Peroxidase	SRBCs: Sheep Red Blood Cells
HSCs: Hematopoietic Stem Cells	STAT: Signal Transducer and Activator of Transcription
ICAM-1: Intercellular Adhesion Molecule 1	TBE: Tris-Borate-EDTA
ICOS: Inducible T-cell Costimulator	TD: T cell-dependent
Id2: Inhibitor of DNA binding 2	T _{FH} : T Follicular Helper cell
IFN: Interferon	T _{FR} : T Follicular Regulatory cell
Ig: Immunoglobulin	Th: T helper
IgH: Immunoglobulin Heavy Chain	TI: T cell-independent
IgL: Immunoglobulin Light Chain	V: Variable
IL: Interleukin	Xbp-1: X-box binding protein-1
ILFs: Isolated Lymphoid Follicles	
I.p. intraperitoneal	

Chapter 1

Introduction

Our knowledge of the mechanisms underlying the antibody-mediated primary immune response; particularly B cell differentiation, has advanced considerably since the publication of Burnet's clonal selection theory. However, we have yet to fully explain how B cells are organised during the humoral response and how this organisation contributes to B cell fate. This information is significant as it will not only enhance our understanding of the mechanisms underlying an efficient humoral response, it will provide insights into disorders of the immune system and aid the generation of therapies in the future. This thesis seeks to define the contribution of the chemokine receptor, CCR6, to B cell differentiation in humoral immunity, and thereby make an original contribution to knowledge. This introduction comprises an overview of the immune system, an introduction to the humoral response, a critical review of the role of CCR6 in the immune system and in the context of autoimmunity and, finally, an outline of the research conducted.

1.1. The Immune System

The mammalian immune system is a complex biological system made up of a network of leukocytes that work together to generate an immune response. The immune system is composed of two subsystems: the innate and adaptive systems. The innate system is responsible for the immediate response to infection, generating a broad inflammatory response against infiltrating pathogens. It is also necessary for initiating the adaptive response, which specifically targets infiltrating pathogens. Both the innate and adaptive immune systems contain cellular and humoral components that fight infection.

There are several different types of leukocytes that defend against infection and disease. The largest population of leukocytes is the granulocytes, which consist of neutrophils, eosinophils and basophils. The granulocytes are involved in the early innate responses where they destroy invading pathogens, including bacteria and parasites. The lymphocytes are the second largest population and consist of largely B and T cells. The lymphocytes reside in secondary lymphoid organs and are essential in adaptive responses, as each one is specific for one epitope of an antigen, and therefore enables the immune system to respond to a diverse range of pathogens. Finally, the monocytes are progenitors of macrophages and dendritic cells. These cells are pivotal in initiating adaptive responses, as they transport antigen to the lymphocytes in secondary lymphoid organs. The innate and adaptive systems are linked together by lymphocyte activation.

A summary of the coordinated actions of the immune system, in response to infection, is depicted in Fig. 1.1.1. The mechanisms underlying innate responses have been reviewed elsewhere¹⁻³. Briefly,

pathogens cause infection by infiltrating host cells such as epithelial cells. Infected cells release inflammatory proteins, such as cytokines and chemokines, which recruit innate cells to the site of infection (Fig. 1.1.1). Tissue resident macrophages are among the first cells to respond to infection. Macrophages can recognise pathogens through receptors that detect repetitive pathogen-associated molecular patterns. As a result, macrophages internalise and destroy pathogens through phagocytosis. Macrophages are also able to release inflammatory proteins, enlisting further help from the innate cells, including granulocytes, monocytes, lymphocytes and toxin releasing natural killer (NK) cells. The complement system, a large family of plasma proteins, is also activated by pathogens and triggers either opsonisation, the recruitment of phagocytes to the site of infection, or the direct lysis of pathogens and infected cells.

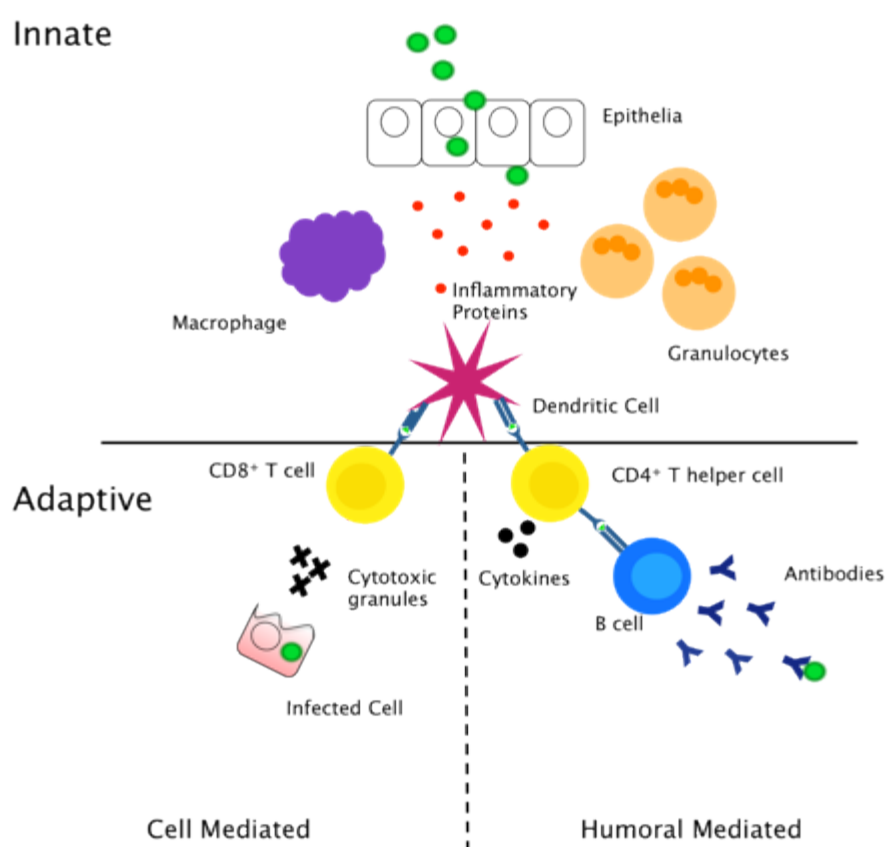


Figure 1.1.1. The mammalian immune response

In response to infection, cells of the innate system, particularly macrophages and granulocytes, are recruited to the sites of infection via inflammatory mediators, where they clear pathogens via phagocytosis. Dendritic cells are also recruited to process antigens. After antigen uptake, dendritic cells migrate to secondary lymphoid organs, where they present antigens to T cells, thereby initiating the adaptive response. Intracellular antigens presented on MHC-I molecules activate CD8⁺ T cells, which release cytotoxic granules that kill the infected cells. Extracellular antigens presented on MHC-II molecules activate CD4⁺ T cells, which support B cells during differentiation and antibody production. Antibodies directly bind antigens, neutralising or opsonising pathogens for destruction.

If pathogens evade the innate system, specialised antigen presenting cells such as dendritic cells, are recruited to the site of infection to initiate the adaptive response (Fig. 1.1.1). These cells are capable of processing pathogens into small antigens, which are then presented to lymphocytes on Major Histocompatibility Complexes (MHC). Depending on whether the infection is caused by an intracellular or extracellular pathogen, a cell- or humoral-mediated response is generated respectively. In the cell-mediated response, dendritic cells present antigens on MHC-I molecules to cluster of differentiation (CD)8⁺ T cells, inducing the lysis of infected cells through the release of cytotoxic granules (Fig. 1.1.1). In contrast, dendritic cells present antigens bound to MHC-II molecules to CD4⁺ T cells, initiating the humoral response. While CD4⁺ T cells become activated, B cells are simultaneously activated by antigen, either directly in soluble form or via antigen presenting cells. The activated CD4⁺ T cell can then support the differentiation of cognate B cells into antibody secreting cells (Fig. 1.1.1). Antibody secreting cells travel to the sites of infection, where they release large amounts of soluble antibody to neutralise pathogens and prevent further damage. Antibodies can also opsonise pathogens, marking them for destruction by phagocytosis. Another important feature of the adaptive response is the generation of memory lymphocytes, which are crucial for mediating responses to re-infection. Further detail on the initiation of adaptive immune responses is provided elsewhere^{4,5}.

1.2. Lymphocytes and Lymphoid Organs

The human body contains approximately 2 trillion lymphocytes, which originate from hematopoietic stem cells (HSCs) in the bone marrow and undergo two developmental stages. The first stage occurs in the primary lymphoid organs. Specifically, T cells develop in the thymus, while B cells continue their development in the bone marrow. After maturation, naïve lymphocytes exit primary lymphoid organs and circulate around the body via secondary lymphoid organs, including the lymph nodes, spleen and mucosa associated lymphoid tissues. Once in the periphery, lymphocytes undergo a second stage of development, in response to antigen. If lymphocytes do not encounter antigen in the periphery, apoptosis will be induced. In this way, there is a constant turnover of lymphocytes. The recognition of antigen and differentiation of lymphocytes occurs within the secondary lymphoid organs, making them crucial components of the adaptive immune system.

All secondary lymphoid organs share a similar structure. They are highly compartmentalised such that B cells are located together in B cell follicles and T cells assemble in adjacent zones. The

secondary lymphoid organs are supplied by the blood and lymph vessels, providing lymphocytes with direct access to antigens and antigen presenting cells (reviewed in^{6,7}).

The spleen is separated into two distinct areas, termed the red and white pulp. The red pulp is responsible for iron recycling and erythrocyte filtration, while the white pulp consists of leukocytes. The white pulp is encased by the marginal zone (MZ), and consists of distinct B cell follicles and T cell zones (Fig. 1.2.1). It is innervated by the central arterioles that branch from the splenic artery, enabling lymphocytes and antigens access via the MZ. The MZ consists of reticular fibroblasts, resident macrophages and B cells, which are capable of detecting and responding to blood-borne antigens.

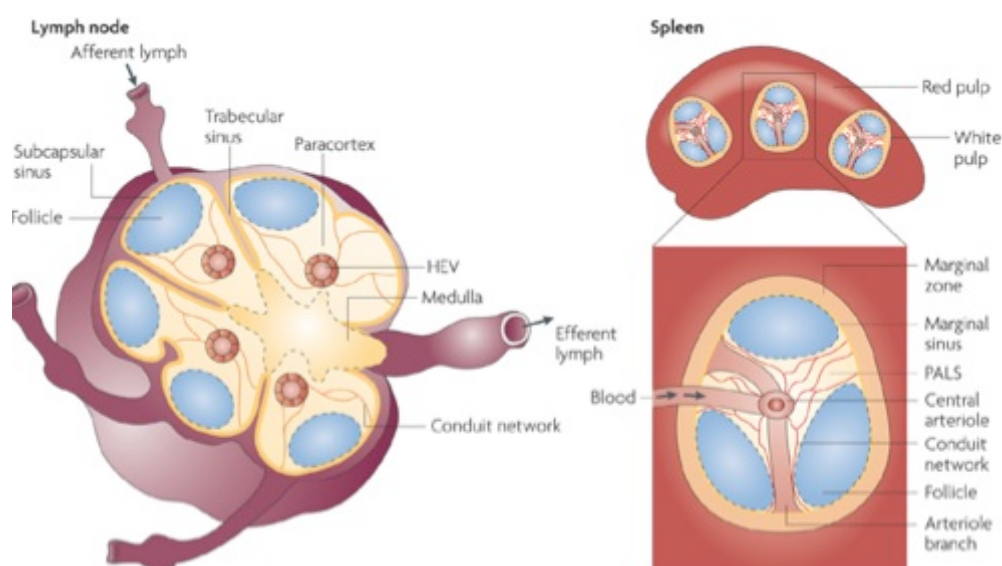


Figure 1.2.1. *The structure of secondary lymphoid organs*

The lymph node contains B cell follicles (blue) adjacent to T cell zones (yellow) in the paracortex. Similarly, the spleen contains white pulp consisting of B cell follicles (blue), as well as T cell zones (yellow) in the periarteriolar lymphoid sheath (PALS). Both organs are innervated by the blood and lymphatic system.

Reproduced from Batista & Harwood (2009) Nature Reviews⁸.

In contrast to the spleen, the lymph nodes are structured in layers. The lymph node is considerably smaller than the spleen and is largely home to T cells. The outer cortex of the lymph node contains B cell follicles, while the paracortex consists of T cell zones, dendritic cells and high endothelial venules (HEVs) (Fig. 1.2.1). Also, the inner medulla region of the lymph node contains macrophages. Lymphocytes and antigens transit through the lymph node via the HEVs, or the lymphatic vessels located at the subcapsular sinus.

The distinct lymphocyte compartments within secondary lymphoid organs provide niches for lymphocytes, facilitating both homeostasis and adaptive immune responses. For instance, the T cell zone is the site of T cell activation by dendritic cells, while the B cell follicle is the site of B cell activation and clonal expansion. The precise organisation of microenvironments within secondary lymphoid organs is essential for the development of a timely and efficient response, and will be discussed in further detail in section 1.5.

1.3. B Lymphocytes

B cells are central to the adaptive immune response and they are most notably known for their ability to produce antibodies. Antibody, also referred to as immunoglobulin (Ig), can be produced as either a membrane bound or soluble form. The membrane bound form acts as the B cell receptor (BCR), inducing B cell activation in response to antigen engagement, while the soluble form directly clears infection through neutralisation, or facilitates phagocytic destruction through opsonisation.

1.3.1. Antibodies

The Ig molecule is a Y-shaped glycoprotein composed of two inner heavy- and two outer light-polypeptide chains, joined together by disulphide bonds. Each heavy and light chain is made up of a constant and variable domain. The constant domain of the heavy chains are fixed to the cell surface in the case of the BCR, while the variable domain of both heavy and light chains function as the antigen-binding site. There are five types of antibodies, which are specified by the constant domain of the Ig. The five heavy chain classes are the IgM (μ), IgD (δ), IgG (γ), IgA (α) and IgE (ϵ) isotypes, and each has a different effector function. IgM is the first antibody to be expressed and secreted. It is expressed on immature B cells as the BCR, as is IgD. IgM is also effective in activating the complement system. IgG is the most abundant antibody produced and it neutralises or opsonises pathogens. IgE is generated during allergic reactions and parasitic infections, inducing the activation of chemical-releasing mast cells. Finally, IgA is secreted at mucosal tissues and is most efficient at neutralisation. In addition to the five classes of antibodies, there are also two types of light chain – the λ or κ light chains – although, the functional significance of this distinction is unknown. Further detail on the structure and function of antibodies is provided elsewhere⁹.

B cells are specialised to respond to an enormous diversity of epitopes from pathogen-derived antigens. This specialisation is accomplished through the recombination of Ig genes. The process of genetic recombination has been reviewed elsewhere⁹⁻¹¹, however a brief summary is provided

below. The heavy chain variable domain is encoded by variable (V), diversity (D) and joining (J) gene segments, while the light chain variable domain is encoded by only V and J gene segments. The constant domain of each chain is encoded by a single exon for each chain class. During B cell development, the Ig heavy chain locus is rearranged first. The D segment is selected and joined to a J segment and this is followed by the joining of a V segment to the newly formed DJ segment. Once the VDJ genes of the heavy chain have been arranged, the light chain VJ arrangement can proceed. Recombination occurs via the VDJ recombinase, a multi-enzyme unit that contains a pair of lymphocyte specific recombination activating gene (Rag) proteins, and is collectively responsible for the cleavage and religation of deoxyribonucleic acid (DNA). Not only is diversity introduced through the selection of gene segments joined together, it is also introduced through junctional diversity, or the insertion or removal of nucleotides between segments, and finally, the combination of a rearranged heavy chain with a rearranged light chain creates further diversity. Upon exposure to antigen in the periphery, B cells can undergo further diversification. Class switch recombination (CSR) allows B cells to switch their heavy chain constant region (exon) from IgM and IgD, to an alternate isotype, whilst maintaining their specificity for antigen. This process also occurs by DNA recombination, and is, hence, irreversible. In addition, somatic hypermutation (SHM) allows B cells to fine tune their genetic recombination to enhance their affinity for antigen.

1.3.2. B cell development





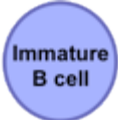
B cell generation begins during embryonic life and continues after birth in two locations. As mentioned previously, B cell development is initiated in the bone marrow and continues in the spleen. Development occurs through a sequence of stages and is controlled by genetic and environmental factors. The intrinsic genetic B cell program, regulated by transcription factors, is essential. However, this program is influenced by environmental cues, such that the migration, localisation and exposure of B cells to antigen, influences the specific subset of B cells generated. Each stage of B cell development can be distinguished by the Ig status and phenotype of the B cell. Several excellent reviews exist that summarise B cell development¹², particularly in the bone marrow¹³, as well as the expansion of B cells¹⁴, their Ig rearrangement¹⁵, and transcriptional regulation¹⁶⁻¹⁸. Key aspects of early B cell development, relevant to this thesis, are described in the following sections.

B cell development in the bone marrow

B cell development in the bone marrow is characterised by several cellular stages, which have been summarised in Table 1.3.2.1. Initially, B cells arise from HSCs, which are identified by the stem cell factor c-kit, as well as the surface markers, CD43 and CD93. The HSC population contains

early lymphoid progenitors that give rise to the interleukin (IL)-7R α^+ CD24 $^+$ common lymphoid progenitors (CLPs). The transcription factors PU.1 and Ikaros are required for commitment to the B cell lineage and this stage marks the beginning of Ig rearrangement, with the joining of D-J immunoglobulin heavy chain (IgH) segments. Specification to the early B cell progenitor, Pro-B cells, requires the expression of the basic helix-loop-helix proteins known as E2A, the early B cell factor-1 (EBF1) and the B cell specific activation protein, also known as Pax5, which leads to CD19 and B220 expression. The Pro-B cells also undergo V-DJ heavy chain Ig rearrangement. Successful expression of the pre-BCR, that is the rearranged heavy chain bound to a surrogate light chain, enables Pre-B cells to proliferate and subsequently arrange their immunoglobulin light chain (IgL) genes. There are several discrete proportions of Pro- and Pre-B cells that can be identified by their cell surface expression. For simplicity however, the characteristics of each fraction have been grouped together. The successful assembly and expression of the BCR, that is IgM, marks the immature B cell stage. Immature B cells with inappropriate BCR specificity or affinity, undergo receptor editing, anergy or apoptosis¹⁹. The remaining immature B cells are then selected to leave the bone marrow and enter the spleen.

Table 1.3.2.1. Stages of B cell development in the bone marrow

Stage					
Immunoglobulin Status		D-J IgH rearrangement	V-DJ IgH rearrangement	Pre-BCR V-J IgL rearrangement	IgM $^+$ IgD $^-$
Transcription Factor Profile	PU.1 Ikaros E2A	E2A EBF1 Pax5			
Surface Phenotype	CD93 $^+$ CD43 $^+$ c-kit $^+$	CD93 $^+$ CD43 $^+$ c-kit $^+$ IL-7R α^+ CD24 $^+$	CD93 $^+$ CD43 $^+$ c-kit $^+$ IL-7R α^+ B220 $^+$ CD19 $^+$ CD24 $^+$	CD93 $^+$ CD43 $^+$ IL-7R α^+ B220 $^+$ CD19 $^+$ IL-2R α^+ CD24 $^+$	CD93 $^+$ B220 $^+$ CD19 $^+$ CD24 $^+$

Adapted from references: ^{13,16-18}.

B cell development in the periphery

Approximately twenty million immature B cells are generated in the bone marrow daily and of these, only 3% are selected to survive as part of the peripheral B cell population in the spleen. Immature B cells generated in the bone marrow enter a transitional stage that links bone marrow development with peripheral development. During this stage, transitional B cells undergo negative selection and the remaining B cells are directed to replenish either the naïve follicular or MZ compartments¹⁹⁻²². The naïve follicular B cells are the largest population of mature B cells and they reside in the B cell follicle, whereas the MZ B cells are located in the MZ of secondary lymphoid organs. The signals that drive transitional B cells to develop into follicular or MZ B cells are unknown.

In the periphery, follicular and MZ B cells must encounter antigen within approximately 24 hours to survive. This encounter induces B cells to differentiate into terminal effector cells inside secondary lymphoid organs. Terminal differentiation occurs through several stages. As the MZ B cells are located adjacent to the blood supply, they are adept at responding to blood borne antigens. For follicular B cells to become activated, antigen needs to be transported to B cells inside follicles. Furthermore, cues from antigen-activated T cells are needed to drive B cell differentiation and antibody production. Consequently, this process is referred to as a T cell-dependent (TD) response. During this process, naïve B cells differentiate into plasma and memory cells via a precursor, germinal centre (GC) and extrafollicular foci (EF) stage. In contrast to follicular B cells, MZ B cells rapidly differentiate into plasma cells in a T cell-independent (TI) manner. A summary of the key surface markers that identify the mature B cell subsets is displayed in Table 1.3.2.2. The process of TD B cell activation will be explored in further detail in the following section.

Table 1.3.2.2. Follicular B cell subsets in the spleen

Naïve	Precursor	Germinal Centre	Plasma	Memory	Marginal Zone	Extrafollicular Foci
B220 ⁺	B220 ⁺	B220 ⁺	B220 ⁺	B220 ⁺	B220 ⁺	B220 ^{lo}
CD38 ⁺	CD38 ⁺	CD38 ⁺	CD38 ⁻	CD38 ^{hi}	CD21 ^{hi}	IgG1 ⁺
GL7 ⁻	GL7 ⁺	GL7 ⁻	GL7 ⁻	GL7 ⁺	CD23 ⁻	CD38 ⁻
IgM ⁺		CD21/35 ⁺	CD138 ⁺	IgM ^{hi}		CD138 ^{hi}
IgD ⁺		CD95 ⁺		IgD ⁺		
IgG ⁻		MHC-II ^{hi}				
CD23 ⁺						
CD21/35 ⁺						
CD95 ⁺						
MHC-II ^{int}						

Table compiled from previous studies:²³⁻²⁸.

As mentioned earlier, to improve antibody affinity for antigen, peripheral B cells undergo CSR and SHM (reviewed in²⁹⁻³²). These processes are directed by the enzyme activation-induced cytidine deaminase (AID), which acts on single-stranded DNA, whilst also reducing cell proliferation and exposing cells to apoptosis³³⁻³⁵. AID is responsible for introducing single nucleotide mutations into Ig genes^{33,36,37}. Specifically, AID converts cytosine to uracil, creating a U-G mismatch in the DNA. Mutations introduced into the variable domain of the IgH and IgL genes, or the switch regions of the constant domain of IgH gene, induce SHM and CSR respectively. Proteins from the Base Excision Repair process repair the mutations introduced by AID, however, these processes are associated with low-fidelity polymerases, and consequently introduce further mutations and single strand breaks. If single strand breaks are converted into double stranded breaks, either spontaneously or via the Mismatch Repair process, then CSR is induced. This occurs when the double strand breaks in the switch regions are resolved and the VDJ sequence is recombined with an alternative switch region, such that the constant μ domain is replaced with either the γ , ϵ or α constant domain.

1.4. The Humoral Immune Response

The TD humoral response consists of a series of discrete cellular events that culminate in the differentiation of antibody secreting plasma cells and memory B cells. These events occur in an ordered fashion, at various locations within the secondary lymphoid organs. The development of the humoral response can be summarised in a five-staged model, as depicted in Fig. 1.4.1. Briefly, naïve B cells encounter antigen through the BCR, inducing activation (Fig. 1.4.1a). Activated B cells then migrate towards the T cell zone to interact with antigen-specific T cells (Fig. 1.4.1b). After receiving T cell help, the activated B cell migrates to the border of the B cell follicle, forming the precursor stage (Fig. 1.4.1c). The precursor B cell will undergo a short burst of proliferation and CSR. Next, the precursor B cell differentiates through one of two pathways. The B cell can leave the follicle and migrate to extrafollicular sites to form EF (Fig. 1.4.1d), or it can enter the follicle to establish GC follicles (Fig. 1.4.1d). Both the GC and EF facilitate clonal expansion. The EF is responsible for the initial production of low-affinity antibodies from short-lived effector B cells, while long-lived effector B cells generated in the GC, produce the more specific high-affinity antibodies. The GC is able to produce these high-affinity B cell clones, as it is specialised to improve antibody affinity through SHM and affinity selection. The cellular and molecular mechanisms of this model, as determined by studies in mice using protein immunisations, are described below.

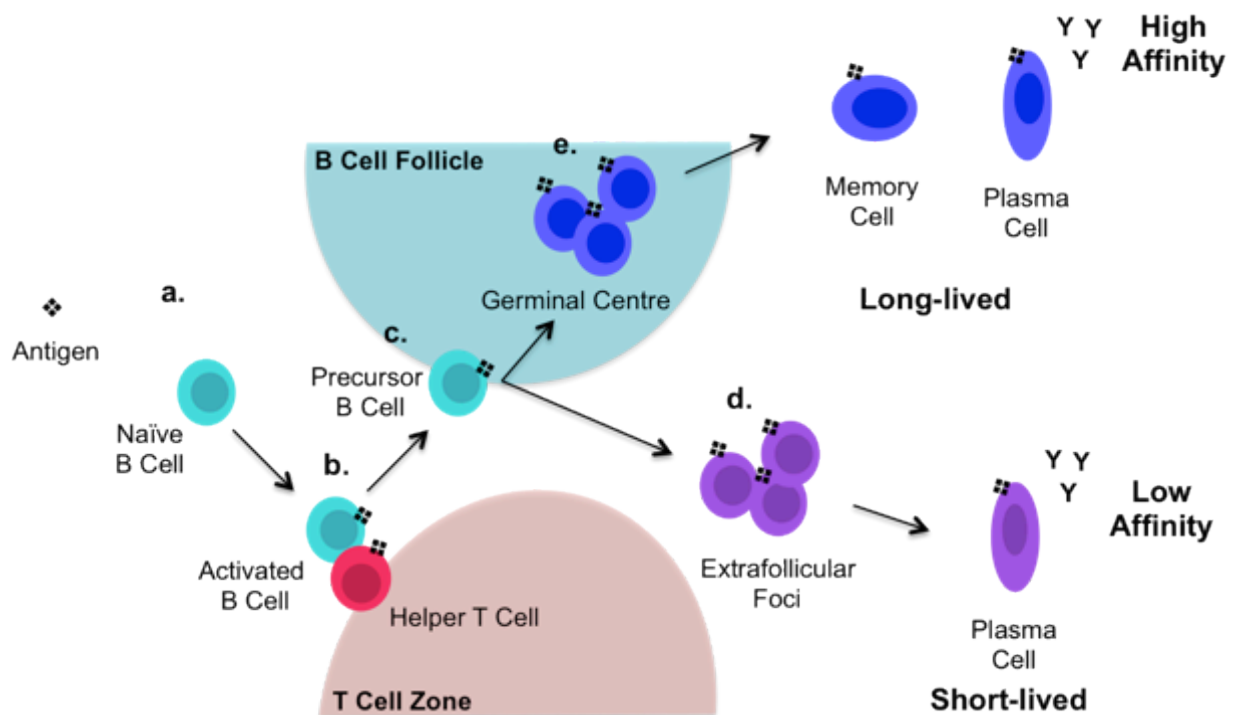
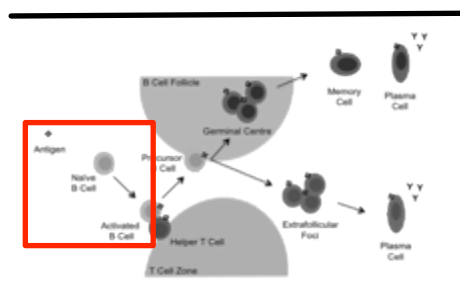


Figure 1.4.1. B cell differentiation in the T cell-dependent humoral immune response

The humoral response can be divided into five discrete stages. Initially, B cells become activated upon antigen exposure (a). Activated B cells then migrate toward the T cell zone, where they form T-B cell conjugate pairs (b). After receiving cognate T cell help, B cells migrate to the border of B cell follicles and enter a precursor state (c). Precursor B cells are then selected to expand into either extrafollicular foci (d) or germinal centres (e), yielding short-lived, low-affinity and long-lived, high-affinity effector B cells respectively.

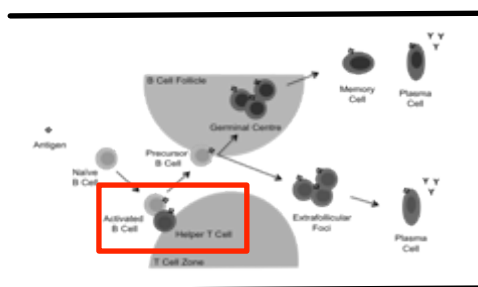
1.4.1. Stage 1: B cell activation



To initiate the humoral response, B cells must encounter antigen. B cells can acquire antigen several ways. Firstly, B cells may encounter soluble antigen directly, after it has diffused across the B cell follicle, or passed through a conduit system of collagen and fibroreticular cells^{38,39}. B cells can encounter larger antigens directly, after they have been broken down by proteases⁴⁰, or by antigen-presenting macrophages^{39,41,42}. Dendritic cells can also present antigen to B cells⁴³. Finally, B cells that are in transit to secondary lymphoid organs can collect soluble antigen directly from circulation³⁸. Despite the multiple ways in which B cells can encounter antigen, antigen acquisition occurs very quickly, with antigen appearing in secondary lymphoid organs within minutes of immunisation, and B cells internalising antigen within a couple of hours^{38,41}.

Once the B cell has bound antigen through its BCR, intracellular signalling is initiated resulting in antigen processing. Initially, the BCR clusters in the cell membrane, mediating the recruitment of intracellular proteins. The immunoreceptor tyrosine-based activation motifs (ITAMs), within the CD79 accessory protein of the BCR, are phosphorylated upon activation, thereby initiating signalling. The CD19/CD21 co-receptor is also activated, amplifying the activation signal. Following antigen binding, a series of protein kinases are activated, initiating the assembly of the signalosome via the B-cell linker protein (Blnk). This signal is then propagated through the signalosome, causing the release of intracellular calcium and activating the mitogen-activated protein kinases (MAPK) pathway. This results in the translocation of the transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1), to the nucleus, facilitating antigen presentation, proliferation and differentiation. Further details on the process of BCR signalling is provided elsewhere⁴⁴⁻⁴⁸. While intracellular signalling is occurring, the BCR-antigen complex is internalised through endocytosis and subsequently degraded by lysozymes. This allows antigen to be collected, loaded onto MHC-II molecules synthesised in the endoplasmic reticulum, and trafficked to the cell surface for presentation to CD4⁺ T cells.

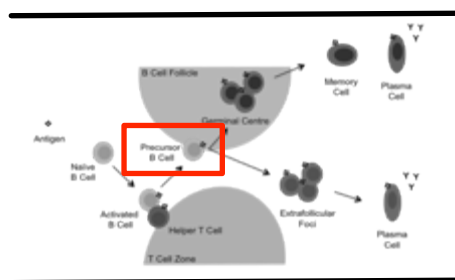
1.4.2. Stage 2: T-B cell interactions



Following activation, B cells stop moving randomly throughout follicles^{49,50}, and migrate directly to the border between the B cell follicle and T cell zone, known as the T-B border^{41,49-52}. Simultaneously, T cells activated by antigen presenting cells within the T cell zone also migrate towards the T-B border⁴⁹. Here, antigen-specific B and T cells physically meet, forming conjugate pairs⁵⁰. Generally conjugates are monogamous, with one B cell interacting with one T cell at any one time, however, conjugates consisting of multiple T cells have been reported^{50,51}.

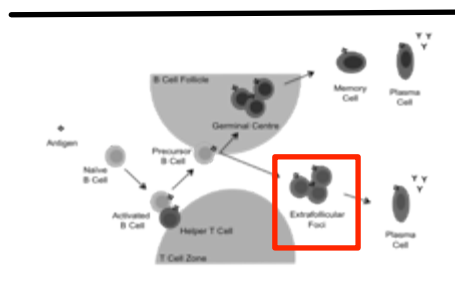
The T-B cell conjugates migrate at the T-B border for approximately an hour, in localised, yet random movements led by the B cell^{50,51}. These interactions generally occur for the first for 2 days following antigen challenge, allowing B cells to consecutively interact with several T helper (Th) cells, prior to the next stage of differentiation^{50,51,53}. During this time, the lymphocytes share signals that cue their subsequent differentiation. Activated B cells present MHC-II bound antigens to T cells, eliciting T cell help through the engagement of the T cell receptor⁵⁴⁻⁵⁷. To facilitate signal transfer, B cells form extensive surface contacts, mediated by integrins, with T cells^{56,58}. For instance, the T cell adhesion molecule, lymphocyte function-associated antigen-1 (LFA-1), binds to intercellular adhesion molecule-1 (ICAM-1) on the B cell^{56,59}. Mutual co-stimulation of B and T cells occurs through the signalling lymphocyte activation molecules (SLAM) and SLAM associated proteins (SAP)^{60,61}. Activated T cells express CD40L, which is bound by CD40 on B cells, not only driving B cell expansion, but also inducing B cells to express CD80/86^{49,62-64}. In turn, CD80/86 binds CD28 on T cells, enabling both T cell expansion and the expression of CD40L, thereby creating a positive feedback loop^{61,65,66}. B cell stimulation of T cells induces T helper (Th) cell function, that is, cytokine secretion, which further supports B cell differentiation. The microtubule-organising centre, golgi apparatus and, the endocytic and exocytic compartments within the T and B cell of a conjugate pair are polarised towards each other at the point of contact^{57,67,68}. This facilitates the efficient delivery of cytokines, including IL-2, IL-4 and interferon (IFN)- γ , which also promote B cell differentiation^{62,69-72}.

1.4.3. Stage 3: Precursor B cell proliferation



After B cells receive T cell help, B cells enter a precursor stage, approximately 48–72 hours after immunisation, prior to GC and EF differentiation^{28,73–75}. Activated B cells migrate from the T-B border to the edge of B cell follicles, where they undergo an initial burst of proliferation and CSR^{74,76}. Following T-B cell interactions, B cells must commit to either GC or EF differentiation – an important event for B cells^{51,77}. However, the signals that drive these B cell fate decisions are poorly understood and remain controversial. It has been suggested that B cell selection is based on their original antigen-BCR interactions, such that high-affinity B cells are directed to differentiate into EF, while low-affinity B cells are directed to form GCs, where their affinity is then greatly enhanced by SHM and affinity selection^{75,78–80}. However, it has also been reported that B cells which present high amounts of antigen-MHC-II complexes to T cells during T-B cell interactions, are selected to differentiate into GCs⁷³. Furthermore, these decisions can also be influenced by cytokines secreted in the local environment⁷⁷. Nonetheless, B cell clones are selected to expand and differentiate through the extrafollicular or GC pathway. As the extrafollicular response has a short life span relative to the GC response, the EF response will be discussed here first.

1.4.4. Stage 4: The expansion of Extrafollicular Foci



Precursor B cells committed to EF differentiation, leave the B cell follicle border to establish EF. The extrafollicular response produces antibody secreting cells that are short-lived and produce low-affinity antibodies, providing an initial, rapid response to infiltrating pathogens (Fig. 1.4.4.1)⁸¹.

Only a few B cell clones are needed to form EF, which then proliferate extensively for 4-7 days following antigen challenge^{27,53,77}. The extrafollicular response peaks at day 8 and dissipates by day 14^{19,2053}. During the extrafollicular response, B cells continue to undergo isotype switching via CSR^{27,53,77}. Interestingly, it is thought that the EF B cells are exposed to selection pressures within the EF. Specifically, dendritic cells have been documented as being associated with extrafollicular plasmablasts, and required for their differentiation into plasma cells⁸². It is likely that these dendritic cells play a similar role to the follicular dendritic cells (FDCs) located in the GC follicle and that is to provide antigen for competitive selection between B cell clones⁵³. In contrast to the GC, it is generally agreed that Th cells are not associated with EF. Although, there is some evidence from the autoimmune Murphy Roths Large (MRL)-lymphoproliferation (lpr) mouse model that suggests the existence of extrafollicular Th cells, which support plasma cell differentiation⁸³. Overall, there is consensus that EF B cells must be able to recognise and respond appropriately to antigen to be selected to survive (Fig. 1.4.4.1). Following selection in the EF, B cells differentiate into plasmablasts and then plasma cells^{26,27,77,84-86}. Plasma cell differentiation requires the transcription factor B lymphocyte maturation protein-1 (Blimp-1), which inhibits activated B cell function, induces proliferation, and the expression of X box binding protein-1 (XBP-1), a transcription factor which also promotes plasma cell differentiation⁸⁷⁻⁹⁰. In this way, plasmablasts transition into plasma cells.

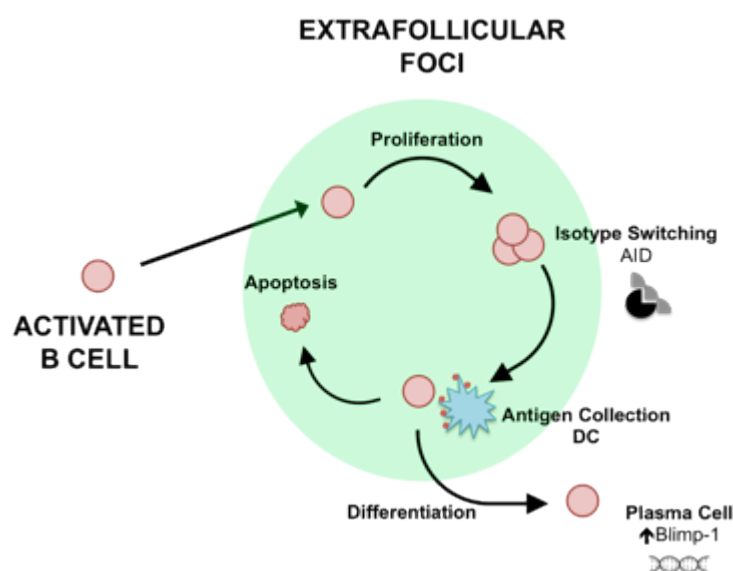
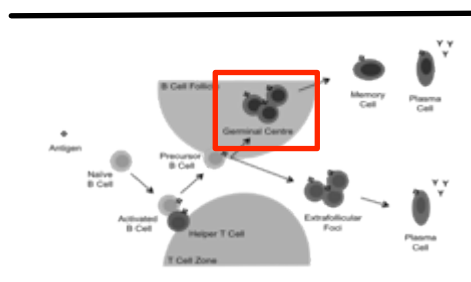


Figure 1.4.4.1. The mechanisms of the Extrafollicular response

Activated B cells are selected to form EF in sites outside the B cell follicle. Here, B cells undergo proliferation, isotype switching and selection into short-lived antibody secreting cells that are of low-affinity. The key proteins associated with the EF response are indicated.

1.4.5. Stage 5: The Germinal Centre reaction



Precursor B cells that are selected to differentiate into GC B cells enter the B cell follicle to establish GC follicles, where they generate long-lived, high-affinity plasma cells and memory B cells^{91,92}. The GC follicle is a highly organised structure compared to EF. It is founded by 1–3 B cell clones and is capable of generating a response to more than one antigen^{77,93}. The GC is specialised to improve antibody affinity through proliferation, SHM and affinity selection⁹⁴⁻⁹⁷. These processes are collectively referred to as the GC reaction. GC follicles appear approximately 3–4 days following antigen challenge, and the response peaks at day 8, before decreasing and persisting until at least day 16 after antigen challenge^{27,49,53,77}. GC-derived plasma and memory B cells are detected in the blood within a week of antigen challenge⁹⁸.

The dynamics of the GC response have been extensively reviewed^{99,100}. Briefly, the GC contains two distinct zones, a dark zone and a light zone, which is encased by a follicular mantle. In order for GCs to be established, precursor B cells must upregulate their expression of the transcription factor B-cell lymphoma 6 (Bcl-6), prior to their migration into the B cell follicle¹⁰¹⁻¹⁰³. Furthermore, the transcription factor, interferon regulatory factor 4 (IRF4) is also required for GC formation and may contribute to Bcl-6 activation¹⁰⁴⁻¹⁰⁶. Once in the dark zone, B cells undergo proliferation, CSR and SHM via AID expression^{27,53}. Newly mutated B cell clones then migrate to the light zone where they undergo affinity selection. It is becoming clear that B cell migration in the GC and the GC reaction is not a simple process, as B cells are capable of migrating back and forth between the dark zone and light zone, undergoing several rounds of proliferation, mutation and selection^{95-97,100}. In this way, B cells are competitively selected to differentiate into plasma and memory B cells (Fig. 1.4.5.1).

The selection of B cells is mediated by the FDCs and a subset of CD4⁺ T cells, known as the T Follicular helper (T_{FH}) cells. In the GC, B cells must collect antigen from FDCs, and present it to T_{FH} cells in order to elicit T cell help (Fig. 1.4.5.1). The development of FDCs and the function of FDC-derived cues have been extensively reviewed^{107,108}, as has the generation of T_{FH} cells¹⁰⁹⁻¹¹¹. As

such, only those aspects relating to B cell differentiation will be highlighted here. The FDCs play an important role in sustaining the GC reaction. They primarily provide a source of antigen to B cells for selection⁴², however, they also secrete cytokines and chemokines, and engage molecules on the surface of B cells, to support the GC reaction¹¹²⁻¹¹⁴. In fact, B cells that don't interact with FDCs are eliminated by apoptosis^{115,116}. Furthermore, the normal cessation of the GC response is due to the gradual exhaustion of antigen supply on the FDCs¹¹⁷. If B cells successfully collect antigen from FDCs and present it to T_{FH} cells, the T_{FH} cells provide (inducible T-cell costimulator) ICOS and CD40L signals to the GC B cell, thereby supporting B cell proliferation, CSR and affinity selection¹¹⁸⁻¹²⁰. In addition, the T_{FH} cells secrete cytokines, such as IL-4, IL-10, IFN- γ and IL-21, which also regulate antibody production through CSR, SHM and differentiation^{115,121-125}. If GC B cells do not receive help from T_{FH} cells, particularly in the form of CD40 interactions, they will undergo apoptosis¹¹⁵.

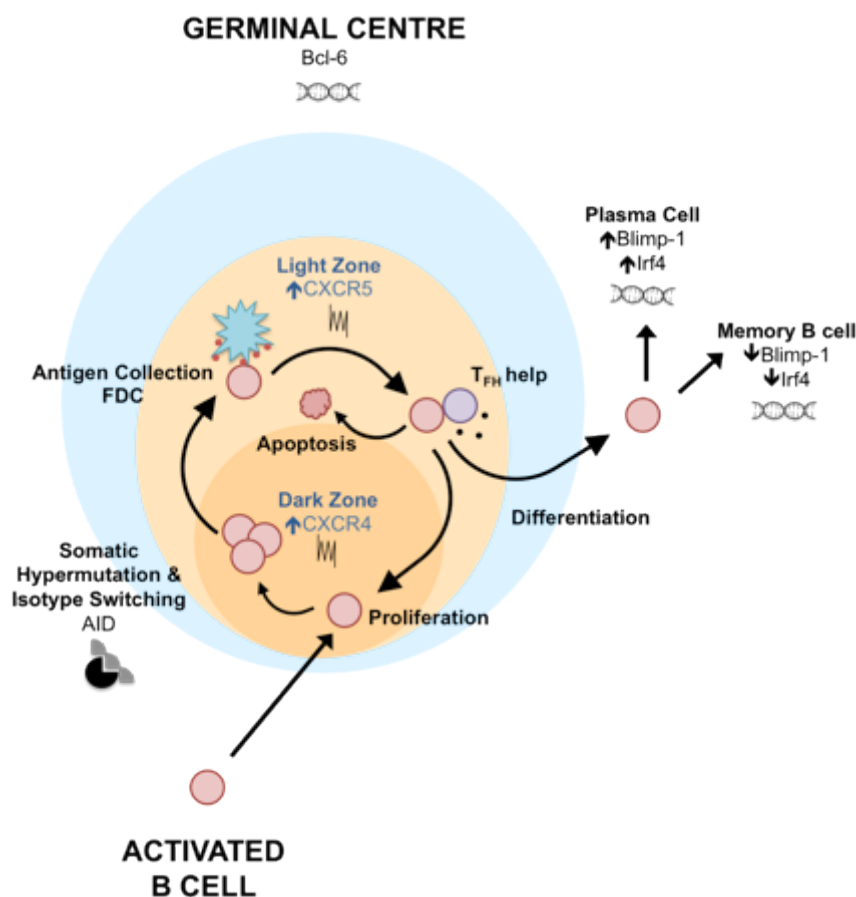


Figure 1.4.5.1. The mechanisms of the Germinal Centre reaction

Activated B cells committed to the GC fate enter the B cell follicle to establish GC follicles. The GC is structurally divided into the dark zone and light zone by chemokine receptors. GC B cells migrate between the dark and light zone, undergoing consecutive rounds of proliferation, SHM, CSR and affinity selection. Interactions with antigen-presenting FDCs and cytokine-secreting T_{FH} cells, are essential for antibody maturation and terminal differentiation. The key proteins associated with the GC reaction, including transcription factors, enzymes and chemokine receptors, are indicated.

Those B cell clones that have acquired mutations that improve antibody affinity, are selected to differentiate into effector plasma and memory B cells^{126,127}. Clones with inappropriate mutations are eliminated by apoptosis and are cleared by tangible body macrophages^{128,129}. The mechanisms responsible for clonal selection remain poorly understood. The evidence to date suggests that B cell selection and differentiation is directed by a combination of signals, including the nature of the antigen, the affinity status of the B cell, the B cell intrinsic transcription program, the cytokine or cellular signals derived from the T_{FH} cells and FDCs, as well as the temporal and spatial organisation of the B cell^{130,131}. Indeed, *in silico* modeling concurs with this theory¹³², however, it remains to be directly established under physiological conditions.

Nonetheless, the GC reaction yields plasma cells and memory B cells, and the characteristics of each terminally differentiated subset are presented here. GC-derived plasma cells are non-proliferating, long-lived cells, that reside in the bone marrow for several months to years after challenge^{85,133,134}. However, a proportion of these cells can also reside in the spleen for approximately 1 year¹³⁴. Plasma cell differentiation peaks approximately 1 week after challenge, and rapidly decreases thereafter^{85,133}. In contrast to plasma cells, memory B cells do not secrete antibody, rather they differentiate into antibody secreting cells upon reinfection, re-initiate the GC response and self-replenish¹³⁵⁻¹³⁷. Memory B cells appear within 3 days of immunisation, peak at day 21 and persist until at least day 56 after immunisation¹³⁵. They can reside in both the spleen and bone marrow, until they are called upon during re-infection¹³⁶. GC-derived memory B cells can be isotype switched, or they can retain IgM^{137,138}. Of course, it is important to note that early TD memory B cells can also be produced independently of the GC^{28,81}. These cells can be isotype switched, but are not mutated and are of low-affinity, and therefore suited to aiding the initial rapid response to infection.

Molecularly, the differentiation of GC B cells is associated with the expression of transcription factors. The transcription factor Blimp-1, encoded by the *Prdm1* gene, is essential for plasma cell differentiation, but not memory formation^{89,139}. In fact, IRF4 expression induces Blimp-1 and AID expression and consequently drives plasma cell differentiation^{105,140,141}. Blimp-1 represses B cell genes associated with proliferation, CSR, B cell phenotype and signalling, including Bcl-6, thereby favouring plasma cell differentiation^{88,102,142}. Blimp-1 also induces XBP-1, a transcription factor required for plasma cell generation but not memory cell generation^{88,143,144}. In addition, Pax5, although required for early B cell development, is repressed during plasma cell differentiation by Blimp-1¹⁴⁵⁻¹⁴⁷. Conversely, Bcl-6 not only supports GC formation, but represses Blimp-1 expression and thereby inhibits plasma cell differentiation¹⁴². Despite the number of transcription

factors identified for plasma cell differentiation, no transcription factor has been identified that specifically instructs GC B cells to differentiate into memory B cells.

1.5. Lymphocyte Migration

The cellular events of the humoral response occur in specific microenvironments within secondary lymphoid organs. This spatial distribution enables cell-cell interactions, which are essential for the initiation and progression of the response. The organisation of lymphocytes in the humoral response must be both accurate and precise – lymphocytes must be at the right place at the right time for an efficient immune response to be generated. Cellular organisation is coordinated by a family of chemokine receptors that exert their function by binding chemotactic cytokines, known as chemokines.

1.5.1. Chemokines and Chemokine Receptors

Chemokines are small 8–12 kDa soluble proteins that direct lymphocyte migration by binding seven-transmembrane $G\alpha_i$ -protein coupled chemokine receptors^{148,149}. Currently, 20 receptors and 50 chemokines have been identified¹⁵⁰⁻¹⁵². Chemokine receptors are made up of an extracellular N-terminal, seven-transmembrane α -helical domain and an intracellular C-terminal domain that is coupled to pertussis toxin sensitive G-proteins (Fig. 6)¹⁵⁰. Upon ligand binding, chemokine receptors undergo a conformational change that activates the G-proteins and triggers Janus Kinase (JAK)/ signal transducers and activators of transcription (STAT) signalling, resulting in calcium mobilisation and chemotaxis of lymphocytes¹⁵³. Chemokine receptors direct lymphocyte migration by binding to chemokines expressed in what is thought to be a gradient in the surrounding environment.

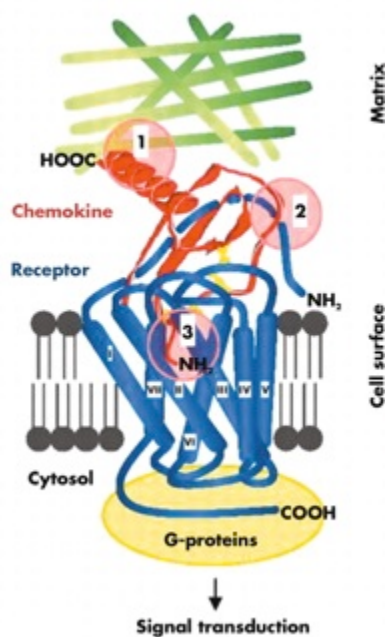


Figure 1.5.1.1. The structure of chemokine receptors

Chemokine receptors are seven transmembrane G-protein coupled receptors that bind chemokines. The binding of chemokines occurs at 3 key sites: (1) the carboxy-terminal α -helix, (2) the N loop region enabling initial receptor contact, and (3) the amino-terminus¹⁵⁴.

Reproduced from Moser and Willmann (2004)¹⁵⁴.

Chemokine receptors can be functionally separated into two broad groups – inflammatory and homeostatic. Inflammatory chemokine receptors mediate the recruitment of monocytes, neutrophils and NK cells to sites of infection, while homeostatic chemokines are constitutively expressed and function in recruiting B cells, T cells, and antigen presenting cells to secondary lymphoid organs. It is important to note that some receptors have both inflammatory and homeostatic functions. Chemokine receptors can be further subdivided based on the frequency and structural arrangement of cysteine groups in their amino terminus – C, CC, CXC, CX3C, where C is a cysteine group and X is an amino acid, and a similar nomenclature exists for chemokine ligands¹⁵¹. A current list of chemokine receptors and their ligands is provided in Table 1.5.1.1. Most chemokine-chemokine receptor interactions are not exclusive and this is particularly true for inflammatory chemokines, which are typically capable of recognising multiple receptors, thus providing versatility when responding to infection.

Table 1.5.1.1. Chemokine Receptors

Classification	Receptor	Ligand/s	Function
C	XCR1	XCL1 & 2	Inflammatory
CC	CCR1	CCL3, 5, 7, 14, 15, 16 & 23	Inflammatory
	CCR2	CCL2, 7, 8, 13 & 16	Inflammatory
	CCR3	CCL5, 7, 8, 11, 13, 15, 16, 24, 26 & 28	Inflammatory
	CCR4	CCL17 & 22	Homeostatic
	CCR5	CCL3, 4, 5, 8, 11, 14 & 16	Inflammatory
	CCR6	CCL20	Homeostatic
	CCR7	CCL19 & 21	Homeostatic
	CCR8	CCL1	Homeostatic
	CCR9	CCL25	Homeostatic
	CCR10	CCL27 & 28	Homeostatic
CXC	CXCR1	CXCL6, 7 & 8	Inflammatory
	CXCR2	CXCL1, 2, 3, 5, 6, 7 & 8	Inflammatory
	CXCR3-A	CXCL9, 10 & 11	Inflammatory
	CXCR3-B	CXCL4, 9, 10 & 11	Inflammatory
	CXCR4	CXCL12	Homeostatic
	CXCR5	CXCL13	Homeostatic
	CXCR6	CXCL16	Inflammatory
	CXCR7	CXCL12	Inflammatory
CX3C	CX ₃ CR1	CX ₃ CL1	Inflammatory

Adapted from references: ^{151,152,155}.

1.5.2. Chemokine Receptors in Humoral Immunity

Lymphocyte migration is imperative for the immune system to maintain homeostasis and generate immune responses upon infection. Lymphocytes are required to migrate from primary lymphoid organs, recirculate throughout the periphery and localise within microenvironments of secondary lymphoid organs. It is clear that the precise positioning of B cells in secondary lymphoid organs is determined by several chemokine receptors. The responsiveness of B cells to chemokines, which define various microenvironments, enables this precise localisation during the humoral response. This migration is essential as it allows B cells to receive appropriate cues that direct terminal differentiation.

Currently, four major chemokine receptors contribute to B cell movement: CXCR5, CCR7, CXCR4 and Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2) (Fig. 1.5.2.1). B cell homing to follicles is dependent on the expression of CXCR5¹⁵⁶. Upon activation, B cells upregulate CCR7, overpowering the CXCR5 signal to the B cell follicle, facilitating their migration to the T-B border, where they can interact with cognate Th cells (Fig. 1.5.2.1)⁵². After B cells receive T cell help, they are selected to differentiate into GC or EF B cells. For the formation of EF, B cells downregulate their expression of CXCR5 and CCR7, and upregulate their expression of EBI2, allowing their

migration to extrafollicular sites (Fig. 1.5.2.1)^{157,158}. Conversely, the downregulation of EBI2 enables GC formation¹⁵⁷. Once in the GC, B cells express high levels of CXCR5 and CXCR4, allowing activated B cells to migrate between the CXCL12⁺ dark zone and CXCL13⁺ light zones of the GC (Fig. 1.5.2.1)^{95-97,159}. After differentiation into plasma cells, B cells downregulate CXCR5 and CCR7 expression, while simultaneously upregulating CXCR4 expression, so that plasma cells can migrate to the periphery to exert their effector functions^{160,161}. However, the chemokine receptors responsible for T-B cell conjugate migration and the migration of precursor B cells remain to be defined. Furthermore, while a number of influential chemokine receptors are known, it is not known whether other chemokine receptors that have not yet been functionally defined, contribute to B cell movement in the humoral response. Thus, B cell movement in the humoral response remains to be fully elucidated.

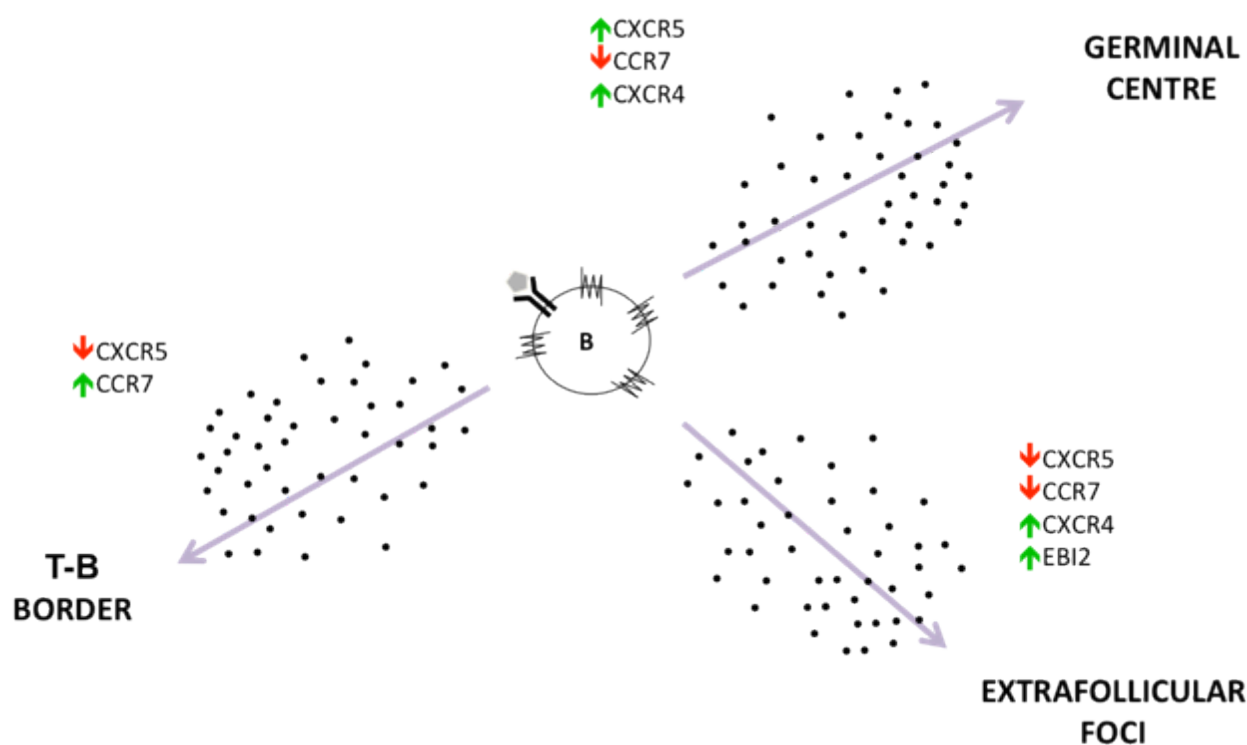


Figure 1.5.2.1. The chemokine receptor-directed positioning of B cells during the T cell-dependent humoral response

At any one time, B cells are exposed to a variety of chemokines (dots) that are constitutively expressed in secondary lymphoid organs and act to define niches within these organs. Depending on the differentiation state of B cells, for instance whether B cells have been activated, or received T cell help, B cells can respond to chemokines through the modulation of chemokine receptor expression. The chemokine receptors and the modulation of their expression required for B cell localisation to the T-B border, extrafollicular foci and germinal centre is depicted.

1.6. The CC-chemokine receptor 6

At the start of this project, the function of the chemokine receptor, CCR6, was unknown. It was clear that CCR6 had a characteristic expression profile on B cells differentiating in response to antigen, indicating that CCR6 may contribute to B cell organisation in the humoral immune response. An overview of CCR6, including its identification, cellular distribution and known interactions, are described below.

1.6.1. The identification of CCR6

CCR6, originally termed STRL22, was first identified in 1997¹⁶². Using a series of cloning and sequencing techniques, CCR6 was identified in human cells by its conserved chemokine receptor sequences that were shared by CXCR1, CXCR2, CXCR4, CCR7 and CXCR5¹⁶². The gene encoding CCR6 was mapped to human chromosome 6 and it was determined that multiple protein variants existed¹⁶². Furthermore, northern blot analysis of human tissue demonstrated that CCR6 was expressed by lymphocytes in lymphoid tissue¹⁶².

1.6.2. The ligand for CCR6

Subsequently, three research groups simultaneously identified the ligand for CCR6. At the time, CCL20 was a novel chemokine without a specific chemokine receptor and consequently research efforts focused on identifying its receptor. Baba and colleagues transfected human cell lines with cloned chemokine receptors, including CCR6, and measured calcium mobilisation after the addition of CCL20 using a fluorescent calcium-indicator dye¹⁶³. This demonstrated that CCR6 responded to CCL20 with high specificity and hence further investigation into CCR6 expression was undertaken. Northern blot analysis showed that human (h) CCR6 messenger ribonucleic acid (mRNA) was expressed in the spleen, lymph node and appendix, and at a lower level in the liver, intestine and testis¹⁶³. CCR6 mRNA was also detected in peripheral blood leukocytes: specifically B cells, CD4⁺ T cells and CD8⁺ T cells, but not granulocytes, monocytes or NK cells¹⁶³. This work suggested that CCR6 may be involved in lymphocyte trafficking within secondary lymphoid organs¹⁶³. Meanwhile, Liao and co-workers had also identified CCL20 as the ligand for CCR6, by analysing calcium mobilisation in human cell lines transfected with CCR6, and PBMCs. This work showed that CCR6 was specific for CCL20, as CCL20 dependent migration was not inhibited by other chemokines¹⁶⁴. Finally, Greaves and colleagues also reported on the identification of the CCR6-CCL20 mediated migration of human CD34⁺-derived dendritic cells¹⁶⁵. In addition, they confirmed that CCR6 was expressed in CD4⁺ and CD8⁺ T cells of the blood, as well as splenic B cells¹⁶⁵.

However, when splenic B cells were activated with anti-CD40 and IL-4, CCR6 mRNA expression was found to be reduced¹⁶⁵.

Although CCR6 bound to CCL20 with high specificity, CCR6 was also reported to bind small antimicrobial peptides, known as β -defensins¹⁶⁶. Antimicrobial peptides, such as the β -defensins, are produced in the innate response and act to recruit cells to sites of infection¹⁶⁷⁻¹⁶⁹. Briefly, β -defensins are expressed by epithelial cells and function in the recruitment of CCR6⁺ dendritic cells, neutrophils, mast cells and T cells. However, this migration does not occur by a calcium flux, as it does in chemokine-chemokine receptor interactions, instead the β -defensins act purely as an inflammatory signal. Thus, it was clear that CCR6 was responsive to a single chemokine, CCL20^{163-165,170}. The hCCL20 protein was mapped to chromosome 2 and found to share only 20–28% homology with other chemokines¹⁷⁰. Initially, CCL20 was largely detected in the liver, lung, and to a lesser extent in the thymus and intestine¹⁷⁰. Subsequently, CCL20 was identified in other lymphoid tissues including the tonsils and appendix¹⁷¹. Furthermore, chemotaxis assays revealed CCL20 was chemotactic for lymphocytes isolated from human peripheral blood¹⁷⁰. Overall, the evidence thus far indicated that CCR6 responded to the chemokine, CCL20, and that the CCR6-CCL20 axis contributed to the migration of leukocytes.

1.6.3. The function of CCR6

To proceed with functional studies of CCR6, an alternative model to humans was required and hence, the murine (m) counterparts of hCCR6 and hCCL20 were cloned¹⁷². The mCCR6 gene encodes a 367 amino acid protein with several mRNA variants and shares 73.8% sequence homology with hCCR6^{172,152}. Northern blot analysis revealed that mCCR6 mRNA was expressed in the spleen and lymph node¹⁷². Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated that murine B cells from the spleen, lymph node and thymus expressed CCR6¹⁷². CCR6 expression was also detected in splenic dendritic cells, as well as CD4⁺ T cells from the spleen and lymph node, while mCCL20 was largely detected in the thymus, colon and small intestine¹⁷². In addition, mCCL20 was shown to induce calcium mobilisation in human cells transfected with mCCR6, in a dose-dependent manner¹⁷². Overall, the similarity observed between murine and human CCR6 demonstrated that *Mus musculus* was a suitable model to study the role of CCR6. From here, research efforts began to focus on examining the role of CCR6 in the mucosal, cutaneous and systemic immune systems.

CCR6 and Mucosal Immunity

CCR6 has been implicated in many areas of the immune system including the mucosa and in particular, the intestine. The first reports that described the generation of CCR6 deficient mice, to study the function of CCR6, provided robust evidence for CCR6 as a regulator of intestinal immunity. Two groups independently generated homozygous null CCR6 mice^{173,174}. These mice were healthy and had no gross abnormalities in the structure of organs and lymphoid tissue, nor in the frequency of major leukocyte populations, indicating that CCR6 was not required for leukocyte development^{173,174}. However, CCR6 appeared necessary for the localisation of dendritic cells to Peyer's Patches (PPs) via CCL20^{173,174}. Following the examination of four independently derived CCR6^{-/-} mice, using a more sensitive immunohistology technique than the previous studies, it was evident that a different chemokine receptor, CCR9, and not CCR6, was responsible for this dendritic cell localisation¹⁷⁵. Despite this setback, research in this area continued to show that CCR6 contributed to intestinal immunity. Both CCR6 and CCL20 were found to be highly expressed in isolated lymphoid follicles (ILFs) of the intestine, collectively known as the gastrointestinal-associated lymphoid tissue (GALT)¹⁷⁶. These sites are essential for antigen interaction and the initiation of adaptive immune responses. Furthermore, ILFs were reduced in CCR6^{-/-} mice, and this corresponded with a reduction in the frequency of intestinal B cells¹⁷⁶. This finding was confirmed in bone marrow reconstitution and adoptive transfer experiments, demonstrating that CCR6 was required for B cell migration in the intestine and was necessary for the establishment of ILFs¹⁷⁶. In addition, reductions in the frequency of PPs B cells in the absence of CCR6, further supported a role for CCR6 in intestinal B cell migration, particularly through antigen uptake into the GALT¹⁷⁶⁻¹⁷⁸. Despite no signs of inflammation, increases in the total lymphocyte population, particularly T cells, was reported in the intestinal mucosa of CCR6^{-/-} mice^{173,174}. Overall, it is clear that CCR6 contributes to the organisation of lymphoid tissue in the intestine.

CCR6 and Cutaneous Immunity

The function of CCR6 has also been examined during inflammation and diseases affecting the skin. While contact hypersensitivity responses between WT and CCR6^{-/-} mice had been reported as normal, there was a reduced response to delayed type hypersensitivity in CCR6^{-/-} mice, indicating a defect in CD4⁺ T cell activation or migration^{173,174}. Also, CCR6 has been implicated in the development of graft versus-host disease, where host T cells selectively damage the skin and intestinal tissues¹⁷⁹. Together, this suggests that CCR6 contributes to T cell migration in the skin. In addition, CCR6 has been implicated in the migration of dendritic cells and B cells in the skin,

potentially aiding antigen uptake, processing and presentation during inflammation^{180,181}. Consequently, CCR6 contributes to leukocyte migration in cutaneous immune responses.

CCR6 and Systemic Immunity

While CCR6 had been implicated in intestinal and cutaneous immunity, evidence of systemic alterations had also been documented. The development and use of CCR6-enhanced green Fluorescent Protein (eGFP) knock-in mice demonstrated that CCR6 is expressed in all mature B cells, splenic CD4⁺ T cells, CD8⁺ T cells and dendritic cells¹⁸¹. Interestingly, altered antibody responses were observed in the absence of CCR6. Specifically, CCR6^{-/-} mice immunised with the protein carrier, keyhole limpet hemocyanin (KLH) generated increased IgM and decreased IgG3 levels compared to WT mice¹⁷⁴, while immunisation with the hapten, 2,4-dinitrophenol (DNP) conjugated to KLH, caused a reduction in IgG2b levels in CCR6^{-/-} mice compared to WT mice¹⁷³. Furthermore, more recent work has demonstrated that CCR6^{-/-} mice produce more low-affinity than high-affinity antibody forming cells than WT mice, in response to TD antigen challenge¹⁸². In addition, preliminary evidence indicates that CCR6^{-/-} mice establish more TD GC follicles than WT mice¹⁸². Overall, these studies indicate that CCR6 contributes to the efficient generation of humoral responses. Indeed, further work in this field implicated CCR6 in B cell differentiation and this evidence is described below.

The research conducted into the CCR6-CCL20 axis during B cell differentiation is limited and conflicting. While CCR6 had been detected on freshly isolated human peripheral B cells, it was shown that these cells do not respond to CCL20 in calcium mobilisation assays¹⁸³. In contrast, it has also been reported that human peripheral B cells do in fact respond to CCL20, but at a lower level compared to other chemokines¹⁸⁴. Furthermore, this migration response remained low after stimulation with either Lipopolysaccharide (LPS) or anti-CD40/IL-4 and was correlated with a reduction in both CCR6 cell surface expression and mRNA levels¹⁸⁴. In agreement with this, Krzysiek and colleagues also documented that CCR6 expression and responsiveness to CCL20 decreased on human B cells, activated by crosslinking¹⁸⁵. However, it was also reported that human peripheral B cells activated by cross-linking, show enhanced responses to CCL20¹⁸⁶. It is important to note, that these studies used B cells isolated from peripheral blood, which were then activated *in vitro*. Therefore, these B cells are inherently different to follicular B cells, which are largely responsible for initiating humoral immune responses. When human tonsillar B cells were cultured without stimuli, CCR6 expression was increased, however this did not correlate with enhanced migration towards CCL20^{184,187}. Overall, due to the contradictory nature of these studies, it was

clear that further investigation was needed to resolve the relationship between CCR6 expression and B cell activation.

Several groups followed this line of inquiry and as such, have established the expression profile of CCR6 on B cells at different developmental stages. It is known that initial bone marrow progenitor B cells do not express CCR6, however CCR6 is expressed on naïve follicular B cells¹⁸⁸. In addition, chemotaxis assays have demonstrated that CCL20 induces the migration of follicular B cells, and to a lesser extent, MZ B cells from the spleen, lymph node and PPs¹⁸⁸. Another study has confirmed that CCR6 is expressed on follicular B cells and shown that only 20% of follicular B cells migrate in response to CCL20, suggesting that only a few subsets of B cells respond to CCL20¹⁸⁹. This was furthered by studies examining tonsillar B cells via flow cytometry and qRT-PCR, which have demonstrated that CCR6 is expressed by naïve B cells, is highly expressed on memory B cells, and expressed at low levels on GC and *in vitro* differentiated plasma cells¹⁸⁵⁻¹⁸⁷. Furthermore, it has been shown that CCL20 induces the migration of naïve and memory B cells, which both express moderate to high levels of CCR6^{185,186}. Finally, evidence that strongly supported the variation in CCR6 expression on B cells during differentiation arose from the transcriptional analysis of murine B cell subsets. This investigation demonstrated that CCR6 is expressed at a moderate level on naïve follicular B cells, that CCR6 expression is reduced on GC and plasma B cells, and that CCR6 is highly expressed on memory B cells (Fig. 1.6.3.1)¹⁹⁰. More recently, CCR6 expression has been detected on precursor B cells, which arise after T-B cell interactions, prior to GC and EF differentiation (Fig. 1.6.3.1)⁷³. However, it has yet to be determined whether CCR6 is expressed during B cell activation, T-B cell conjugate movement and EF development. Overall, the research to date has provided valuable insight into the characteristics of CCR6 expression and responsiveness to CCL20 in the B cell compartment. However, studies investigating the function of CCR6 are lacking. Further work is required to establish the purpose of modulating CCR6 expression during B cell differentiation in the humoral response.

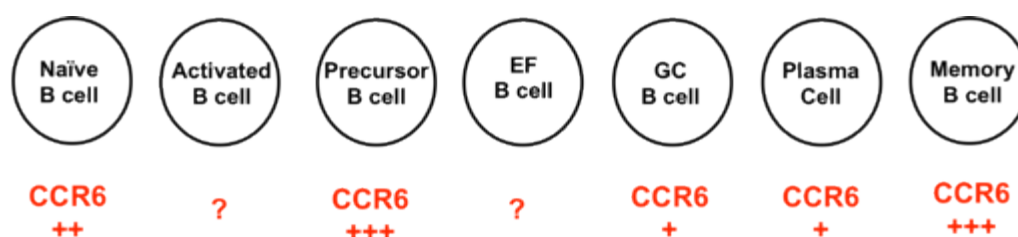


Figure 1.6.3.1. The expression profile of CCR6 on B cells during the T cell-dependent humoral response

CCR6 is moderately expressed on naïve follicular B cells. Following TD antigen activation, CCR6 is expressed on precursor cells, at low levels on germinal centre (GC) and plasma B cells, and high levels on memory B cells. It is currently unknown whether CCR6 is expressed on activated B cells and extrafollicular foci (EF) B cells.

1.7. Autoimmunity

Models of humoral responses are extremely valuable in studying the mechanisms underlying the development of humoral immunity. However, considerable insight can also be gained from dysregulated immune responses, as is the case in autoimmunity. Autoimmunity is defined as a state of self-reactivity, where the immune system loses tolerance to self-antigens and mounts a response against the host. This is particularly devastating if B cells are affected. If B cells specific for host antigens are selected to survive and subsequently differentiate in the periphery, they can generate a targeted antibody response against the host. Under normal conditions, the immune system prevents the onset of autoimmunity through B cell survival decisions, where B cells are either selected to survive or are eliminated by apoptosis, based on the affinity status of their BCR during development in the bone marrow and in the periphery^{191,192}. Given the incredible diversity of B cell clones produced through gene arrangement in the bone marrow, it is inevitable that B cells specific for host pathogens are also generated. The majority of these host specific B cells are normally eliminated and the few that enter the periphery are unlikely to encounter antigen and are unresponsive to antigen. Unfortunately though, auto-reactive B cells can escape elimination and can, consequently, cause autoimmune disease that can greatly vary in onset and severity.

1.7.1. CCR6 and Autoimmunity

It is interesting to consider the role of CCR6 in a dysregulated model of humoral immunity, given its role in mediating systemic lymphocyte migration and the excessive production of auto-reactive lymphocytes that occurs in autoimmune disease. In fact, CCR6 has been implicated in the pathogenesis of multiple autoimmune diseases, including Rheumatoid Arthritis (RA), Multiple

Sclerosis (MS) and of particular interest to this thesis, Systemic Lupus Erythematosus (SLE). Several studies have demonstrated associations between the expression of CCR6 at the gene, mRNA and surface level, with several autoimmune diseases. A comprehensive list of these associations has been compiled and is displayed in Table 1.7.1.1. This evidence provides unquestionable support that CCR6 contributes to the efficient generation of humoral immune responses.

Table 1.7.1.1. CCR6 autoimmune disease associations

Disease	Characteristics	Model	Effect
Ankylosing Spondylitis (AS)	Inflammation of axial skeleton and sacroiliac joints	Human	Increased frequency of CCR6 ⁺ Th cells in blood during AS ¹⁹³
Autoimmune Hepatitis (AIH)	Liver damage	Mouse	Dysregulated CCR6 dependent migration of splenic T cells to CCL20 ⁺ liver induces AIH ¹⁹⁴
Discoid Lupus Erythematosus (DLE)	Skin damage	Human	CCR6 ⁺ dendritic cells identified in skin lesions of DLE patients and correlated with disease activity ¹⁹⁵
Granulomatosis with Polyangitis (GPA)	Inflammation of respiratory tract and vasculitis		Increased frequency of CCR6 ⁺ memory T cells ¹⁹⁶
Multiple Sclerosis (MS)/ Experimental Autoimmune Encephalomyelitis (EAE)	Central nervous system (CNS) inflammation	Mouse	CCR6 ⁺ cells including CD4 ⁺ T cells and dendritic cells, infiltrate the CNS, contributing to EAE severity ¹⁹⁷⁻²⁰¹ CCR6 and CCL20 expression increased in CNS during EAE ^{202,203} Increased CCL20 mRNA expression in CNS and lymph nodes correlates with EAE onset and severity ²⁰⁴ CCL20 expressed by astrocytes in the CNS during EAE and acts to recruit T cells ²⁰⁵
Sjögren's Syndrome (SS)	Inflammation of lacrimal and salivary glands	Human	Unchanged expression of CCR6 on B cells in SS ²⁰⁶ Increased frequency of circulating CCR6 ⁺ Th17 cells in SS ²⁰⁷
Psoriasis	Chronic skin disease	Human	CCR6, CCL20 and β -defensin-2 expression increased in skin lesions during Psoriasis ^{208,209} Increased frequency of CCR6 ⁺ T cells in skin lesions compared to blood during Psoriasis ²¹⁰ CCL20 expressed by keratinocytes and PBMCs ²⁰⁹ Memory T cells have increased CCR6 expression ²⁰⁹
Rheumatoid Arthritis (RA)	Joint inflammation	Human	CCR6 gene polymorphisms, rs3093024, rs3093023, rs59466457, rs1571878, associated with RA susceptibility ²¹¹⁻²¹⁴ CCR6 ⁺ mononuclear cells, including B cells and Th17 cells, are recruited to CCL20 ⁺ joints during RA ²¹⁵⁻²¹⁸ CCR6 and CCL20 expressed in bone tissue of RA patients ²¹⁹ Increased CCR6 and CCL20 mRNA in RA synovial tissue ²²⁰ Reduced frequency of CCR6 ⁺ B cells in RA ²²¹ Increased frequency of CCR6 ⁺ memory B cells in blood during RA ²²⁰ Unchanged CCR6 expression on CD3 ⁺ T cells during RA ²²²
Systemic Lupus Erythematosus (SLE)	Systemic disease	Human	Positive correlation between CCR6 mRNA in PBMCs and SLE severity ²²³ Increased frequency of T _{FH} cells and memory T cells correlates with SLE severity ^{224,225} Reduced CCR6 expression on circulatory T cells in SLE ²²⁶ No alteration in frequency of CCR6 ⁺ B cells and Th17 cells in SLE ^{221,224}
Systemic Sclerosis (SSc)	Systemic disease	Human	CCR6 dinucleotide polymorphisms, rs3093023 & rs10946216, associated with SSc susceptibility ²²⁷

1.7.2. CCR6 and SLE

SLE is a complex autoimmune disease that affects multiple organs and is difficult to diagnose and subsequently treat due to its various clinical and immunological manifestations²²⁸. Symptoms can range from cutaneous rashes, hair weakness and ulcers, to joint synovitis or tenderness, haemolytic anaemia, as well as renal and neurological problems²²⁸. Immunologically, it can be characterised by leukopenia, auto-antibody production against nuclear proteins, DNA and phospholipids, and a reduction in complement proteins²²⁸. The auto-antibodies generated can form immune complexes that are then deposited in organs throughout the body, causing widespread inflammation and tissue damage²²⁹⁻²³¹. SLE is also associated with a reduction in lymphocyte activity and impaired lymphocyte migration^{232,233}. It is currently unknown as to how these immune defects arise. Whilst a number of cellular factors are known to contribute to the development of SLE, it appears that B cells play an essential role. The murine MRL^{lpr} model displays an SLE-like disease characterised by increased antibody production, auto-reactive antibody production and inflammation. In this mouse model, B cells cause dysregulation in antigen presentation, lymphocyte interactions, proliferation, differentiation and antibody production²³⁴⁻²³⁸. Furthermore, studies in SLE patients have shown that B cells are in a hyperactive state, inducing higher calcium responses following activation and thereby contributing to disease pathogenesis²³⁹. In addition, reductions in the frequency of circulatory B cells have also been documented in SLE²⁴⁰, whereas increased populations of GC and memory B cells have been reported in the secondary lymphoid organs of SLE participants²⁴¹. Collectively, it is clear that the B cell compartment is substantially altered during SLE. Although the precise mechanism underlying disease development is unknown, it has been proposed that the defective selection of auto-reactive B cells into the GC response, likely due to a breakdown in B cell tolerance during early development, is responsible for the abnormal B cell repertoire observed in SLE²⁴²⁻²⁴⁴. Currently, SLE therapies used to treat disease are targeted towards inhibiting the signalling, proliferation and differentiation of B cells, and T-B cell interactions²⁴⁵.

Along with the alterations in the B cell compartment described above, SLE has also been associated with an altered chemokine receptor profile. Recently, there has been great interest in defining the contribution of chemokine receptors to disease, due to their pivotal role in lymphocyte organisation; however, few studies have examined CCR6 expression in SLE and most of these studies have examined CCR6 expression on T cells^{224,246-249}. Studies examining the expression of CCR6 on B cells during SLE are beginning to emerge. In particular, one study has analysed several chemokine receptors, including CCR6, on B cell subsets in peripheral blood samples of SLE participants. Despite the limited number of participants, the study showed a trend of reducing CCR6⁺ B cell frequency in participants diagnosed with SLE, compared to controls²²¹. Furthermore, a significantly

higher level of CCR6 mRNA has been detected in peripheral blood mononuclear cells (PBMCs) of SLE participants compared to controls and this correlated with disease progression²²³. In addition, a significant reduction in CXCR5⁺ plasma and memory B cells, as well as CXCR4⁺ naïve and memory B cells was also observed in SLE participants compared to controls²²¹. This data indicates that CCR6 may contribute to the hyperactive B cell phenotype observed in SLE. Thus, further work is necessary to clearly elucidate whether CCR6 expression is associated with SLE and would indicate if chemokine receptors, including CCR6, can be used as biological markers of disease or therapeutic targets.

1.8. Scope of Study

Recently, interest in defining chemokine receptor mediated positioning of lymphocytes during the humoral response has intensified. Whilst the major chemokine receptors essential for the migration of B cells within the spleen have been identified, it is expected that other chemokine receptors contribute to this process. Such information is necessary to enhance our understanding of the initiation and progression of efficient humoral immune responses, and can be applied to vaccinations and immune system disorders. It is also important as it may lead to the development of diagnostic tools and pharmaceuticals to treat disease. Therefore, there is a need to fully comprehend the chemokine receptor driven cellular organisation of the humoral immune system.

CCR6 is a strong candidate for B cell localisation in the humoral response for several reasons. First, the expression of CCR6 on B cells is modulated during their differentiation in response to TD antigens: CCR6 is expressed at moderate levels on naïve B cells, low levels on GC and plasma B cells, and high levels on memory B cells. Such a specific expression profile is consistent with the idea that CCR6 has a role in GC and EF B cell fate decisions. In addition, the altered GC formation and antibody generation detected in CCR6 deficient mice, suggests a substantial role for CCR6 in the efficient generation of humoral immune responses. Thus, the research to date indicates that CCR6 contributes to efficient B cell differentiation and antibody production. Consequently, I hypothesised that the expression of CCR6 on B cells is necessary for the efficient generation of TD humoral responses, specifically B cell fate decisions. To investigate this hypothesis, I aimed to:

- 1) Define the expression of CCR6 during B cell activation
- 2) Characterise the TD humoral immune response in the absence of CCR6
- 3) Identify the potential molecular mechanisms that underlie B cell dysregulation in the absence of CCR6 and finally

4) Examine the relevance of CCR6 in the B cell-mediated autoimmune disease, Systemic Lupus Erythematosus

To accomplish this, I have used a combination of gene deficient mouse models and clinical samples to examine the properties governing B cell differentiation during TD humoral responses. These protocols are described in the following chapter. In Chapter 3, I quantify CCR6 expression during TD B cell activation. In the next chapter, the effect of the distinct CCR6 expression profile is examined on B cell differentiation. Here, I demonstrate that CCR6 is required for efficient B cell differentiation in the early stages of humoral immunity. In Chapter 5, I investigate the potential cellular and molecular mechanisms that underlie CCR6 regulation of B cell differentiation. In Chapter 6, I examine whether there is an association between CCR6 expression on B cells and systemic autoimmune disease. The results arising from this work will demonstrate that CCR6 contributes to efficient B cell differentiation in the TD humoral response, and this novel finding is critically discussed in the closing chapter. Overall, this is the first study to characterise the role of CCR6 in B cell differentiation, providing a compelling explanation as to how changes in CCR6 expression may regulate B cell differentiation in the humoral response and ultimately, it provides great insight into the development of efficient humoral responses.

Chapter 2

Materials and Methods

2.1. Animals

Mice were purchased from The Jackson Laboratory (U.S.A.) and either transferred from James Cook University, Queensland (Australia) or imported directly to the University of Tasmania Cambridge Farm Facility. Mice were bred at this location before being transferred to our specific pathogen-free facility at the Menzies Institute for Medical Research, at the University of Tasmania. The MD4 x CCR6-eGFP mice were bred at University of Tasmania Cambridge Farm Facility. A complete list of animals used is provided in Table 2.1.1. Mice were used for the experiments described in this chapter, at 6–8 weeks of age, unless otherwise stated. All animal procedures were approved by the Animal Ethics Committee of the University of Tasmania (Ethics Number: A11694 & A11626), in accordance with the Australian Code of Practice and Guidelines, set out by the National Health and Medical Research Council.

Table 2.1.1: Strains of Mus musculus used

Strain	Common Name	JAX® Stock Number	Description
C57BL/6J	WT	000664	Wild-type
B6.SJL-<i>Ptprc</i>^a <i>Pepc</i>^b/BoyJ	Ly5.1	002014	C57BL/6 congenic strain variant
B6.PL-<i>Thy1</i>^a/CyJ	Thy1.1	000406	C57BL/6 congenic strain variant
B6.129P2-<i>Ccr6</i>^{tm1Dgen}/J	CCR6 ^{-/-}	005793	Targeted knock-out
B6.129S6-<i>Ccr6</i>^{tm1(EGFP)Irww}/J	CCR6-eGFP	013061	Targeted eGFP knock-in that abolishes CCR6 function
C57BL/6-Tg(IghelMD4)4Ccg/J	MD4	002595	BCR (predominantly IgM & IgD) specific for HEL (transgene)
B6.Cg-Tg(TcraTcrb)425Cbn/J	OT-II	004194	CD4 TCR α- & β-chain is specific for OVA (transgene)
B6(Cg)-<i>Rag2</i>^{tm1.1Cgn}/J	Rag	008449	Targeted knock-out that arrests B & T cell development in pro-stages
B6.129P2(C)-<i>Ccr7</i>^{tm1Rfor}/J	CCR7 ^{-/-}	006621	Targeted knock-out
B6.129S2(Cg)-<i>Cxcr5</i>^{tm1Lipp}/J	CXCR5 ^{-/-}	006659	Targeted knock-out
B6Smm.C3-<i>Fas</i>^{gld}/J	<i>gld</i>	001021	Fas-ligand point mutation causing generalized lymphoproliferative disease (<i>gld</i>)
C57BL/6-Tg(IghelMD4)4Ccg/J x B6.129S6-<i>Ccr6</i>^{tm1(EGFP)Irww}/J	MD4.CCR6-eGFP	-	HEL-specific BCR & targeted eGFP knock-in that abolishes CCR6 function

2.2. Participants

All participants were recruited from Tasmania. A total of 14 participants diagnosed with SLE were recruited, including 7 from Launceston and 7 from Hobart. In addition, 20 healthy volunteers were recruited, including 5 from Hobart and 15 from Launceston. The inclusion criteria was a diagnosis, or not, of SLE and an age between 18–80 years. The details of each cohort are provided in Table

2.2.1. All participants provided informed and voluntary written consent prior to being included in this study. This study was approved by the Human Ethics Committee of the University of Tasmania (Ethics Number: H001067). Participants provided one blood sample of 9–18 mL for flow cytometry analysis.

Table 2.2.1: Characteristics of Participants

	Participants (n)	Female/Male Ratio	Age (yrs) Mean \pm SD	Age at Diagnosis (yrs) Mean \pm SD
Control	20	12/8	45.25 \pm 15.17	-
SLE	14	13/1	54.71 \pm 15.62	40 \pm 15.89
Total	33			

2.3. Standard Solutions

A list of frequently used solutions is provided in Table. 2.3.1.

Table 2.3.1: Frequently used Solutions

Solutions	Components
10 x Phosphate Buffered Saline (PBS)	80 g NaCl (Sigma-Aldrich [®] , S5886) 14.4 g Na ₂ HPO ₄ H ₂ O (Sigma-Aldrich [®] , S5136) 2.4 g KH ₂ PO ₄ (Sigma-Aldrich [®] , P0662) 2 g KCl (Sigma-Aldrich [®] , P9541) H ₂ O to 1 L (pH 7.4 on dilution)
FACS Buffer	100 mL 10 x PBS 1 g Bovine Serum Albumin (BSA) 2 mL 10 % Sodium Azide (Sigma-Aldrich [®] , S2002) H ₂ O to 1 L
Red Blood Cell (RBC) Lysis Buffer	4.55 g NH ₄ Cl (Sigma-Aldrich [®] : A0171) 10 mL 1 M HEPES (Sigma-Aldrich [®] , H3375) H ₂ O to 500 mL
MACS Buffer	150 mL 10 x PBS 7.5 g BSA 3 mL 10 % Sodium Azide 6 mL 0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8 H ₂ O to 1.5 L
10 x Tris-Borate-EDTA (TBE)	108 g Tris (hydroxymethyl)aminomethane (Sigma-Aldrich [®] , T87602) 55 g Boric Acid (Sigma-Aldrich [®] , B6768) 40 mL 0.5 M EDTA pH 8 H ₂ O to 1 L
Carbonate Buffer	4.2 g NaHCO ₃ (Sigma-Aldrich [®] , S6014) 1.78 g Na ₂ CO ₃ (Sigma-Aldrich [®] , 223484) H ₂ O to 500 mL (pH 9.5)
Conjugation Buffer	0.35 M Mannitol (Sigma-Aldrich [®] , M4125) 0.1 M NaCl H ₂ O to 500 mL

2.4. Antigens and Immunisations

Several antigens were used to induce a humoral immune response in mice. The hapten, 4-hydroxy-3-nitrophenyl acetyl (NP), coupled to the protein carrier, KLH, at a conjugation ratio of 23–27 (Biosearch Technologies, N-5060-25) was prepared by precipitation with 10 % alum (Sigma-Aldrich®, 237086-100G) and Dulbecco's Modified Eagle's medium (DMEM) (Life Technologies™, 11960-044) containing 1 M HEPES (Sigma-Aldrich®, H3375-100G), at a ratio of 1:2:1 (v/v/v). The pH was adjusted to 6–7 and the antigen was washed 4 times in sterile 1 x Phosphate Buffered Saline (PBS) for 10 minutes at 4 °C and 1500 rpm, using the Allegra® X15R centrifuge (Beckman Coulter, U.S.A.). Mice were then intraperitoneally (i.p.) immunised with 50–100 µg of alum precipitated NP-KLH in sterile 1 x PBS.

Alternatively, mice received 10^8 sheep red blood cells (SRBCs) (IMVS, SHBA0100) via i.p. immunisation or 10^8 hen egg lysozyme (HEL) (Sigma-Aldrich®, A5503) conjugated SRBCs subcutaneously (s.c.) in the thigh. For the preparation of HEL-SBRCs, 5 mL of SRBCs was washed with sterile 1 x PBS and resuspended in conjugation buffer with 250 µg of HEL. It was then incubated on ice, on a laboratory rocker for 10 minutes. Next, 50 mg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (Sigma-Aldrich®, E7750) was added and the antigen was incubated for a further 30 minutes. The antigen was then washed in sterile 1 x PBS until cell lysis had stopped and finally, resuspended in sterile 1 x PBS for immunisations.

2.5. Flow Cytometry and Cell Sorting

Mouse cell suspensions were prepared by isolating and homogenising the spleen or inguinal lymph nodes in FACS Buffer. Cells were collected after centrifuging at 1500 rpm for 5 minutes at 4 °C. The red blood cells (RBCs) were removed by resuspending the samples in 10 mL of RBC Lysis Buffer and incubating at 37 °C for 10 minutes. Lysed samples were then washed in FACS Buffer. For the analysis of murine PBMC, blood was collected into tubes containing 50 µL of the anti-coagulant, ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich®, FDS) and the lymphocytes were subsequently isolated using Histopaque 1083 (Sigma-Aldrich®, 10831), as per the manufacturer's instructions.

To detect specific cell subsets, 1.5×10^6 cells were stained with 50 µL of antibodies for 25 minutes on ice. Antibodies used to detect B cells, chemokine receptors and T cells are detailed in Tables 2.5.1, 2.5.2, and 2.5.3 respectively. The secondary antibodies used are listed in Table 2.5.4. For CCR6 DyLight™ 488, purified CCR6 (R&D) was labeled using the DyLight™ 488 Microscale

antibody labeling kit (THERMO Scientific™, 53025). To detect extrafollicular B cells, HEL (Sigma-Aldrich®, A5503) was labeled with DyLight™ 650 using the DyLight™ 650 Microscale antibody labeling kit (THERMO Scientific™, 84536). Samples were washed in 2 mL of FACS Buffer as per the centrifuge settings above. Where purified antibodies were used, samples were blocked with 10 % rat serum (Sigma-Aldrich®, R9759) prior to the addition of directly labeled antibodies, to prevent non-specific background. For intracellular staining, the Forkhead box P3 (FOXP3) Fix/Perm Buffer Set was used as per the manufacturer's instructions (BioLegend®, 421403).

Table 2.5.1: Antibodies used to detect murine B cells

Antibody	Working Dilution	Clone	Manufacturer	Catalogue Number
B220 (CD45R) APC Cy™7	1/200	RA3-6B2	BD	552094
CD38 PerCP-eFluor® 710	1/200	90	eBioscience	46-0381-82
GL7 eFluor® 450	1/100	GL-7	eBioscience	48-5902-82
CD95 PE-Cy™7	1/200	Jo2	BD	557653
IgM^b FITC	1/200	AF6-78	BD	553520
CD73 PE-Cy™7	1/400	TY/11.8	eBioscience	25-0731
IgD APC	1/200	11-26	eBioscience	17-5993
CD138 PE	1/200	281-2	BD	553714
CD21/35 FITC	1/200	7G6	BD	553818
CD23 Biotin	1/200	B3B4	BD	553137
Ig λ Biotin	1/200	R11-153	BD	553431
Purified Ig λ1, λ2, λ3 light chain	1/200	R26-46	BD	553432

Table 2.5.2: Antibodies used to detect chemokine receptors in murine samples

Antibody	Working Dilution	Clone	Manufacturer	Catalogue Number
CCR6 PE	1/100	29-247	BioLegend®	129-804
Purified CCR6	1/1000	140706	R&D	MAB590
Purified CXCR5	1/100	2G8	BD	551961
Purified CXCR4	1/200	2B11	BD	551852
CCR7 supernatant	1/2	4B12.2	-	-

Table 2.5.3: Antibodies used to detect murine T cells

Antibody	Working Dilution	Clone	Manufacturer	Catalogue Number
PD-1 PE	1/100	J43	eBioscience	12-9985-81
TCRαβ PB	1/200	H57-597	Life Technologies™	HM3628
CD4 PerCP-Cy™5.5	1/400	RM4-5	BD	550954
CD8a APC-Cy™7	1/200	53-6.7	BD	557654
CD44 PeCy™7	1/400	IM7	BioLegend®	103029

Table 2.5.4: Secondary antibodies used for flow cytometry in murine samples

Antibody	Working Dilution	Clone	Manufacturer	Catalogue Number
Streptavidin V500	1/200	-	BD	561419
Donkey α-Rat IgG Alexa Fluor® 647	1/200	-	Jackson ImmunoResearch Laboratories, Inc.	712-606-153
Goat α-Rat IgG Alexa Fluor® 488	1/200	-	Life Technologies™	A-11006

Human PBMCs were isolated from blood samples using Histopaque (Sigma-Aldrich®, 10771), as per the manufacturer's instructions. Cells were stained with antibodies to detect B and T cells, as described in Tables 2.5.5 and 2.5.6.

Table 2.5.5: Antibodies used to detect human B cells by flow cytometry

Antibodies	Working Dilution	Clone	Manufacturer	Catalogue Number
CCR6 PE	1/40	11A9	BD	559562
CD27 PE-CF594	1/40	M-T271	BD	562297
CD19 Alexa Fluor® 488	1/100	HIB19	BD	557697
CD38 APC	1/5	HIT2	BD	555462
IgD BV421	1/100	IA6-2	BD	562518
CD95 PE-Cy™7	1/20	DX2	BD	561633

Table 2.5.6: Antibodies used to detect human T cells by flow cytometry

Antibodies	Working Dilution	Clone	Manufacturer	Catalogue Number
CCR7 PE-CF594	1/20	150503	BD	562381
CXCR5 BV421	1/20	RF8B2	BD	562747
PD-1 Alexa Fluor® 647	1/20	EH12.1	BD	560838
CD4 PerCP-Cy™5.5	1/10	L200	BD	552838

All samples were filtered through 48 μ m nylon mesh (Sefar, 03-50/31) prior to being acquired on the CyAn™ ADP Flow Cytometer (Beckman Coulter, U.S.A.) and the MoFlo® Astrios™ cell sorter (Beckman Coulter, U.S.A.). Flow cytometry data was analysed with FlowJo version 9 (Tree Star Inc., U.S.A.) for Macintosh. Cell sorting was carried out by Ms. Jocelyn Darby.

2.6. Immunofluorescence

Secondary lymphoid organs from mice, including the spleen, were frozen in Tissue-Tek[®] O.C.T. (ProSciTech, IA018) using liquid nitrogen vapour. The Leica CM1850 cryostat (Leica Biosystems, Germany) was used to prepare 10 µm cross-sections which were fixed to Flex IHC microscope slides (Dako, K8020) with Acetone (Sigma-Aldrich[®], 270725) for 10 minutes at -20 °C. A PAP pen (Sigma-Aldrich[®], Z672548) was used to create a hydrophobic barrier around sections and the slides were allowed to air-dry prior to being blocked with 1 x PBS and 1 % Bovine Serum Albumin (BSA) for 15 minutes at 4 °C. Slides were placed in a humidifying chamber and stained for 25 minutes at room temperature with 100 µL of antibodies. The primary and secondary antibodies used for immunofluorescence are listed in Tables 2.6.1 and 2.6.2 respectively. Sections were washed thoroughly in 1 x PBS and 0.1 % BSA in-between stains. Coverslips (Dako, C5704) were fixed to slides using polyvinyl alcohol mounting medium with DABCO[®] (Sigma-Aldrich[®], 10981). To quantify GC follicles, stained sections were visualised with the Leica DM LB2 Microscope (Leica Biosystems, Germany) using the 5 x air objective. All other images were acquired with the LSM 510 Meta Confocal Microscope (Carl Zeiss, Germany) using the Plan-Apochromat 20 x NA 0.75 air objective. Images were processed using ImageJ version 1.45s (ImageJ, U.S.A.).

Table 2.6.1: Primary antibodies used for immunofluorescence

Antibodies	Application	Working Dilution	Clone	Manufacturer	Catalogue Number
PNA Biotin	IH	1/300	-	Vector Laboratories	B-1075
B220 Biotin	IH	1/300	RA3-6B2	BioLegend [®]	103204
CD4 purified	IH	1/200	RM4-5	BD	553043
B220 purified	IH	1/300	RA3-6B2	BD	553084

Table 2.6.2: Secondary antibodies used for immunofluorescence

Antibodies	Application	Working Dilution	Clone	Manufacturer	Catalogue Number
Streptavidin Alexa Fluor[®] 594	IH	1/300	-	Life Technologies [™]	S-11227
Goat α-Rat IgG Alexa Fluor[®] 633	IH	1/300	-	Life Technologies [™]	A-21094
Goat α-Rat IgG Alexa Fluor[®] 488	IH	1/300	-	Life Technologies [™]	A-11006

For the examination of T-B cell conjugates, splenocytes were first labelled with flow cytometry antibodies and then fixed for 30 minutes in 0.2 % (v/v) Paraformaldehyde (Sigma-Aldrich®, 158127). Forward scatter parameters were used to isolate T-B cell conjugates using the MoFlo® Astrios™ cell sorter. Sorted conjugates were then air-dried onto Flex IHC microscope slides for visualisation with the confocal microscope.

2.7. Magnetic Cell Separation

Anti-CD43 (Ly-48) MACS® MicroBeads (Miltenyi Biotec, 130-049-801) were used to purify B cells from splenocytes by depletion, with the aid of the AutoMACS Pro Separator (Miltenyi Biotec, Germany) as per the manufacturer's instructions. B cells were subsequently used for adoptive transfer, tissue culture or quantitative (q) RT-PCR.

2.8. Tissue culture

B cells were cultured at 10^6 cells/well in 200 μ L of Gibco® RPMI-1640 medium (Life Technologies™, 21870-092) supplemented with 5 mL of Gibco® Penicillin/Streptomycin/Glutamine (Life Technologies™, 10378-016) and 10 % (v/v) Gibco® fetal calf serum (FCS) (Life Technologies™, 10099141), at 37 °C and 5 % CO₂. To examine CCR6 expression, B cells were stimulated with 5 μ g/ mL of HEL for 24 hours and analysed by flow cytometry at 2, 4, 8, 12 and 24 hours after the addition of HEL.

2.9. Adoptive Transfers

For mixed adoptive transfers, 10^7 naïve B cells from either CCR6^{-/-} or WT mice were transferred into recipient Rag mice, along with either naïve WT or CCR6^{-/-} splenocytes containing an equivalent number of B cells, as approximated by flow cytometry. The donor cells were allowed to settle for 3 days before the recipient mice were challenged with NP-KLH. The response was subsequently analysed 5 days later by flow cytometry and immunofluorescence.

2.10. Quantitative Real-Time Polymerase Chain Reaction

For samples containing 1000 cells, RNA was extracted using an in-house lysis buffer containing 1 μ L of 10 % (v/v) Igepal® (Sigma-Aldrich®, 1000862047), 2 μ L of Oligo-dT (GeneWorks),

0.4 µL of 0.1 M dithiothreitol (DTT) (Invitrogen, Y00147), 1 µL of rRNASEOUT™ (Invitrogen, 100000840), 2 µL of 1 x PBS and Gibco® UltraPure™ distilled H₂O (Life Technologies™, 10977-015) to 11 uL. Alternatively, RNA was extracted from samples containing 1000 cells using the iScript™ RT-qPCR Sample Preparation Reagent (BIO-RAD, 170-8898), as specified.

For samples containing 10⁶ cells, pellets were re-suspended in TRIzol® Reagent (Life Technologies™, 15596-018) and RNA was isolated according to the manufacturer's instructions. Complimentary DNA (cDNA) was synthesised using the QuantiTect® Reverse Transcription Kit (Qiagen, 205313). The primers (GeneWorks) detailed in Table 2.10.1 were used to amplify 2 µL of cDNA via the SensiFAST™ SYBR No-ROX Kit (Bioline, 98005). Gene expression was analysed using the Rotor-Gene qRT-PCR cycler (Qiagen, Germany). Data was displayed as the difference in cycle threshold (CT) between the gene of interest and the reference gene, 18S ribosomal RNA (rRNA).

Table 2.10.1: Primers used for quantitative Real-time Polymerase Chain Reaction

Gene	Sequence (5'-3')		Product (bp)
	Forward	Reverse	
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	151
Bcl-6	CTGCAATGGAGCATGTTGT	CGGCTGTTTCAGGAAGTCTTC	106
IL-21	TGAAAGCCTGTGGAAGTGCAAACC	AGCAGATTCATCACAGGACACCCA	108
CCR6	TGTCCTCACCTACCGTTCTG	TACAGGCCAGGAGCAT	131
CXCR5	CGACATCAGACAGTGACCAGCC	GTCCTGTAGGGGAATCTCCGTG	151
AID	TGCTACGTGGTGAAGAGGAG	TCCCAGTCTGAGATGTAGCG	119
EBI2	CAGCTTTACCACTCGGATA	AAGAAGCGGTCTATGCTCAA	148
Id2	TCAGCCTGCATCACCAGAGA	CTGCAAGGACAGGATGCTGATA	92
IRF4	CTGAGTGGCTGTATGCCAGA	ATCAGCAATGGGAAAGTTTCG	194
CCL20	ATAGGATCCACTCCTGGAGCTGAGAATGG	ATAGGATCCTTACATCTTCTTGACTCTT AGGCTGA	328
CCR6	CCTCACATTCTTAGGACTGGAGC	GGCAATCAGAGCTCTCGGA	151
CCR7	CCAGCAAGCAGCTCAACATT	GCCGATGAAGGCATACAAGA	92
Pax5	CAGACCACAGAGTATTCAGCCATG	AGGGACTTCCAGAAAATTCCTCC	259
Blimp-1	TCTGTTCAAGCCGAGGCATCC	TCTTGGAAGTGTGTCATTAG	163

2.11. Preparation for Next Generation Sequencing

RNA was extracted from samples containing 1000 cells using the iScript™ RT-qPCR Sample Preparation Reagent as per the manufacturer's instructions. The RNA was subsequently precipitated by incubating on ice for 30 minutes in 0.3 M Sodium Acetate (Sigma-Aldrich®, S2889), 2 x (v/v) of

Absolute Ethanol (Sigma-Aldrich[®], 459844) and 20 µg of Glycogen (Life Technologies[™], AM9510). The precipitated RNA pellet was collected after centrifuging for 20 minutes, 4 °C at maximum speed and washed in 70 % ethanol for 3 minutes, 4 °C at maximum speed. The pellet was then air-dried before being resuspended in 20 µL of Gibco[®] UltraPure[™] distilled H₂O. All precipitated RNA was used to synthesise cDNA, as previously described. Of this cDNA, 3 µL was used as the template to amplify the variable region of the Ig heavy chain in the J558 region by PCR.

The PCR reaction consisted of 5 x Green GoTaq[®] Reaction Buffer (Promega, M7911), 25 mM MgCl₂ (Promega, 83511), 10 mM deoxynucleotide triphosphates (dNTPs) (Bioline, BIO-39025), 20 µM each of both the forward and reverse sequencing primers, GoTaq[®] hot start polymerase (Promega, M5005), and Gibco[®] Ultrapure H₂O to 25 µL. The primers were provided by Professor Thomas Winkler's laboratory. The forward primer, 5'- CGTATCGCCTCCCTCGCGCCATCAG-MID-GRGCCTGGGRCTTCAGTGAAG-3' binds the variable Ig region, whilst the reverse primers, 5'-RCTCAGGGAARTARCCYTTGAC-3' and 5'-GTGCTGGGCAGGAAGTCCCG-3' bind the constant γ or μ region respectively. The various MID sequences located on the forward primer, used to identify distinct samples, are listed in Table 2.11.1. This protocol required optimisation and as such, alternate reverse primers were generated: IgM Rv 5'-TATGCGCCTTGCCAGCCCGCTCAGGTGCTGGGCAGGAAGTCCCG-3' and IgG Rv 5'-CTATGCGCCTTGCCAGCCCGCTCAGRCTCAGGGAARTARCCYTTGAC-3'.

The PCR was incubated in the Veriti thermal cycler (Applied Biosystems[®], U.S.A.) under the following conditions: 95 °C for 5 minutes, 40 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds and 72 °C for 1 minute, followed by 72 °C for 7 minutes and 4 °C for ∞. The samples were then analysed on a 1 % agarose (Bioline, BIO-41025) gel containing GelRed[™] (Biotium, 41003), in 1 x Tris-Borate-EDTA (TBE) buffer. The DNA was extracted using the Wizard SV[®] Gel & PCR Clean-up System (Promega, A9282). The concentration of DNA was determined using the Qubit 2.0 Fluorometer (Life Technologies[™], U.S.A.). For analysis, 200 ng of DNA was precipitated as previously described and sent as a dried pellet to Dr. Andreas Hiergeist in Germany for 454 sequencing using the GS Junior system (Roche: Switzerland).

Table 2.11.1: Next Generation MID Sequences

MID Name	Sequence (5'-3')
2	ACGCTCGACA
3	AGACGCACTC
4	AGCACTGTAG
5	ATCAGACACG
6	ATATCGCGAG
7	CGTGTCTCTA
8	CTCGCGTGTC
9	TCTCTATGCG
10	TGATACGTCT
11	CATAGTAGTG
14	TCACGTACTA
15	CGTCTAGTAC

2.12. Serum Isolation

Blood was collected from the tail vein of mice. To isolate the serum, samples were incubated at 37 °C for 30 minutes, followed by 30 minutes at 4 °C, and then centrifuged for 5 minutes at 1500 rpm in the microcentrifuge (Thermo Scientific, U.S.A.). Serum was stored at -20 °C until it was analysed by enzyme-linked immunosorbent assay (ELISA).

2.13. Enzyme-linked immunosorbent assay

To detect NP-specific antibodies in mouse serum, 96 well round-bottom plates (Sarstedt, 82.1582) were coated with 5 µg/ mL NP-BSA with a conjugation ratio >20 (Fisher Biotec, N-5050H-10) in carbonate buffer overnight at 4 °C. The plate was then washed with 1 x PBS and 0.05 % Tween (Sigma-Aldrich[®], P1379) and subsequently blocked in 1 x PBS and 1 % BSA (Sigma-Aldrich[®], A7906) for 1 hour at 4 °C. Following washing, serum was diluted 1/1000 and incubated in the plate at ambient temperature for 2 hours. If necessary, samples were diluted further. Afterwards, the plate was washed and incubated with biotinylated IgM, IgG1^b or IgG2a^b for 1 hour. NP-specific antibodies were detected with streptavidin bound Horseradish Peroxidase (HRP), which was incubated for 30 minutes. Details of the antibodies used for the ELISA are shown in Table 2.13.1. TMB substrate (Sigma-Aldrich[®], T0440) was used to develop the HRP. Once the standards had developed, 2N Sulfuric Acid (Sigma-Aldrich[®], 320501) was added to stop the reaction. The plate was washed between antibody labeling steps and substrate addition. The light absorbance was measured at 450 nm on the Spectra Max Plus³⁸⁴ plate reader (Molecular Devices, U.S.A.). The

standard curve was generated by combining and subsequently diluting serum collected at Days 7 and 21 post alum-precipitated NP-KLH immunised WT mice.

Table 2.13.1: Antibodies used for Enzyme-linked Immunosorbent Assay

Antibodies	Application	Working Dilution	Clone	Manufacturer	Catalogue Number
IgM Biotin	ELISA	1/1000	R6-60.2	BD	553406
IgG1^b Biotin	ELISA	1/2500	B68-2	BD	553533
IgG2a^b Biotin	ELISA	1/5000	5.7	BD	553504
Streptavidin HRP	ELISA	1/1000	-	BD	554066

2.14. Statistical Analysis

All data are expressed as the mean \pm standard error of the mean (SEM). Data were analysed using GraphPad Prism 5.0a (GraphPad Software Inc., U.S.A.). Comparisons between two groups were performed using the non-parametric Mann-Whitney two-tail t-test. Comparisons between 3 or more groups were performed using the Kruskal-Wallis One-way analysis of variance (ANOVA). A p-value of ≤ 0.05 was considered significant.

Chapter 3

CCR6 expression on activated B cells during the humoral response

The overall purpose of this thesis was to investigate the function of CCR6 in the humoral immune response. The first part of this work involved determining whether CCR6 is expressed during B cell activation. Previous reports have demonstrated a unique expression profile for CCR6 during B cell differentiation. Specifically, it is known that CCR6 is expressed on naïve B cells, downregulated on GC and plasma cells, and upregulated on memory B cells¹⁹⁰. The fact that CCR6 is expressed by naïve and effector B cells suggests that CCR6 expression is altered during B cell activation. However, this has yet to be investigated. Previous unpublished observations from the Körner laboratory have indicated that CCR6 is upregulated on B cells within 24 hours of stimulation *in vitro*, in response to both B cell specific (HEL) and generic (anti-CD40) stimuli (Fromm and Körner, unpublished results). Furthermore, chemotaxis assays from the same laboratory indicate that B cells activated with the Toll-like receptor ligand, LPS, display enhanced migration towards CCL20, when compared to naïve B cells (Fromm and Körner, unpublished results). However, the precise expression of CCR6 on B cells after antigen-specific activation remains to be established. Therefore, I set out to determine the cellular expression of CCR6 during TD B cell activation.

3.1. CCR6 expression is upregulated upon T cell-dependent B cell activation *in vitro*

To quantify CCR6 expression on the surface of B cells, an appropriate antibody that specifically detected CCR6 by flow cytometry had to be selected. Three antibodies were tested: monoclonal CCR6 (R&D) purified from hybridoma supernatant and detected with anti-rat Alexa Fluor® 647 (Jackson), purified CCR6 (R&D) conjugated to DyLight 488 and CCR6 PE (BD). These antibodies were used to examine CCR6 expression on splenic B cells from WT and CCR6^{-/-} mice challenged for 5 days with NP-KLH – a standard immunogen used to study TD humoral immunity. CCR6 PE and purified CCR6 were found to be the most appropriate antibodies as they clearly distinguished CCR6 expression on WT B cells from CCR6^{-/-} B cells (Fig. 3.1.1). CCR6 DyLight 488 provided a weak signal that could not be clearly separated from background (Fig. 3.1.1).

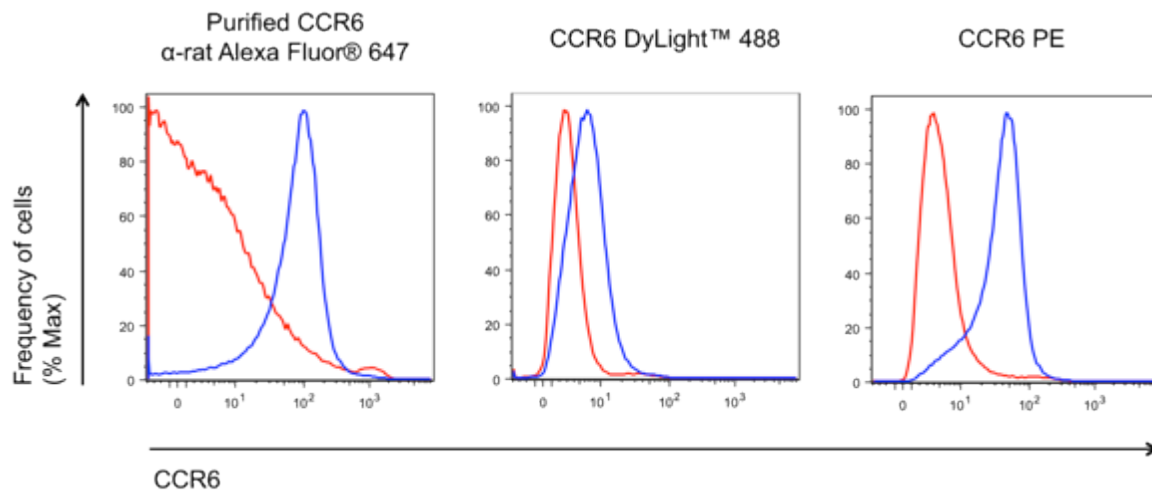


Figure 3.1.1. Optimisation of CCR6 detection on B cells

WT (blue) and CCR6^{-/-} (red) mice were i.p. immunised with 50µg of NP-KLH for 5 days. The mean fluorescence intensity of CCR6 expression was quantified by flow cytometry on splenic B220⁺ cells using purified CCR6 detected with anti-rat Alexa Fluor® 647, CCR6 conjugated to DyLight™ 488 and CCR6 PE (n=1).

Following the identification of suitable antibodies to detect CCR6, I defined the kinetics of CCR6 expression during TD B cell activation *in vitro* using flow cytometry. Naïve B cells were isolated from HEL-Ig transgenic MD4 mice using anti-CD43 MicroBeads and activated *in vitro* for 24 hours with the antigen HEL. Throughout this time, CCR6 expression was regularly examined on B cells, as was their expression of CD38 and GL7 – two cell surface markers used to identify differentiated B cell subsets^{23,24,28}. Naïve B cells were CD38⁺ GL7^{lo} CCR6⁺ (Fig. 3.1.2a). After 8 hours of activation, as demonstrated by the significant upregulation of CD69 on the surface of B cells, a significant increase in CCR6 expression was observed, which corresponded with significant increases in both CD38 and GL7 expression (Fig. 3.1.2a, b). *In vivo*, CD38⁺ GL7⁺ B cells define precursor B cells that directly arise after B cell activation and T cell help^{28,73}. The results from this study suggest that B cells encounter antigen, upregulate CCR6 surface expression during activation and maintain this expression as CD38⁺ GL7⁺ precursor B cells.

When CCR6 expression was examined at the mRNA level using qRT-PCR, the upregulation of CCR6 surface expression did not correspond with an increase in CCR6 mRNA. In fact, CCR6 mRNA expression was significantly reduced 8 hours after activation (Fig. 3.1.2c). It should be noted that the primers used to quantify CCR6 mRNA expression were validated in WT and CCR6^{-/-} mice. Also, the mRNA quantification of 18S and other chemokine receptors in the same samples,

provides an intrinsic control for the quality of the RNA. Thus, the most likely explanation for the discrepancy between surface and mRNA expression is that the upregulation of CCR6 mRNA is transient, while the increased surface expression of CCR6 8 hours after activation is the result of an accumulation of CCR6 on the surface over time. It is also possible that post-transcriptional and post-translational mechanisms contribute to this increased surface expression of CCR6. Furthermore, it has been shown that the downregulation of CCR6 from the cell surface depends on the presence of its ligand, CCL20¹⁸⁹, which these B cells did not have access to *in vitro*.

Subsequently, CCR6 mRNA expression was quantified at an earlier time point in an attempt to capture this transient response. However, CCR6 mRNA expression remained low 2 hours after activation (Fig. 3.1.2c). Consequently, it is likely that CCR6 mRNA expression peaks quickly after activation and begins to decline soon thereafter, prior to 2 hours after activation, and therefore is not captured by this experiment. Alternatively, it is possible that the increased CCR6 surface expression and reduced mRNA expression may occur simultaneously, facilitating both the current and imminent needs of the B cell.

As the chemokine receptors, CXCR5, CCR7, CXCR4 and EBI2, are known to coordinate B cell positioning during the humoral response^{52,159,188}, their expression was also analysed on MD4 B cells during activation *in vitro*. As expected, activated B cells upregulated CXCR5, CXCR4 and CCR7 expression on the cell surface (Fig. 3.1.2a, b). In contrast to surface expression though, CXCR5 mRNA was significantly decreased in activated B cells at both 2 and 8 hours after activation (Fig. 3.1.2c). CXCR4 mRNA expression was significantly upregulated 2 hours after activation, before a strong reduction was observed 6 hours later (Fig. 3.1.2c). Interestingly, the upregulation of CCR7 surface expression correlated with a significant upregulation in CCR7 mRNA 8 hours after activation (Fig. 3.1.2c). This is different to the mRNA levels observed for CCR6, CXCR5 and CXCR4, and may be due to the fact that high sustained CCR7 expression is required by B cells immediately after activation *in vivo*, for interaction with Th cells⁵². While EBI2 surface expression could not be examined, EBI2 mRNA expression was, and a significant decrease in EBI2 mRNA was detected in MD4 B cells 8 hours after activation (Fig. 3.1.2c).

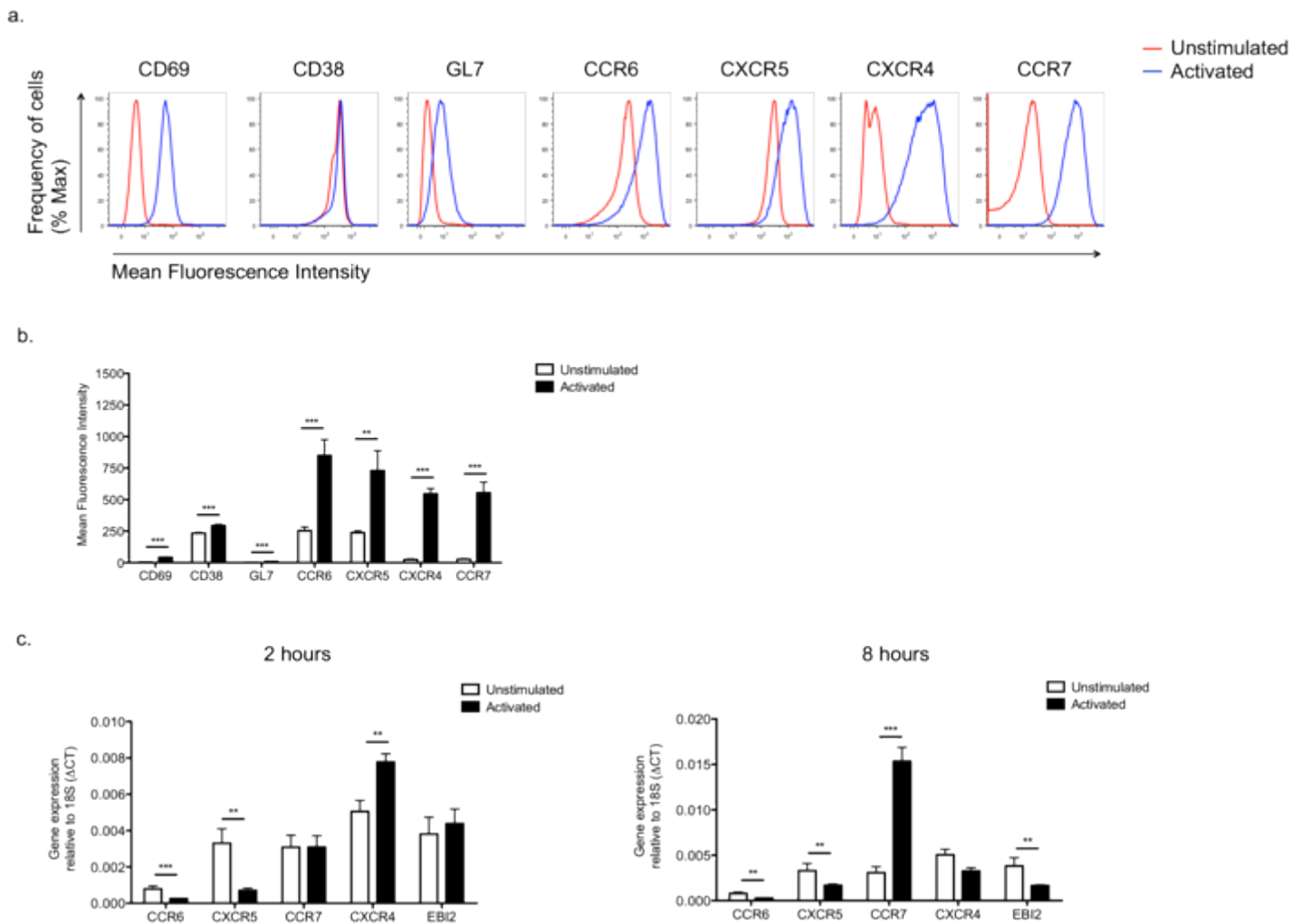


Figure 3.1.2. CCR6 expression during T cell-dependent B cell activation *in vitro*

B cells were isolated from MD4 mice using anti-CD43 MicroBeads and stimulated *in vitro* for 8 hours with 5 µg/mL of HEL. The B cell surface expression of CD69, CD38, GL7, CCR6, CXCR5, CXCR4 and CCR7 was monitored by flow cytometry. Representative images of surface protein expression on unstimulated and activated B cells are shown (a). The mean fluorescence intensities were quantified in unstimulated B cells and B cells cultured with HEL for 8 hours (activated) (b). The relative gene expression of CCR6, CXCR5, CXCR4, CCR7 and EBI2 was quantified by qRT-PCR in unstimulated and activated B cells at 2 and 8 hours following antigen challenge (c). Figure represents data collected from 2 independent experiments (n=8 mice).

Mann – Whitney two-tail t-test, *significant at $p < 0.05$; **significant at $p < 0.01$; ***significant at $p < 0.001$

Next I examined B cell activation in the absence of CCR6. Unactivated MD4.CCR6^{-/-} B cells had a significantly higher expression of CD69 compared to MD4 B cells. However, this did not appear to enhance their activation or differentiation into CD38⁺ GL7⁺ B cells, when compared to MD4 B cells (Fig. 3.1.3). In fact, MD4.CCR6^{-/-} B cells tended to have significant reductions in CD38 expression compared to MD4 B cells following stimulation (Fig. 3.1.3). This was also true for GL7 expression, but was only significant 2 hours after activation (Fig. 3.1.3). In addition, significant reductions in CXCR5, CXCR4 and CCR7 surface expression were detected after activation in MD4.CCR6^{-/-} B cells compared to MD4 B cells (Fig. 3.1.3), suggesting a possible connection between the

chemokine receptors. Overall, MD4.CCR6^{-/-} B cells displayed similar trends to MD4 B cells, in that they too had increased expression levels of the chemokine receptors, as well as CD69, CD38 and GL7, after activation with HEL (Fig. 3.1.3).

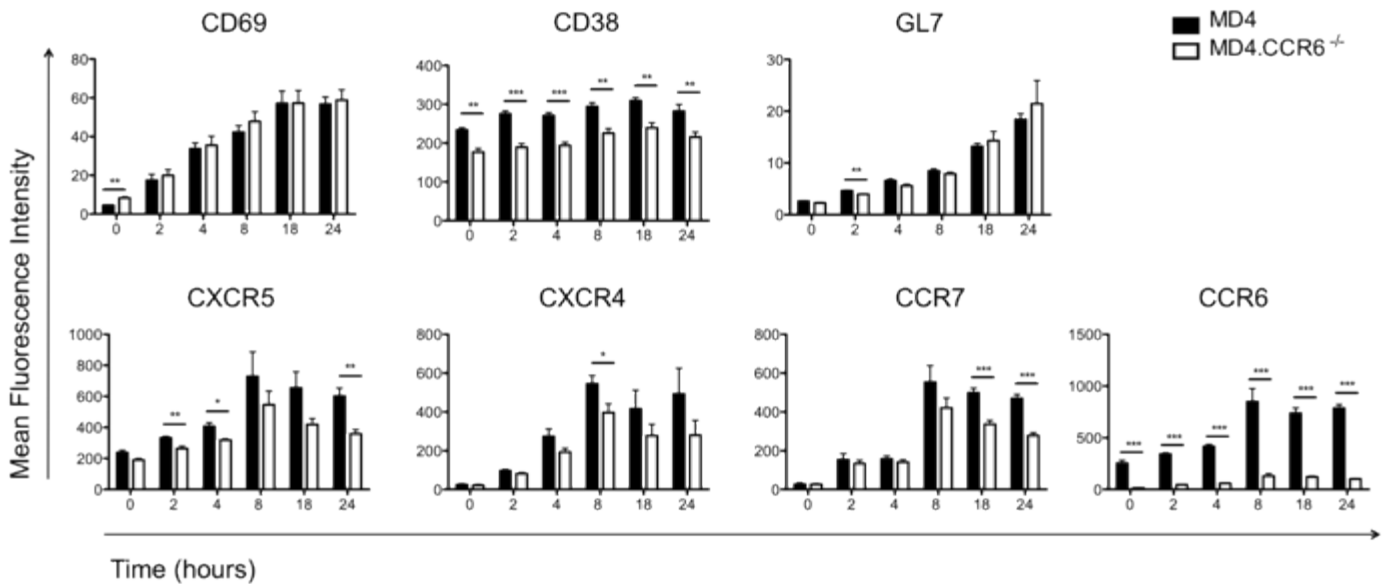


Fig. 3.1.3. *In vitro* T cell-dependent B cell activation in the absence of CCR6

B cells were isolated from MD4 and MD4.CCR6^{-/-} mice using anti-CD43 MicroBeads and cultured *in vitro* for 24 hours with 5ug/ml of HEL. Throughout this period, the mean fluorescent intensity of CD69, CD38, GL7, CXCR5, CXCR4, CCR7 and CCR6 expression, was quantified by flow cytometry. Figure represents data collected from 2 independent experiments (n=8 mice/genotype).

Mann – Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01; ***significant at p<0.001

3.2. CCR6 is expressed by T cell-dependent activated B cells *in vivo*

To validate the finding that CCR6 is upregulated on activated B cells *in vitro*, CCR6 expression was analysed on B cell subsets *in vivo*. WT mice were immunised with NP-KLH and the splenic B cell population was examined seven days later by flow cytometry, at the peak of the humoral response. Rare antigen-specific responding B cells were easily identified from the remaining peripheral B cell population using anti- λ Ig antibodies, as NP specifically induces λ -IgL antibody production^{250,251}. As mentioned previously, CD38 and GL7 expression identifies peripheral B cells. Specifically, five distinct subsets can be identified: CD38⁺ GL7⁻ naïve B cells, CD38⁺ GL7⁺ precursor B cells, CD38⁻ GL7⁺ GC B cells, CD38⁻ GL7⁻ plasma cells and CD38^{hi} GL7⁻ memory B cells^{23,24,28}. The gating strategy used to identify these subsets is displayed in Fig. 3.2.1a. Briefly, lymphocytes and singlets were selected based on the forward and side scatter properties of the cells. From here, λ^+ B cells were selected and separated into the five B cell subsets based on their expression of CD38 and GL7. Using this strategy, CCR6 expression was quantified on each B cell subset, as shown in Fig. 3.2.1b.

This analysis revealed that naïve B cells expressed a moderate level of CCR6 (Fig. 3.2.1b, c). The GC B cells had a significantly lower expression of CCR6 compared to both naïve B cells and memory B cells (Fig. 3.2.1b, c). Plasma cells were also found to express low to moderate levels of CCR6 (Fig. 3.2.1b, c). This expression profile concurs with that previously documented in the literature^{185,186,190}. Interestingly, CCR6 expression was significantly higher on precursor B cells than on GC and plasma cells (Fig. 3.2.1b, c). The high expression of CCR6 on precursor B cells, which arise after naïve B cells become activated, supports the previous *in vitro* finding that CCR6 is upregulated on activated B cells.

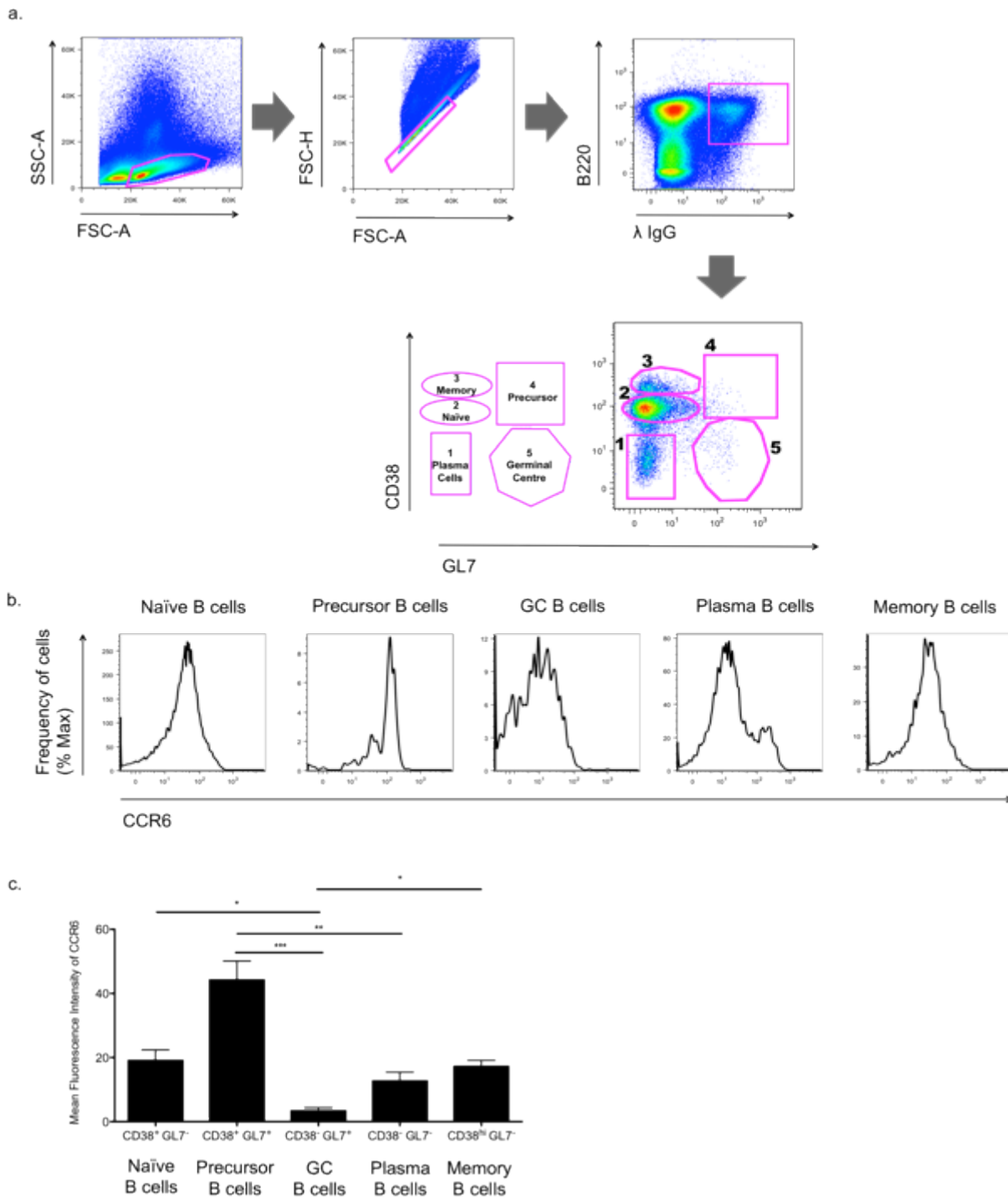


Figure 3.2.1. CCR6 is highly expressed by CD38⁺ GL7⁺ precursor B cells in vivo

WT mice were i.p. immunised with 100µg of NP-KLH for 7 days. Responding B cells were identified by their expression of λ-Ig and delineated based on their expression of CD38 and GL7: 1) CD38⁺ GL7⁻ plasma cells, 2) CD38⁺ GL7⁻ naïve B cells, 3) CD38^{hi} GL7⁻ memory B cells, 4) CD38⁺ GL7⁺ precursor B cells and 5) CD38⁻ GL7⁺ GC B cells (a). Representative images of CCR6 expression on B cell subsets are displayed (b). The mean fluorescence intensity of CCR6 expression on each B cell subset was quantified by flow cytometry (c). Figure represents data collected from 2 independent experiments (n=9 mice/genotype).

Kruskal – Wallis one-way ANOVA, *significant at p<0.05; **significant at p<0.01; ***significant at 0.001

Having observed that CCR6 is expressed by CD38⁺ GL7⁺ precursor B cells *in vivo*, I next examined whether CCR6 is expressed by B cells during interactions with Th cells. To achieve this, a combination of cell sorting and immunofluorescence was used. To visualise CCR6 expression, a CCR6.eGFP knock-in mouse was used, which expresses GFP where CCR6 would normally be expressed. Mice were immunised for six days with SRBC, a potent inducer of TD humoral immune responses. T-B cell conjugates were then isolated using forward scatter properties, along with B220 and CD4 expression. Despite the low frequency of conjugates present *in vivo* during a normal humoral response, it was possible to isolate intact conjugates after mild fixation using the MoFlo® Astrios™ cell sorter. Once sorted, conjugates were examined for GFP expression using the confocal microscope. GFP expression was observed on the B cell of T-B cell conjugates, as predicted, but also, surprisingly, on the T cell of T-B cell conjugates (Fig. 3.2.2). The fact that CCR6 is expressed by T-B cell conjugates suggests that CCR6 has a broader function than simply B cell activity. However, it is important to note that not all observed conjugates expressed GFP, indicating that CCR6 is not an absolute requirement for T-B cell conjugate formation and the subsequent differentiation of B cells. In further support of this, is the previous finding that CCR6^{-/-} B cells are still capable of becoming activated and upregulating their expression of CD38 and GL7 *in vitro*.

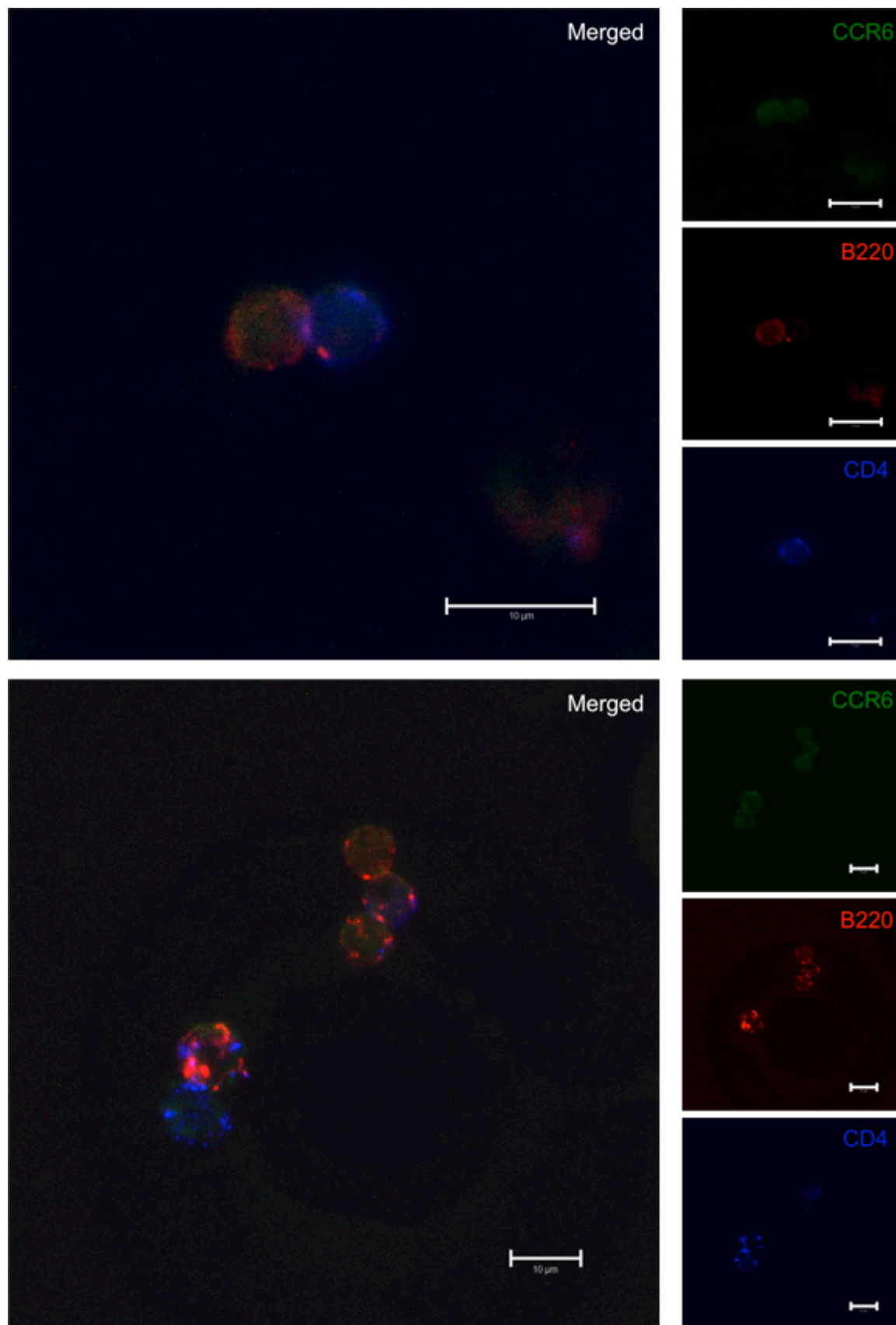


Figure 3.2.2. *CCR6 is expressed by T-B cell conjugates in vivo*

CCR6-eGFP mice were i.p. immunised with SRBC. T-B cell conjugates were isolated 6 days later using the MoFlo® Astrios™ cell sorter. Conjugates were selected using the forward scatter parameters, as well as B220 and CD4 expression. Prior to sorting, samples were fixed in 2% formaldehyde for 30 minutes. Purified conjugates were air-dried onto slides and imaged using the confocal microscope (n=1). Images show 3 distinct T-B cell conjugates. Objective magnification: top panel 40x, bottom panel 20x; Scale bars are 10μm.

To investigate the formation of T-B cell conjugates in the absence of CCR6, WT and CCR6^{-/-} mice were immunised with NP-KLH for 42 days and T-B cell conjugates were quantified by flow cytometry throughout this period. As before, T-B cell conjugates were identified using forward scatter parameters, B220 and CD4 expression (Fig. 3.2.3a). This analysis demonstrated that T-B cell conjugates are still formed in the CCR6^{-/-} mice (Fig. 3.2.3a, b). In fact, at days 14 and 21 post antigen challenge, CCR6^{-/-} mice had a significantly higher frequency of T-B conjugates than WT mice (Fig. 3.2.3b). However, for the remaining response, the CCR6^{-/-} mice produced an equivalent number of T-B cell conjugates to WT mice (Fig. 3.2.3b). Overall, these results demonstrate that CCR6 is not essential for T-B cell conjugate formation, but may contribute to B cell differentiation in the early humoral response.

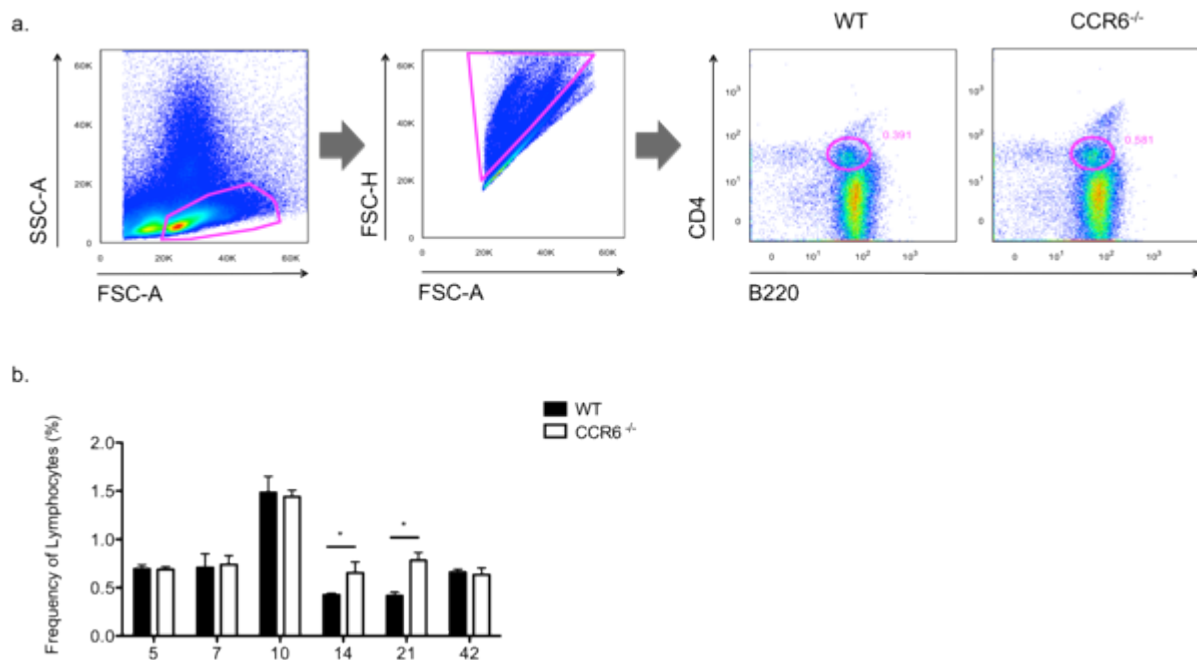


Fig. 3.2.3. Quantification of T-B cell conjugates in vivo

WT and CCR6^{-/-} mice were i.p. immunised with 100ug of NP-KLH for 42 days. Flow cytometry was used to examine T-B cell conjugates, which were identified by their forward scatter parameters, as well as B220 and CD4 expression (a). The frequency of T-B cell conjugates was quantified at days 5, 7, 10, 14, 21 and 42 following challenge. Figure represents data collected from 1 experiment (n=4 mice/genotype).

Mann – Whitney two-tail t-test, *significant at p<0.05

3.3. Concluding Remarks

In this chapter I have quantified CCR6 expression on activated B cells. Initially, an *in vitro* model was employed to directly investigate the phenotype of naïve B cells as they underwent activation.

This model established that CCR6 is upregulated on activated B cells. Further investigation *in vivo* demonstrated that CCR6 is highly expressed on precursor B cells, which arise from CCR6⁺ naïve B cells and appear prior to CCR6^{lo} GC B cells. Moreover, CCR6 expression was detected on T-B cell conjugates. However, to directly demonstrate that CCR6 is upregulated *in vivo*, it will be necessary to isolate and transfer antigen-specific naïve B cells into recipient mice, which are then immunised with TD antigens. In this way, CCR6 expression can be analysed on activated cells derived from the naïve donor cells. While a similar experiment has already been carried out¹⁸², the authors transferred whole splenocytes and not naïve B cells directly, thus they did not exclude activated or differentiated B cell subsets already present in the donor population. In addition, regular examination of the B cell population is needed, so that the activated B cells can be identified as they appear in response to antigen challenge. This improved model would also be useful to visualise the localisation of B cells deficient in CCR6, as well as the migration of T-B cell conjugates. Such insights would be useful to assess the impact of CCR6 on B cell migration. Overall the data presented within this chapter demonstrates that CCR6 is upregulated on follicular B cells upon antigen activation continues to be expressed as B cells interact with Th cells and is maximal on precursor B cells, prior to their differentiation into GCs and EF. Taken together, this data suggests that CCR6 may aid B cell migration during their activation, while B cell fate decisions are made.

Chapter 4

The cellular kinetics of the humoral response in the absence of CCR6

The next step in addressing the role of CCR6 in the humoral response was to characterise B cell differentiation in the absence of CCR6. As demonstrated in the previous chapter, activated B cells express high levels of CCR6, suggesting an important role for the chemokine receptor in the subsequent differentiation of B cells. It was therefore expected that if CCR6 is required for B cell differentiation, then B cell differentiation would be halted, or at least reduced in the absence of CCR6. To determine this, a TD humoral response was induced in CCR6^{-/-} mice using NP-KLH. As noted earlier, NP-KLH induces a TD humoral response characterised by the production of λ -specific B cells. In addition to this, the NP-KLH induced model of humoral immunity is a valuable study model, as the spatial and temporal organisation of B cells in this TD response have been defined^{53,77,252}, and consequently provide an excellent framework for this study.

4.1. CCR6 counterbalances excessive splenic Germinal Centre formation

The development of GCs is considered the hallmark feature of the humoral response because of their prominent appearance and specialised antibody maturation capacity. Therefore, GCs are a good indicator of the functionality of the humoral response. As such, I began analysing splenic B cell differentiation by quantifying the GC response in both CCR6^{-/-} and WT mice immunised with NP-KLH.

Initially, the frequency of GC B cells was determined by flow cytometry. Antigen-specific GC B cells were identified by their expression of CD38 and GL7, as shown in Fig. 4.1.1a. WT GC B cells were detectable throughout the primary immune response, from days 3–42 post antigen challenge (Fig. 4.1.1a, b). The beginning of the GC response was detected at day 3 with approximately 0.3% of λ^+ B cells, it peaked by day 7 with approximately 8% of λ^+ B cells and gradually declined by day 42 to approximately 1% of λ^+ B cells. This GC B cell kinetic correlates with the TD GC response described previously^{53,77}. However, substantial alterations were detected early in the GC response of CCR6^{-/-} mice. A significantly higher frequency of GC B cells was observed in CCR6^{-/-} mice at days 3 and 5 post antigen challenge, compared to WT mice (Fig. 4.1.1a, b). This increase was only short-lived, as there were similar quantities of GC B cells between CCR6^{-/-} and WT mice for the remaining response, except at day 21, where the CCR6^{-/-} mice appeared to have fewer GC B cells than WT mice (Fig. 4.1.1a, b). Furthermore, when all antigen-specific B cells were examined, the increased frequency of GC B cells detected in CCR6^{-/-} mice at day 5, corresponded with a significant increase in the frequency of all antigen-specific B cells (Fig. 4.1.1c). In addition, the total antigen-specific B cell population was observed to increase at day 3 and decrease at day 21 in

the CCR6^{-/-} mice compared to the WT mice, correlating with the increased frequency of GC B cells observed in the CCR6^{-/-} mice (Fig. 4.1.1ac).

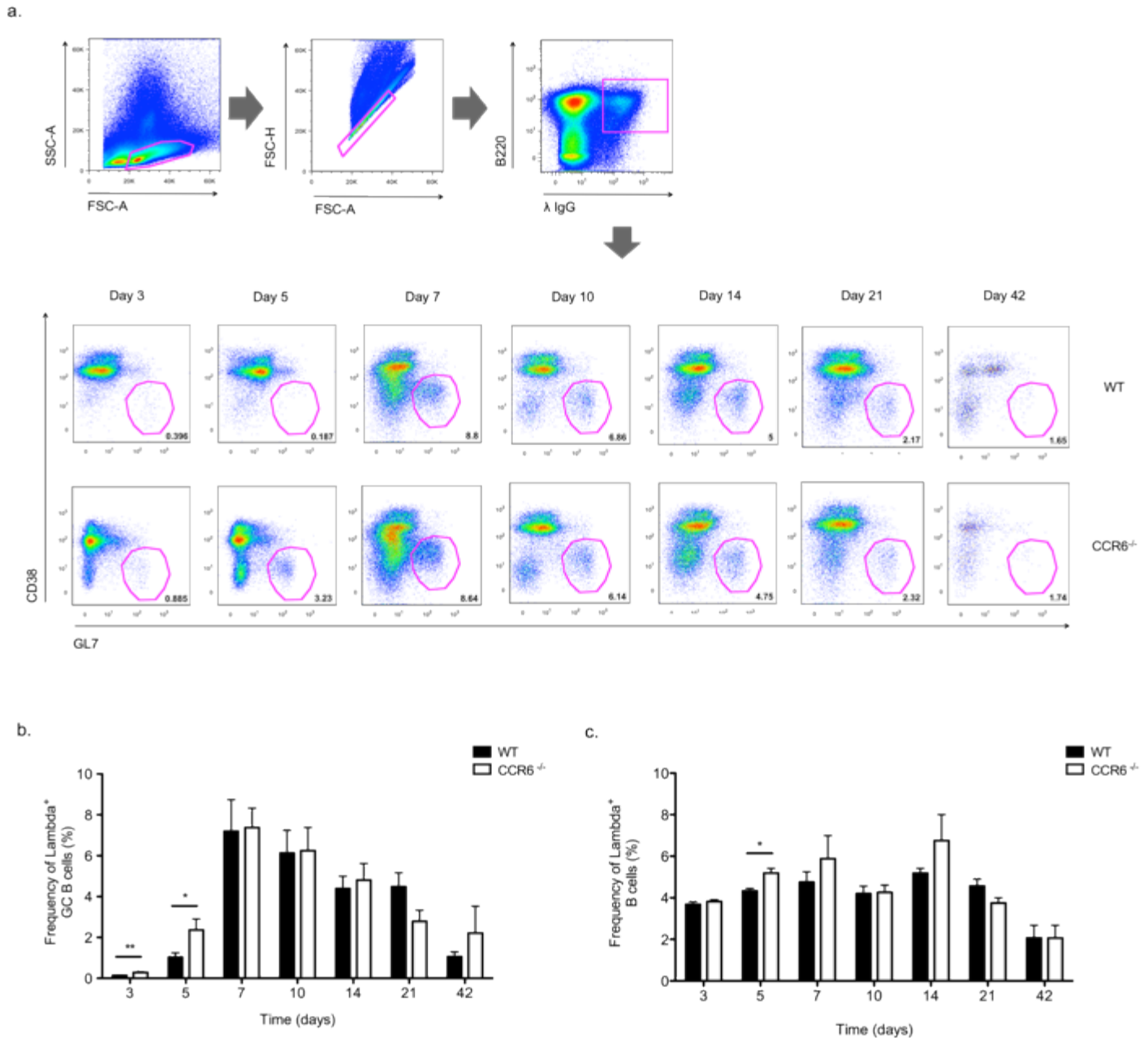


Figure 4.1.1. The T cell-dependent Germinal kinetics in the absence of CCR6

WT and CCR6^{-/-} mice were i.p. immunised with 50–100μg of NP-KLH for 42 days. Representative images of the GC population (CD38⁺ GL7⁺), indicated by the gate, are displaced (a). The frequencies of λ-specific GC B cells (b) and total λ-specific B cells (c) were quantified 3, 5, 7, 10, 14, 21 and 42 days after antigen challenge. Data is indicative of 1–2 independent experiments (Day 3 n=8 WT mice, 6 CCR6^{-/-} mice; Day 5 n=5 mice/genotype; Day 7, 21 & 42 n=9 mice/genotype; Day 10 n=8 WT mice, 9 CCR6^{-/-} mice; Day 14 n=4 mice/genotype).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

To determine whether the increased frequency of early GC B cells in the CCR6^{-/-} mice results in an overall increase in the number of GC follicles established, splenic GC follicles were quantified during the humoral response using immunofluorescence. Two antibodies were used to identify GC follicles: B220 and PNA. PNA is a protein specifically expressed by GC B cells, such that GC follicles are identified by a bright PNA cluster encapsulated by a B220⁺ border^{91,253}. Quantification of GC follicles revealed that the CCR6^{-/-} mice had significantly more follicles at days 3 and 5 following antigen challenge than WT mice (Fig. 4.1.2a). The CCR6^{-/-} GC follicle response then peaked at day 7 and declined as per normal (Fig. 4.1.2a). During this time, the frequency of GC follicles between CCR6^{-/-} and WT mice were similar. Representative images of the GC follicle response in the absence of CCR6 are displayed in Fig. 4.1.2b. Overall, the frequency of CCR6^{-/-} GC B cells correlates with the number of CCR6^{-/-} GC follicles formed, demonstrating that the increased population of GC B cells results in more GC B cells seeding follicles and consequently more GC follicles being established. The increased GC response observed at days 3 and 5 post antigen challenge in the CCR6^{-/-} mice, suggests that the cause of dysregulation occurs early, during the initiation of the humoral response. As the GC response in CCR6^{-/-} mice peaks at day 7 as per normal, it seems that more B cells are initially recruited into the GC B cell differentiation pathway. Furthermore, the CCR6^{-/-} GC response is not reduced beyond the WT GC response after day 5, indicating firstly that there is not a constant supply of B cells differentiating into GC B cells and secondly, that the early GCs do not persist for an extended period of time. Consequently, CCR6 appears necessary for initial B cell activation and selection in the humoral response.

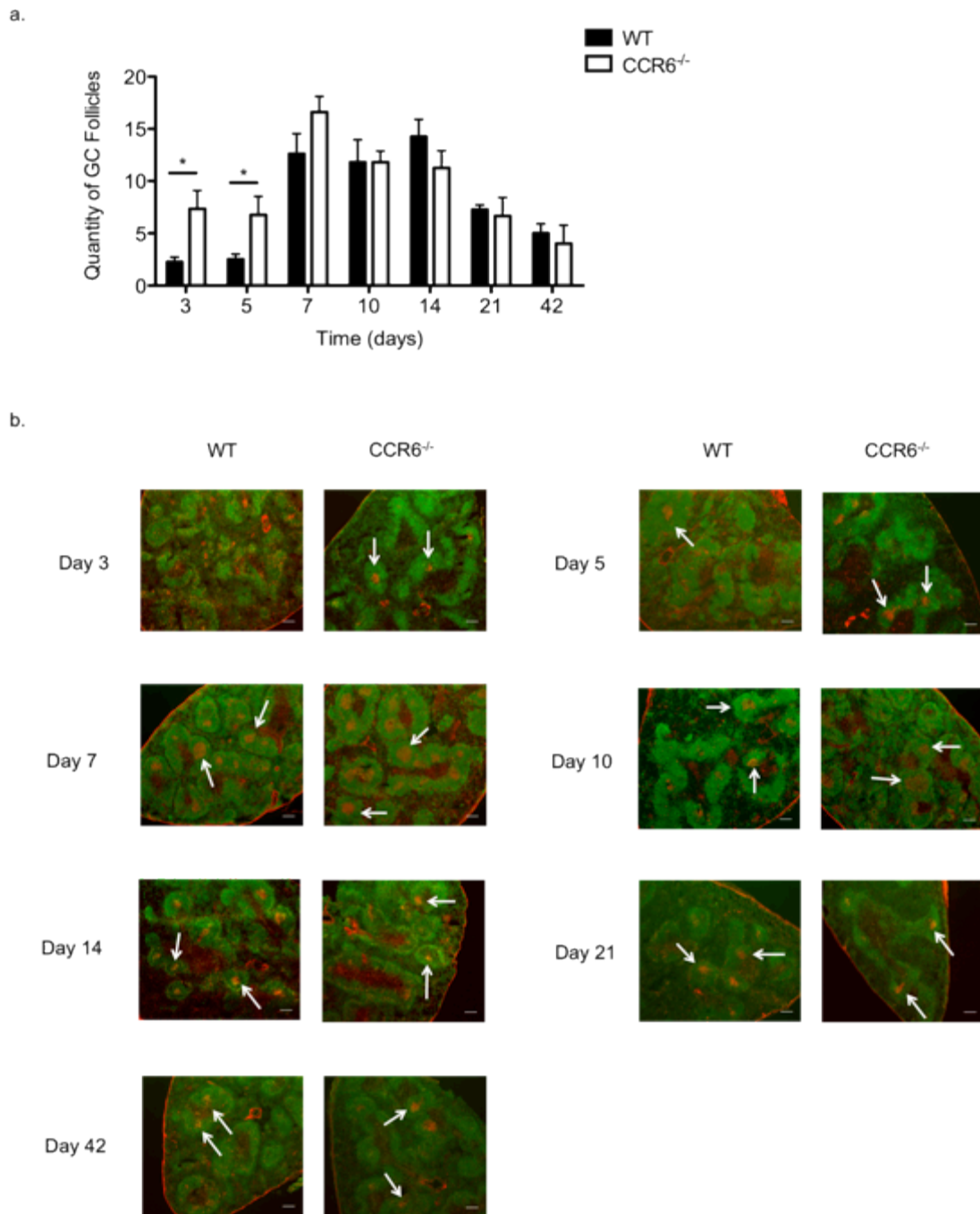


Figure 4.1.2. *The frequency of splenic Germinal Centre follicles during the T cell-dependent humoral response in the absence of CCR6*

Spleen sections of WT and CCR6^{-/-} mice immunised with 50–100µg of NP-KLH, were examined for GC follicles at 3, 5, 7, 10, 14, 21 and 42 days following antigen challenge. GC follicles were identified as B220⁺ PNA⁺ clusters and quantified in 1 spleen cross-section/mouse (a). Representative images of the GC follicle response in WT and CCR6^{-/-} mice are shown (b). Arrows indicate GC follicles. Data was collected from 1–2 independent experiments (Day 3 & 21 n=4 WT mice, 3 CCR6^{-/-} mice; Day 5, 14 & 42 n=4 WT mice, 4 CCR6^{-/-} mice; Day 7 & 10 n=5 WT mice, 5 CCR6^{-/-} mice).

Mann-Whitney two-tail t-test, *significant at p<0.05

4.2. CCR6 regulates efficient effector B cell differentiation

After establishing that the GC response was increased in CCR6^{-/-} mice, the frequency of other B cell subsets including naïve, precursor, memory and plasma cells, were also examined in these mice. Again, CCR6^{-/-} and WT mice were immunised with NP-KLH for 42 days and the λ -specific B cell subsets identified by CD38 and GL7 expression, were quantified by flow cytometry. The gating strategy employed to quantify these distinct B cell subsets is displayed in Fig. 4.2.1a. As the loss of CCR6 triggers GC expansion in the early stages of the humoral immune response, I examined whether this was due to an initial increase in activated B cells, and therefore a reduction in the naïve B cell population. Throughout the course of the humoral response, the naïve B cell population (CD38⁺ GL7⁻) generally appeared reduced in the CCR6^{-/-} mice compared to the WT mice (Fig. 4.2.1b). The frequency of naïve B cells was significantly reduced in CCR6^{-/-} mice at days 3, 5 and 14 post antigen challenge compared to WT mice (Fig. 4.2.1b). This result correlates with the increased GC response observed at days 3 and 5 in the CCR6^{-/-} mice and implies that more B cells are being recruited into the humoral response in CCR6^{-/-} mice than in WT mice. The increased population of activated B cells would potentially account for the increased GC response, however these B cells would also have to be selected to differentiate into GC B cells. Given the reduction in naïve B cells, it was hypothesised that the frequency of precursor B cells that have just been activated, would also increase. Interestingly, when the frequency of the precursor B cell population was examined, a similar quantity of precursor B cells (CD38⁺ GL7⁺) was detected between CCR6^{-/-} and WT mice throughout the humoral response (Fig. 4.2.1c). Though their existence has only recently been described and their complete function remains to be established, this population does arise before GC formation, and is thought to be capable of differentiating directly into GC and EF B cells^{28,73}. The precursor stage is quick and it is possible that no difference in the frequency of precursor cells is detected, as B cells are quickly entering and leaving this stage.

After examining the B cell populations responsible for initiating the humoral response, which appear prior to GC B cells, I next investigated whether the absence of CCR6 affected the outcome of the humoral response by quantifying the plasma and memory B cell populations. The plasma cell population (CD38⁻ GL7⁻) was detected 3 days after antigen challenge in both CCR6^{-/-} and WT mice (Fig. 4.2.1d). It then increased rapidly to day 5 and plateaued until day 14, where the frequency dropped off at day 21, only to rise again by day 42 (Fig. 4.2.1d). Throughout the humoral response though, the CCR6^{-/-} mice generated a similar frequency of plasma cells to WT mice (Fig. 4.2.1d). However, it is important to note that it is unclear whether these plasma cells are derived from the GC or EF, as there are no distinguishing markers available.

Next, memory B cells were examined. Memory B cells are thought to share a similar CD38⁺ GL7⁻ phenotype to naïve B cells. However in this study, I identified a discrete CD38^{hi} GL7⁻ population, for the first time, believed to be memory B cells. This population was significantly increased in CCR6^{-/-} mice compared to WT mice at 3, 5 and 10 days following antigen challenge (Fig. 4.2.1e). Of course, it is possible that the memory B cell population may have a bimodal expression of CD38. However, further analysis of this population based on the expression of IgM and IgD, supported the notion that the CD38^{hi} GL7⁻ B cells were part of the memory B cell population. The CD38^{hi} GL7⁻ population expressed high levels of IgM, while IgD expression was similar to that of the CD38⁺ GL7⁻ naïve B cells (Fig. 4.2.2a, b). Also, the expression level of both IgM and IgD was higher on the CD38^{hi} GL7⁻ cells, compared to the GC and plasma B cells (Fig. 4.2.2a). In contrast, IgM expression appeared similar between the GC and naïve B cells, while IgD expression was higher on the naïve cells than on the GC B cells, consistent with a naïve phenotype (Fig. 4.2.2a). In agreement with these observations is the existence of IgM^{hi} memory B cells²⁸. Furthermore, the temporal and cellular kinetics of the naïve and memory B cell population lends further support to their delineation. Both the naïve (CD38⁺ GL7⁻) and memory (CD38^{hi} GL7⁻) B cell populations are present 3 days after NP-KLH immunisation in WT mice, although the memory B cell population is substantially smaller than the naïve subset, as would be expected (Fig. 4.2.1b, e). Overall, it appears that the increased early GC response observed in the absence of CCR6 is due to an increase in activated B cells and results in an increased early memory B cell response.

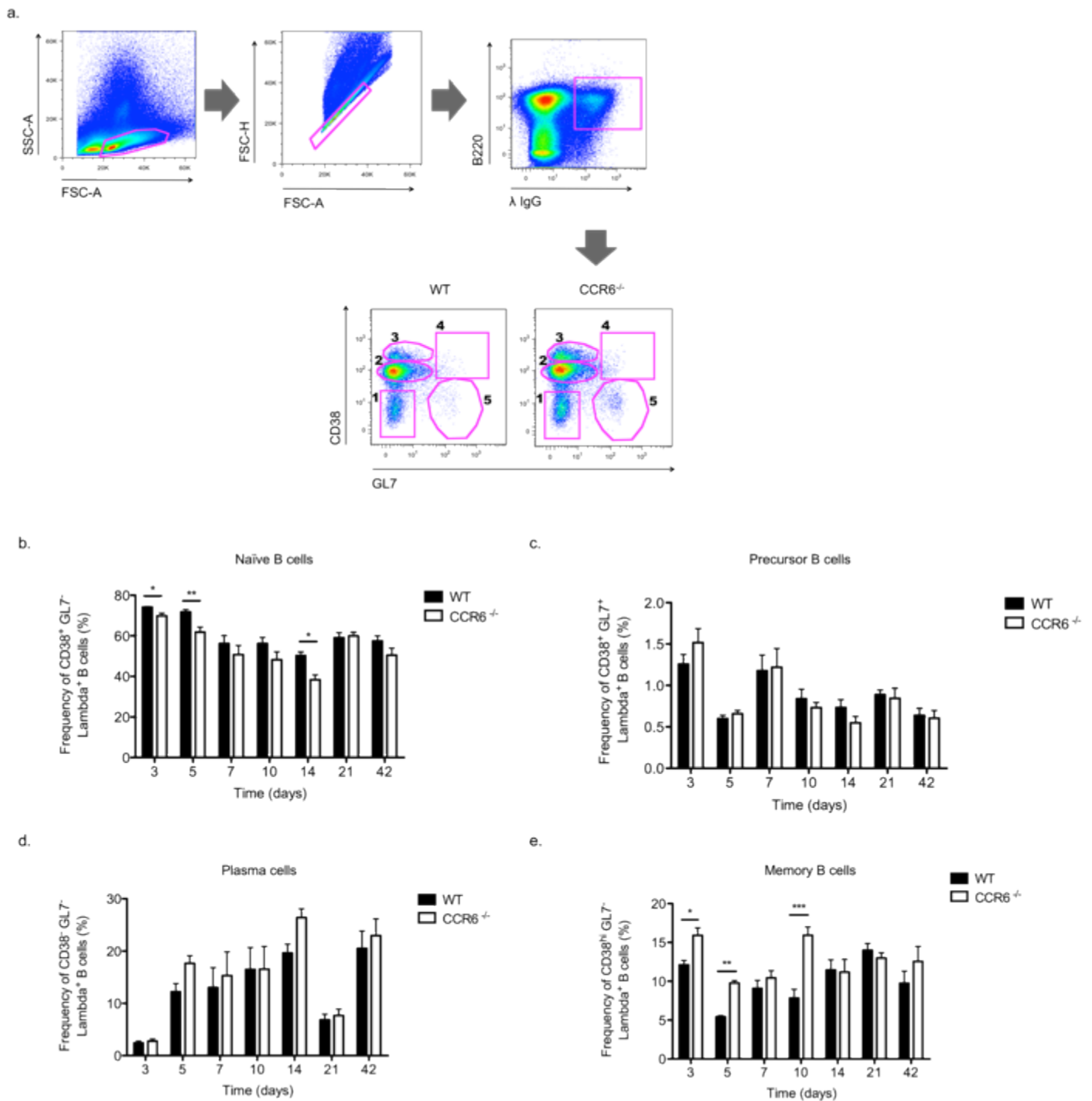


Figure 4.2.1. Effector B cell differentiation during the T cell-dependent humoral immune response in the absence of CCR6

WT and CCR6^{-/-} mice were immunised with 50-100µg of NP-KLH and the splenic B cell subsets were examined 3–42 days following challenge by flow cytometry. B cell populations were identified by their expression of CD38 and GL7, and representative images of WT and CCR6^{-/-} mice at day 5 are shown (a): 1) CD38⁻ GL7⁺ plasma B cells, 2) CD38⁺ GL7⁻ naïve B cells, 3) CD38^{hi} GL7⁻ memory B cells, 4) CD38⁺ GL7⁺ precursor B cells and 5) CD38⁻ GL7⁺ GC B cells. The frequencies of naïve (b), precursor (c), plasma (d) and memory (e) B cells were quantified. Data was collated from 1–2 independent experiments (Day 3 n=8 WT mice, 6 CCR6^{-/-} mice; Day 5 n=5 mice/genotype; Day 7, 21 & 42 n=9 mice/genotype; Day 10 n=8 WT mice, 9 CCR6^{-/-} mice; Day 14 n=4 mice/genotype).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01; ***significant at p<0.001

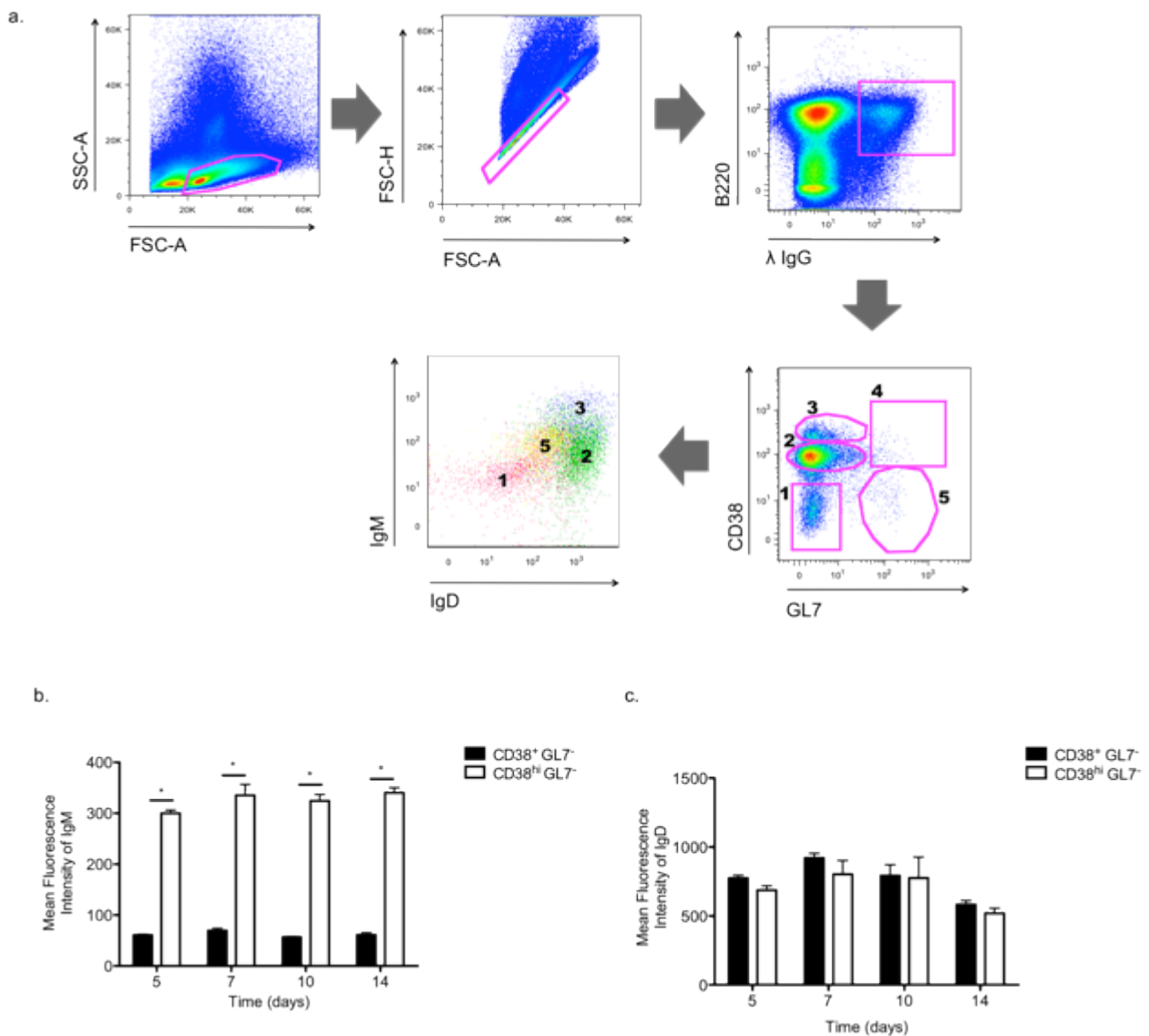


Figure 4.2.2. Characterisation of the CD38^{hi} GL7⁻ B cell population in the T cell-dependent humoral response

WT mice were immunised with 100µg of NP-KLH and the splenic CD38^{hi} GL7⁻ (memory) and CD38⁺ GL7⁻ (naïve) B cells were examined by flow cytometry at 5, 7, 10 and 14 days after immunisation. The representative image indicates the expression of IgM and IgD on 1) CD38⁻ GL7⁺ plasma cells, 2) CD38⁺ GL7⁻ naïve B cells, 3) CD38^{hi} GL7⁻ memory B cells and 5) CD38⁻ GL7⁺ GC B cells (a). CD38⁺ GL7⁺ precursor B cells are also indicated in the gating strategy (a). The mean fluorescence intensity of IgM (b) and IgD (c) expression was quantified on naïve and memory B cells. Figure represents data collected from 1 experiment (Day 5, 7 & 14 n=4 mice/genotype; Day 10 n=3 mice/genotype). Mann-Whitney two-tail t-test, *significant at p<0.05

4.3. CCR6 contributes to humoral responses in multiple secondary lymphoid organs

Having established that the absence of CCR6 causes B cell dysregulation in TD humoral responses in the spleen, I wanted to determine if this alteration is systemic, that is, if it also occurs in other

secondary lymphoid organs. As such, B cell differentiation was analysed in the draining lymph node of CCR6^{-/-} and WT mice. Mice were subcutaneously immunised with SRBC, to allow lymph node B cells to directly respond to antigen, based on their proximity to the site of immunisation. Responding lymph node B cells were then examined six days after immunisation, which represents the peak of the humoral response. The frequency of B cells was quantified by flow cytometry using antibodies against CD38 and GL7. However, the response measured is not antigen specific, as it is not possible to identify SRBC specific B cells. The gating strategy used to examine B cells in the lymph node is shown in Fig. 4.3.1a.

Initially, the frequency of the entire B cell population was quantified. In the CCR6^{-/-} mice, the B cell population appeared increased compared to WT mice (Fig. 4.3.1b). A significant increase in the frequency of GC B cells (CD38⁻ GL7⁺) was detected in CCR6^{-/-} mice compared to WT mice (Fig. 4.3.1c) and this finding parallels the GC response observed in the spleen. Also, the naïve B cells (CD38⁺ GL7⁻) were significantly reduced in the CCR6^{-/-} mice compared to the WT mice, again correlating with the splenic B cell response (Fig. 4.3.1d). Interestingly, the frequency of precursor B cells (CD38⁺ GL7⁺) was found to be significantly higher in the CCR6^{-/-} mice than in the WT mice (Fig. 4.3.1e). This finding differs to that in the spleen and is perhaps a result of the higher sensitivity in detecting this population in the lymph node, due to the lower frequency of B cells that reside in this organ. Furthermore, the prominent memory B cell population (CD38^{hi} GL7⁻), previously detected in the spleen, could not be detected in the lymph node and the reason for this is unknown (Fig. 4.3.1a). Finally, a similar frequency of plasma cells (CD38⁻ GL7⁻) was observed between CCR6^{-/-} and WT mice (Fig. 4.3.1f). Overall, the results obtained from the draining lymph node strengthen the conclusion that CCR6 plays a role in the regulation of B cell differentiation in the early humoral response. It is clear that the absence of CCR6 causes B cell dysregulation, such that more naïve B cells become activated and are subsequently selected to differentiate into GC B cells. Therefore, this work demonstrates that the absence of CCR6 affects the ability of follicular B cells to generate an efficient systemic humoral response.

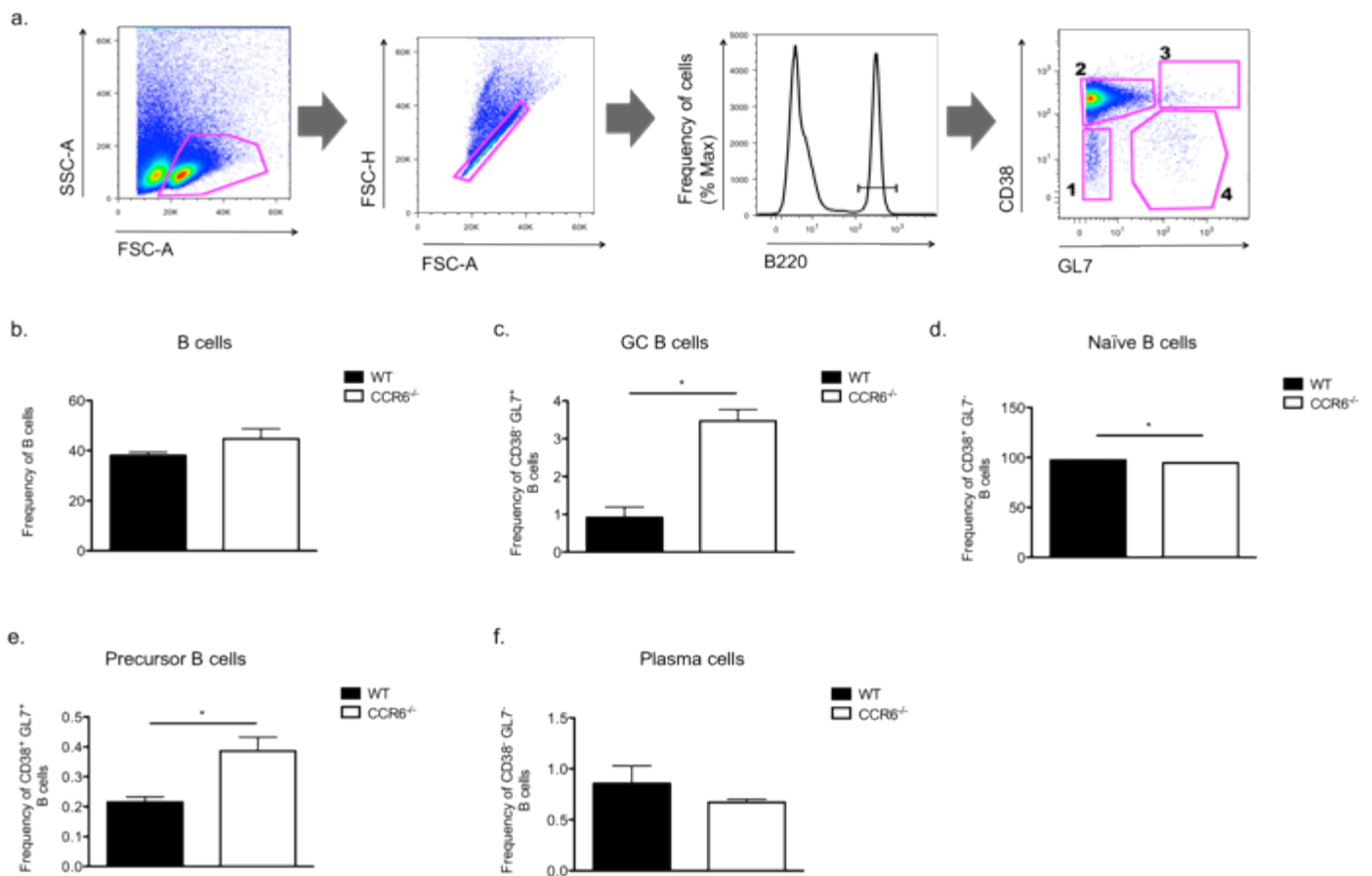


Figure 4.3.1. The T cell-dependent humoral response of lymph node B cells

WT and CCR6^{-/-} mice were s.c. immunised with SRBCs for 7 days. The draining lymph node B cell subsets, delineated by CD38 and GL7 were quantified by flow cytometry: 1) CD38⁺ GL7⁺ plasma cells, 2) CD38⁺ GL7⁻ naïve B cells, 3) CD38⁺ GL7⁺ precursor B cells, 4) CD38⁻ GL7⁺ GC B cells (a). The frequency of all B cells (b), GC B cells (c), naïve B cells (d) precursor B cells (e) and plasma cells (f) were quantified. Figure represents data generated from 1 experiment (n=5 WT mice, 4 CCR6^{-/-} mice).

Mann-Whitney two-tail t-test, *significant at p<0.05

4.4 CCR6 expression is linked to the formation of Extrafollicular Foci

Given that the GC B cell differentiation pathway is increased in the absence of CCR6, I wanted to determine whether the EF pathway was also affected in the CCR6^{-/-} mice. In considering that the plasma cell population was not altered in the absence of CCR6, it was expected that EF B cell differentiation occurred normally. However, a previous report has shown that CCR6^{-/-} mice have a higher frequency of low-affinity antibody forming cells¹⁸². As EF are the major producers of low-affinity plasma cells, an increase in the extrafollicular response would potentially account for this published finding.

To evaluate the influence of CCR6 on EF development, the antigen-specific HEL-Ig transgenic MD4 mice and MD4.CCR6^{-/-} mice were immunised with HEL-SRBC. The extrafollicular B cell

population, identified as B220^{lo} cells that produce antibodies against HEL, was quantified by flow cytometry six days later, as shown in Fig. 4.4.1a. This analysis revealed a significant reduction in the frequency of extrafollicular B cells in the CCR6^{-/-} mice compared to the WT mice (Fig. 4.4.1a, b). This suggests firstly that CCR6 is expressed by EF B cells, as its loss results in a reduction of this population and secondly, that CCR6 expression by B cells destined to differentiate into EF, may direct their migration to extrafollicular sites. Overall, the reduced EF response and increased GC response observed in the absence of CCR6 is perplexing, as it is unclear whether the alterations in EF and GC differentiation are separate events, or whether they are linked. It is possible that the excessive GC response may actually compensate for the impaired EF response in the absence of CCR6.

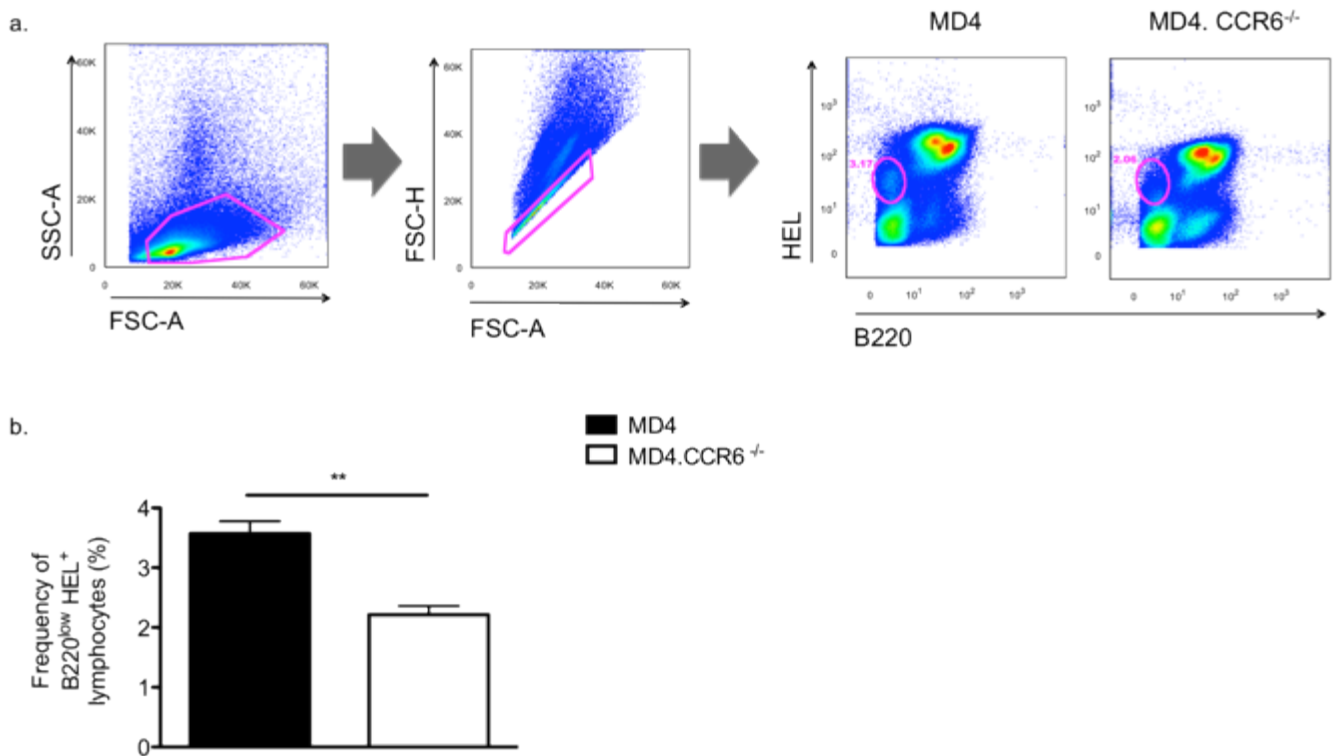


Figure 4.4.1. CCR6 deficiency impairs the Extrafollicular response

The frequency of splenic EF B cells was analysed by flow cytometry in MD4 and MD4.CCR6^{-/-} mice, 6 days after i.p. immunisation with HEL-SRBC. Representative flow cytometry images show the EF (B220^{lo} intracellular HEL⁺) population, as indicated by the gate (a). The frequency of B cells was quantified by flow cytometry (b). Data was compiled from 2 independent experiments (n= 7 MD4 mice, 8 MD4.CCR6^{-/-} mice). Mann-Whitney two-tail t-test, **significant at p<0.01

4.5. CCR6 influences Marginal Zone B cells during T cell-dependent humoral responses

Having assessed the consequences of CCR6 deficiency on the follicular B cell subset in TD humoral responses, the influence of CCR6 on MZ B cells was subsequently examined. Although MZ B cells are primarily involved in innate and TI immune responses^{254,255}, they are also capable of contributing to TD humoral responses. For instance, MZ B cells have been shown to aid cognate CD4⁺ T cell activation and contribute to the maintenance of FDC antigen levels²⁵⁶. For this reason, the response of MZ B cells in the CCR6^{-/-} mice was investigated, in the NP-KLH induced humoral response. The frequency of MZ B cells was quantified by flow cytometry between days 5–42, using antibodies against CD21 and CD23. The MZ B cell population was identified by their high CD21 and low CD23 expression (Fig. 4.5.1a)^{257,258}. A significant reduction in the frequency of MZ B cells was detected 21 days following antigen challenge in the CCR6^{-/-} mice compared to the WT mice (Fig. 4.5.1b). There also appeared to be an increase in the frequency of MZ B cells in CCR6^{-/-} mice 10 days after antigen challenge compared to WT mice, however this was not significant due to the varying responses in individual mice. Similar frequencies of MZ B cells were observed between CCR6^{-/-} and WT mice for the remaining response. Therefore, the findings from this work suggest that MZ B cells do not contribute to the induction of the humoral response, but rather play a greater role in the later stages of the responses, potentially in the cessation of the GC response.

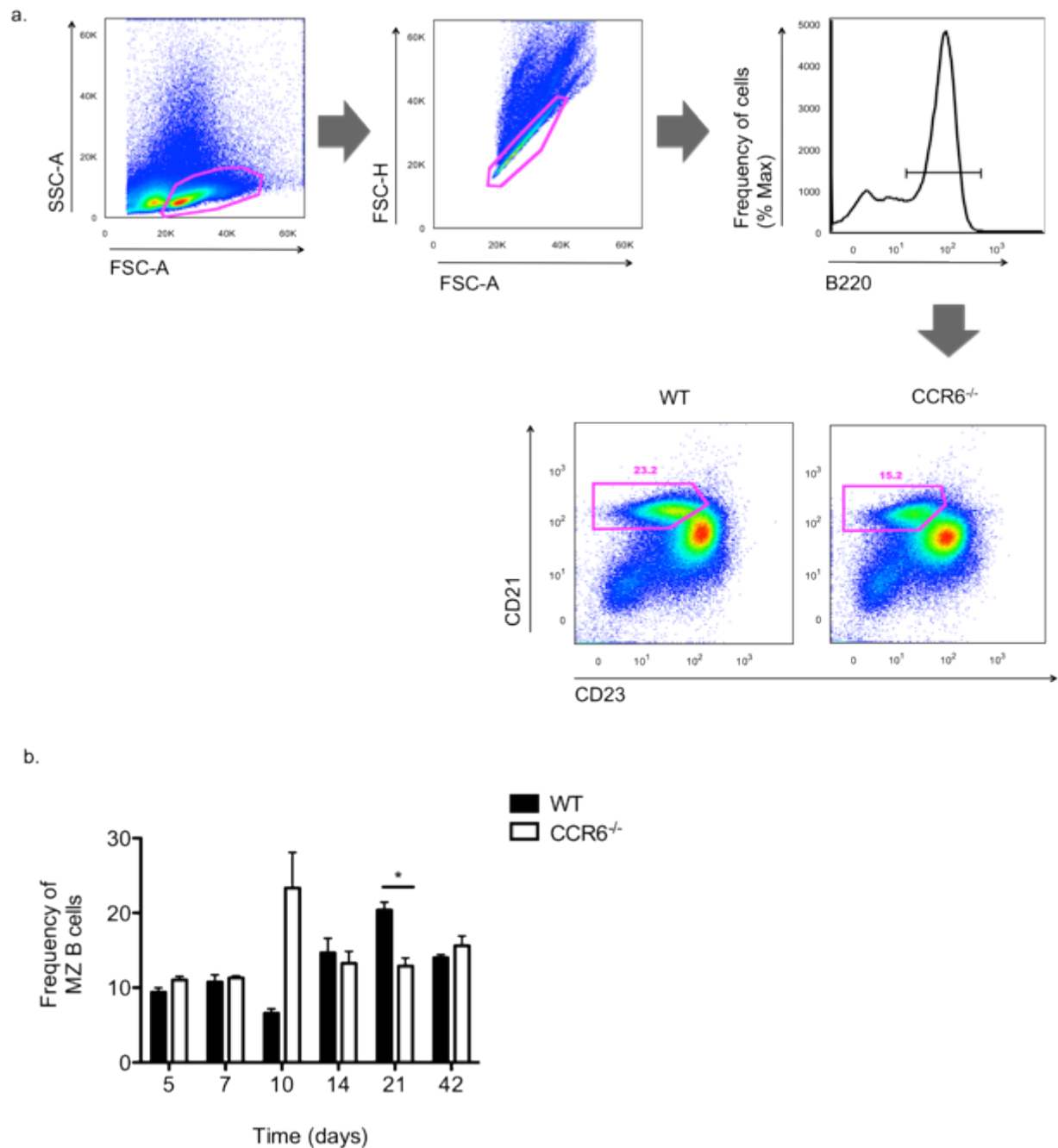


Figure 4.5.1. *The contribution of CCR6 to Marginal Zone B cell differentiation in the T cell-dependent humoral response*

WT and CCR6^{-/-} mice were immunised with 100µg of NP-KLH and the splenic MZ B cell population was quantified by flow cytometry 5, 7, 10, 14, 21 and 42 days following immunisation. Representative flow cytometry images indicate the CD21^{hi} CD23^{lo} MZ B cell population in WT and CCR6^{-/-} mice at day 21 (a). The frequency of MZ B cells was quantified (b). Data was collected from 1 experiment (Day 5, 21 & 42 n=4 WT mice, 4 CCR6^{-/-} mice; Day 7 n=4 WT mice, 3 CCR6^{-/-} mice; Day 10 n=3 WT mice, 4 CCR6^{-/-} mice; Day 14 n=5 WT mice, 4 CCR6^{-/-} mice).

Mann-Whitney two-tail t-test, *significant at p<0.05

4.6. Concluding Remarks

In summary, I have directly examined the temporal kinetics of B cell differentiation in the absence of CCR6. The major TD antigen used to induce humoral immunity was NP-KLH. This model of humoral immunity was advantageous as it was possible to identify the expansion of NP-specific λ -Ig B cells. The λ^+ B cell population could be subdivided into five distinct subsets, based on the expression level of CD38 and GL7: naïve ($CD38^+ GL7^-$), precursor ($CD38^+ GL7^+$), GC ($CD38^- GL7^+$), plasma ($CD38^- GL7^-$) and memory ($CD38^{hi} GL7^-$) B cells. The cell surface phenotype of these subsets is consistent with the literature. For example, GL7 is not expressed by the follicular B cell subsets, except following activation, in which it is upregulated on precursor and GC B cells^{28,259,260}. In addition, CD38 is expressed on naïve B cells and precursor B cells, downregulated on GC B cells, absent from plasma cells and upregulated on memory B cells^{24,28,261,262}. Furthermore, the upregulation of both CD38 and GL7 on activated B cells concurs with the *in vitro* findings documented in Chapter 3. Subsequent analysis of the $CD38^{hi} GL7^-$ memory B cells confirmed its identity, corresponding with the previously reported existence of IgM^{hi} memory B cells^{98,137,138}.

The B cell response of WT mice to NP has been well defined^{26-28,50,53,77,85,135}. Briefly, naïve B cells become activated and subsequently interact with Th cells within 2 days of antigen challenge. B cells then enter a precursor stage at day 3, which is quickly followed by GC differentiation. The GC B cell response peaks at day 7, declines and persists until at least day 16. Meanwhile the EF response is initiated early, peaks by day 8 and dissipates by day 14. During this time, plasma and memory B cells are generated, peaking approximately 1 week and 21 days after antigen challenge, respectively. Hence, these studies have provided an excellent reference for TD B cell kinetics. In this study, I detected GC B cells 3 days after antigen challenge. The GC B cell population peaked at day 7, gradually declined by day 21 and persisted at low levels, as observed at day 42. This correlated with a reduction in the naïve B cells and the appearance of memory and plasma cells. The memory B cells remained at a similar level for the course of the response, while the plasma cells increased at day 6, and declined at day 21 before rising again at day 42. Also, a high frequency of precursor B cells was observed at day 3 and 7, before declining and plateauing for the remaining response. Thus, the results from this study correlate with the literature.

The best method available to quantify the extrafollicular response is to examine the B220^{lo} cells that produce HEL-specific antibodies. In doing this, the EF B cell population was found to be significantly reduced in the absence of CCR6. However, it would also be valuable to examine the EF response in the *s_w*HEL mouse model, which differs from the MD4 model used here, in that it is capable of B cell isotype switching^{78,263}. The investigation of the EF B cell response to HEL–

SRBCs, a potent inducer of TD responses, has been published previously⁷⁵. This study has provided valuable insight at the peak of the response in CCR6 deficient mice compared to WT mice. It used unimmunised mice as reference points, and as such mice without SRBCs were not included in this study. It would be appropriate in future studies though, to document the initiation and progression of SRBC-mediated responses, including experiments without SRBCs.

Although MZ B cells are involved in innate-like immune responses^{254,255}, they have also been implicated in TD immune responses. Indeed in NP studies, it has been proposed that MZ B cells share a similar function to follicular B cells, in that they too are responsible for the early generation of antibody forming cells, particularly plasma cells, IgM⁺ memory B cells, and GC B cells²⁶⁴. Whilst the potential dysregulation of the MZ B cell compartment in the CCR6^{-/-} mice could account for the increased production of IgM^{hi} memory B cells and the increased GC B cell response, a consistent MZ B cell population was observed for most of the humoral response in CCR6^{-/-} and WT mice, effectively ruling out this possibility. However, there was a significant decrease in MZ B cells 21 days following antigen challenge in the CCR6^{-/-} mice, which corresponded with a reduction in GC B cells at day 21 in the CCR6^{-/-} mice. It has previously been established that MZ B cells migrate to the B cell follicle to deliver antigen to FDCs, thereby supporting the GC response²⁶⁵⁻²⁶⁷. This published data, in combination with the results of this investigation, indicate that the CCR6^{-/-} mice may effectively maintain antigen supply to the GC via the MZ B cells, until its cessation at day 21, at which point both a decrease in MZ and GC B cells is detected.

Overall, this chapter has demonstrated that CCR6 performs a substantial role in regulating B cell differentiation during the initial stages of the humoral immune response. The absence of CCR6 appears to inhibit EF development, which may be overcome by excessive GC formation. Furthermore, this results in an increased early memory response, while the plasma cell response appears unaffected. The functional significance of this dysregulation will be investigated in the next chapter.

Chapter 5

The mechanisms underlying CCR6 deficient B cell differentiation

In this chapter, I report on the biological consequences of CCR6 impairment, particularly in regards to the heightened GC response, and shed light onto the potential mechanisms underlying the B cell dysregulation observed in the CCR6^{-/-} mice. Understanding the CCR6 mediated regulation of B cell differentiation is crucial, as it will enhance our knowledge of the mechanisms underlying efficient humoral responses and conversely, how humoral responses can be manipulated for therapeutic applications.

5.1. CCR6 contributes to the Germinal Centre reaction

Considering the substantial increase in the early GC response of CCR6^{-/-} mice, the quality of the GC reaction was evaluated. As there is a clear structure-function relationship between B cell positioning in secondary lymphoid organs and the function of B cells, the structure of GC follicles was examined in the absence of CCR6. Within GC follicles, B cells are positioned in the light or dark zone depending on their expression of CXCR5 and CXCR4¹⁵⁹. This spatial division enables SHM and affinity selection to occur in discrete specialised microenvironments. Specifically, clonal expansion and SHM of B cells occurs in the dark zone, whilst affinity selection occurs in the light zone⁹⁵⁻⁹⁷.

The structure of GC follicles in CCR6^{-/-} and WT mice was assessed by immunofluorescence five days following NP-KLH challenge. The structure of GCs was examined using antibodies against IgD, PNA and CD35. These antibodies were selected, as IgD identifies B cell follicles but not GC B cells^{23,91}, PNA identifies GC B cells^{91,253} and CD35 identifies FDCs located in the light zone²⁶⁸. Upon examination of spleen sections, no gross abnormalities were observed in the GC follicles between CCR6^{-/-} and WT mice (Fig. 5.1.1). Consistent with the findings in the previous chapter, noticeably more GC follicles were observed in CCR6^{-/-} mice than WT mice (Fig. 5.1.1). In addition, both the size of the GC follicles and the size of the light zone within the GC were comparable between CCR6^{-/-} and WT mice (Fig. 5.1.5). However, the PNA⁺ dark zone appeared more prominent in the CCR6^{-/-} mice than the WT mice (Fig. 5.1.5). This would suggest that the WT mice are in the early stages of GC follicle establishment.

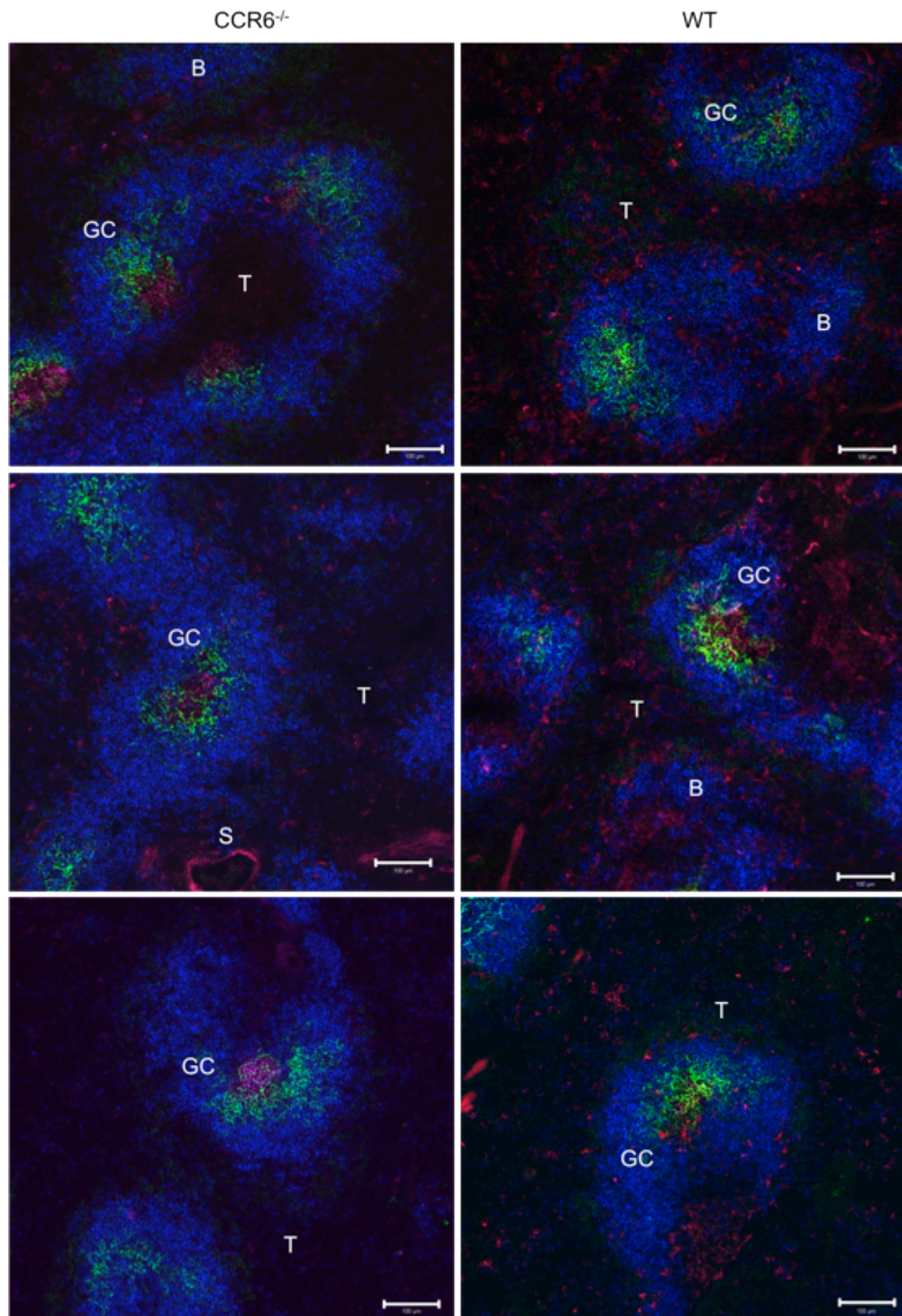


Figure 5.1.1. *The structure of Germinal Centre follicles in the CCR6 deficient humoral response*

$CCR6^{-/-}$ and WT mice were immunised with 100 μ g of NP-KLH for 5 days. Spleen sections were stained with antibodies against IgD, PNA and CD35. GC follicles were identified as IgD⁻ PNA⁺ CD35⁺. Samples were imaged using the confocal microscope (n=3 mice/genotype). Images indicate (GC) germinal centres, (B) B cell follicles, (T) T cell zones and (S) Sinus.

Objective magnification: 10x; Scale bars are 100 μ m

Having observed that the structure of GC follicles in CCR6^{-/-} mice is largely intact, the antibody maturation and differentiation capacity of CCR6^{-/-} GC B cells was analysed at the molecular level. Specifically, qRT-PCR was used to examine the expression of the chemokine receptors that contribute to GC organisation, as well as the transcription factors and enzymes that direct the GC reaction. GC B cells were isolated from CCR6^{-/-} and WT mice that had been immunised with NP-KLH for 5 days, using the MoFlo® Astrios™ cell sorter. All samples were normalised by cell number and gene expression was analysed using the relative Δ CT method, which compares physiological changes in gene expression at the mRNA level relative to a reference gene.

Initially, the transcription factor Bcl-6 was examined, which is essential for GC formation¹⁰². A significant increase in Bcl-6 expression was detected in CCR6^{-/-} GC B cells compared to WT GC B cells (Fig. 5.1.2). This increase was not due to an increase in the frequency of GC B cells, as all samples were normalised by cell number. Thus, Bcl-6 expression is inherently higher in CCR6^{-/-} GC B cells. As Bcl-6 is essential for GC formation and proliferation, it offers an explanation for the increased GC response observed in the CCR6^{-/-} mice in Chapter 4. The next protein examined was inhibitor of DNA-binding 2 (Id2). Id2 is a protein that negatively regulates splenic B cell differentiation and in the GC, suppresses AID activity and therefore SHM^{35,269,270}. Interestingly, a significant increase in the mRNA expression of Id2 was also detected in the CCR6^{-/-} GC B cells compared to the WT GC B cells (Fig. 5.1.2). Hence, it was possible that the increased Id2 expression was inhibiting AID expression and consequently SHM, potentially accounting for the increased frequency of low-affinity antibody-forming cells documented in CCR6^{-/-} mice¹⁸². However, when AID expression was examined, it was not downregulated in CCR6^{-/-} GC B cells, but expressed at a similar level to GC B cells in WT mice (Fig. 5.1.2). This indicates that SHM is not impaired in CCR6^{-/-} mice. Therefore, I investigated whether terminal B cell differentiation was affected, by examining the expression of Blimp-1 and IRF4. These proteins are transcription factors that are essential for plasma cell, but not memory B cell, differentiation¹³⁹. Again, both the CCR6^{-/-} and WT GC B cells produced similar levels of Blimp-1 and IRF4 mRNA, illustrating that terminal differentiation into plasma cells is not affected by CCR6 expression (Fig. 5.1.2). This finding concurs with the similar frequency of plasma cells observed between CCR6^{-/-} and WT mice in Chapter 4. In addition, Pax5 expression was analysed. Pax5 is required for B cell lineage commitment and GC formation, while its downregulation facilitates plasma cell differentiation^{145,147,271,272}. Thus, it was possible that Pax5 may contribute to the increased GC response detected in CCR6^{-/-} mice. However this was not the case, as equivalent levels of Pax5 expression were detected between CCR6^{-/-} and WT GC B cells (Fig. 5.1.2). Finally, the expression of the chemokine receptors: CXCR5, CXCR4, CCR7 and EBI2 were examined, due to their pivotal

role in B cell positioning during the humoral response. Specifically, CXCR5 and CXCR4 are essential for B cell positioning within the light and dark zones of the GC, while the downregulation of CCR7 and EBI2, is required for B cells to migrate in a CXCR5-dependent manner to the B cell follicle^{52,156,158,159}. Consequently, I wanted to rule out the possibility that chemokine receptors other than CCR6 were responsible for the increased GC seeding in CCR6^{-/-} mice. This analysis showed no significant variation in the expression of CXCR5, CXCR4, CCR7 or EBI2, between CCR6^{-/-} and WT GC B cells (Fig. 5.1.2). Therefore, CCR6^{-/-} GC B cells do not have any alterations in the chemokine receptors that play pivotal roles in the GC response.

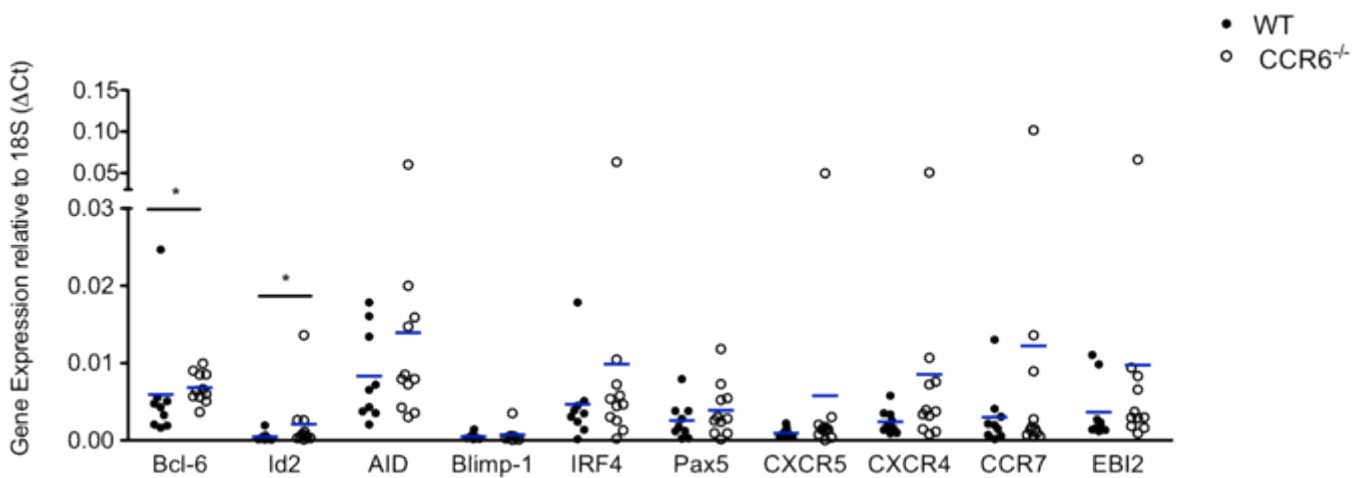


Figure 5.1.2. *The molecular characteristics of CCR6 deficient Germinal Centre B cells*

WT and CCR6^{-/-} mice were i.p. immunised with 100μg of NP-KLH for 5 days. 1000 GC B cells, identified as λ⁺ CD38⁻ GL7⁺ B cells, were isolated using the MoFlo® Astrios™ cell sorter. RNA was extracted and cDNA subsequently synthesised. Gene expression of Bcl-6, Id2, AID, Blimp-1, IRF4, Pax5, CXCR5, CXCR4, CCR7 and EBI2, relative to 18S, was analysed by qRT-PCR. Data is displayed as ΔCt and represents 3 independent experiments (n=9 WT mice, 11 CCR6^{-/-} mice; except for Blimp-1 where n=8 mice/genotype, and CXCR5 & CXCR4 where n=9 WT mice, 10 CCR6^{-/-} mice).

Mann-Whitney two-tail t-test, *significant at p<0.05

In continuing with the analysis of the GC response, the T_{FH} cell and FDC populations were also examined in the CCR6^{-/-} mice. The T_{FH} cells are crucial in supporting the GC reaction as they secrete proteins that support B cell proliferation and the affinity selection of B cells. As such, the T_{FH} cells of CCR6^{-/-} and WT mice immunised with NP-KLH for five days were isolated and prepared for qRT-PCR analysis. The T_{FH} cells were isolated using the MoFlo® Astrios™ cell sorter, based on their high expression of CXCR5 and PD-1^{119,273,274}. Three populations of T cells were identified: CXCR5⁻ PD-1⁻ non-T_{FH} cells, CXCR5^{int} PD-1^{int} intermediate T_{FH} cells and CXCR5^{hi} PD-1^{hi} T_{FH} cells. Intermediate T_{FH} cells must upregulate CXCR5 and PD-1 to become T_{FH}

cells²⁷⁴⁻²⁷⁶. As per the analysis of GC B cells, all T_{FH} cell samples were normalised by cell number and the samples prepared for qRT-PCR.

Initially, CCR6 and CCL20 expression was quantified in WT T_{FH} cell subsets. Given that CCR6 expression was detected on the surface of T cells in T-B cell conjugates in Chapter 3, CCR6 mRNA expression was examined on T_{FH} cell subsets in WT mice by qRT-PCR. This analysis showed that all three populations: non-T_{FH} cells, intermediate T_{FH} cells and T_{FH} cells, expressed similar levels of CCR6 mRNA (Fig. 5.1.3a). The possibility that T cells were responsible for CCL20 production arose, as T cells are known to produce cytokines that support B cell differentiation. Hence, CCL20 expression was examined on the T_{FH} cell subsets of WT mice. A significantly higher amount of CCL20 mRNA was detected in the intermediate T_{FH} cells, compared to the T_{FH} cells (Fig. 5.1.3b). This is interesting as the T_{FH} cells are thought to arise from precursor T cells at the T-B border, which are in the process of upregulating CXCR5 and PD-1 expression⁵¹. Thus, the finding that intermediate T_{FH} cells produce CCL20 indicates that CCL20 may act in an autocrine manner to retain T cells at the T-B border, and potentially also retain activated B cells, which upregulate CCR6 expression, as demonstrated in Chapter 3, and localise to the T-B border.

Next, the expression of proteins associated with the differentiation and function of T_{FH} cells were examined. Given that IL-21 production by T_{FH} cells is fundamental for T_{FH} cell commitment through an autocrine mechanism²⁷⁷, and consequently GC formation, B cell differentiation and antibody production²⁷⁷⁻²⁷⁹, IL-21 expression was examined in CCR6^{-/-} and WT mice. A significantly higher amount of IL-21 mRNA was detected in CCR6^{-/-} T_{FH} cells than in WT T_{FH} cells (Fig. 5.1.3c). However, this increase in IL-21 expression did not induce significant increases in Bcl-6 nor CXCR5 expression (Fig. 5.1.3c), proteins that are essential for T_{FH} cell development and the GC response^{119,273,275,280}. Nonetheless, it has been demonstrated that IL-21 secreted by T_{FH} cells also acts directly on GC B cells, through Bcl-6 and thereby controls GC formation and terminal B cell differentiation^{121,122,279}. Therefore, it is highly likely that the increased IL-21 production by T_{FH} cells in the CCR6^{-/-} mice, directly induces the increased Bcl-6 expression of GC B cells and is consequently responsible for the increased early GC response observed in these mice. When IL-21 was examined in intermediate T_{FH} cells, precursor to T_{FH} cells, IL-21 expression was found to be similar between CCR6^{-/-} and WT mice (Fig. 5.1.3d, e).

As mentioned earlier, no significant variation in CXCR5 expression was detected in CCR6^{-/-} T_{FH} cells compared to WT T_{FH} cells, and this was also true for CCR7 (Fig. 5.1.3c). Both of these chemokine receptors are required for T_{FH} cell localisation to the GC^{274,281}, and therefore the results

indicate no impairment in the localisation of T_{FH} cells to GC follicles. Likewise, there was no significant variation in either CXCR5 or CCR7 expression between CCR6^{-/-} and WT intermediate T_{FH} cells and non-T_{FH} cells (Fig. 5.1.3c, d, e). In addition, FoxP3 expression was quantified in T_{FH} cells. FoxP3 expression was quantified, as it is expressed by follicular regulatory T cells (T_{FR}) in the GC, which phenotypically resemble the T_{FH} cells, but are instead responsible for suppressing the GC response²⁸². As predicted, there was no significant variation in FoxP3 expression between CCR6^{-/-} and WT T_{FH} cell subsets, demonstrating that the increased GC response was not due to a loss in T_{FR} cells (Fig. 5.1.3c). Finally, there was no significant variation in the expression of FoxP3 or Bcl-6 in the intermediate T_{FH} cells and T_{FH} cells, between CCR6^{-/-} and WT mice (Fig. 5.1.3d, e).

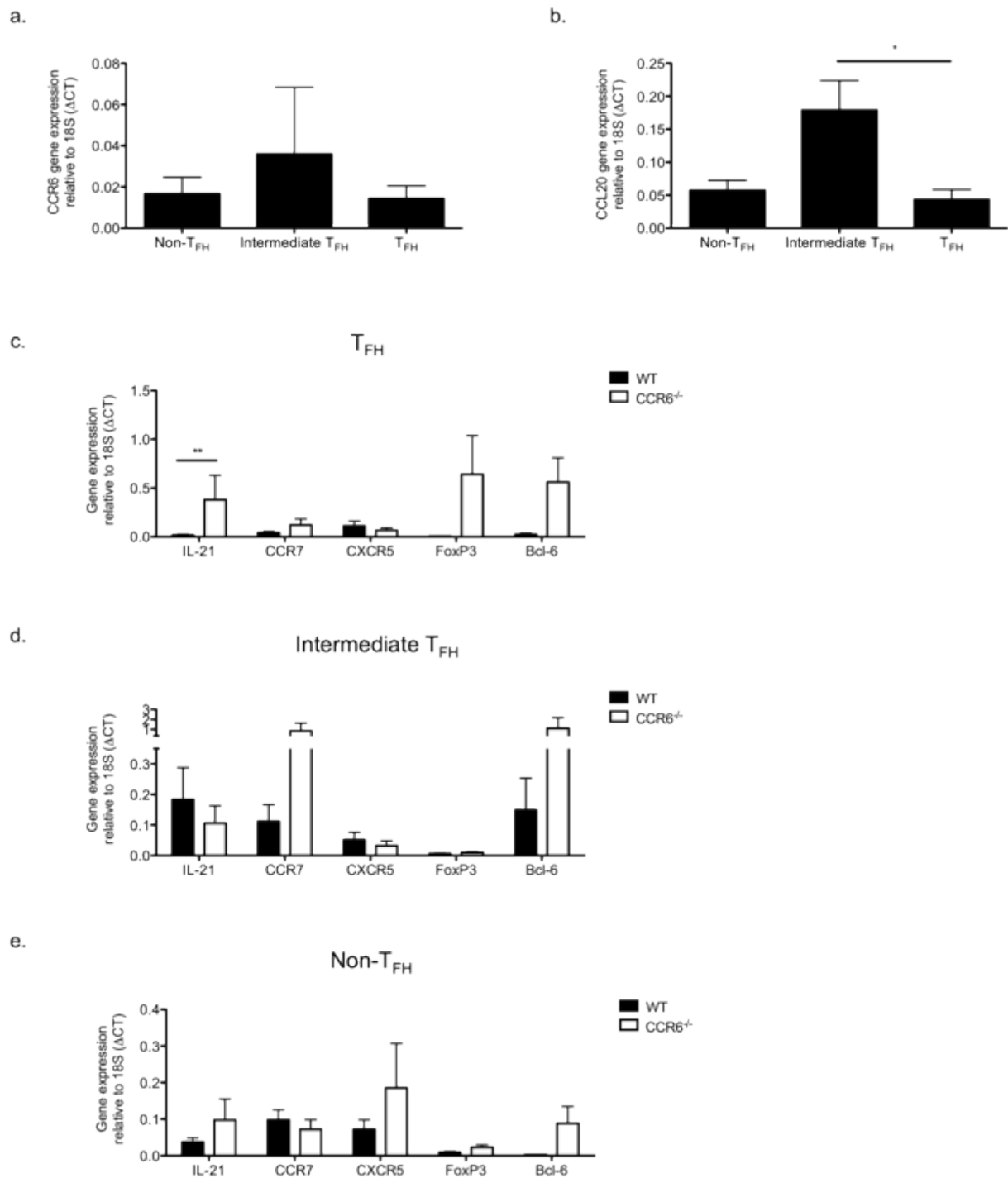


Fig. 5.1.3. The molecular characteristics of CCR6 deficient T Follicular Helper cells

WT and CCR6^{-/-} mice were i.p. immunised with 100 μ g of NP-KLH for 5 days. 1000 cells from the T_{FH} cell subsets: CXCR5^{hi} PD-1^{hi} T_{FH} cells, CXCR5^{int} PD-1^{int} intermediate T_{FH} cells and CXCR5⁺ PD-1⁻ non-T_{FH} cells, were isolated using the MoFlo® Astrios™ cell sorter. RNA was extracted and cDNA synthesised. The gene expression of CCR6 (a) and CCL20 (b) was quantified in T_{FH} cell subsets of WT mice. Also, the gene expression of IL-21, CCR7, CXCR5, FoxP3 and Bcl-6 was quantified in T_{FH} cells (c), intermediate T_{FH} cells (d) and non-T_{FH} cells (e). Figure represents data collected from 2 independent experiments (n=6 mice/genotype). Inefficient reactions were excluded.

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

In addition to examining the molecular characteristics of T_{FH} cells, the T_{FH} cell response was also examined by flow cytometry. WT and CCR6^{-/-} mice were immunised with NP-KLH and the T_{FH} cells were quantified 5–42 days after antigen challenge. As mentioned earlier, T_{FH} cells are identified by their high expression of CXCR5 and PD-1. The gating strategy used to detect T_{FH} cells is displayed in Fig. 5.1.4a. The WT T_{FH} cell response peaked at day 7, as did the GC response, before declining (Fig. 5.1.4b). It was hypothesised that the increased GC response observed in CCR6^{-/-} mice at day 5, would be associated with an increased frequency of T_{FH} cells. However, the CCR6^{-/-} mice had a similar frequency of T_{FH} cells compared to WT mice, 5 days after antigen challenge (Fig. 5.1.4b). While there was no change in the frequency of T_{FH} cells between CCR6^{-/-} and WT mice, the GC response is still increased in CCR6^{-/-} mice, and thus, the T_{FH} cell population is reduced compared to WT mice. This finding suggests that at this time point, CCR6^{-/-} GC follicles are either under more stringent selection pressure due to the lower number of T_{FH} cells, or the reduced number of T_{FH} cells may directly impair B cell affinity selection. Surprisingly, a significant increase in the frequency of T_{FH} cells was observed in CCR6^{-/-} mice at days 10 and 42 after antigen challenge, compared to WT mice (Fig. 5.1.4a), and perhaps reflects a delayed response to the increased early GC B cell population. Next, I examined the intermediate T_{FH} cell population, which is thought to be the precursor of T_{FH} cells. Similar to the T_{FH} cells, the intermediate T_{FH} cell population peaked at day 7 before declining (Fig. 5.1.4c). During this time, the frequency of intermediate T_{FH} cells was generally similar between CCR6^{-/-} and WT mice (Fig. 5.1.4c). However, a significant reduction in the frequency of intermediate T_{FH} cells was detected at day 21, and a significant increase was also detected at day 42 following antigen challenge, in the CCR6^{-/-} mice compared to the WT mice (Fig. 5.1.4c). The frequency of non-T_{FH} cells was also similar between CCR6^{-/-} and WT mice, except at day 21, where a significant increase was detected (Fig. 5.1.4d). The reasons for these late stage alterations in the T_{FH} cell compartment are unknown and probably reflect the effects of GC cessation in the absence of CCR6. Overall, these results suggest that the unchanged T_{FH} cell population in the CCR6^{-/-} mice, which have an increased GC response, may impair the affinity selection of B cells.

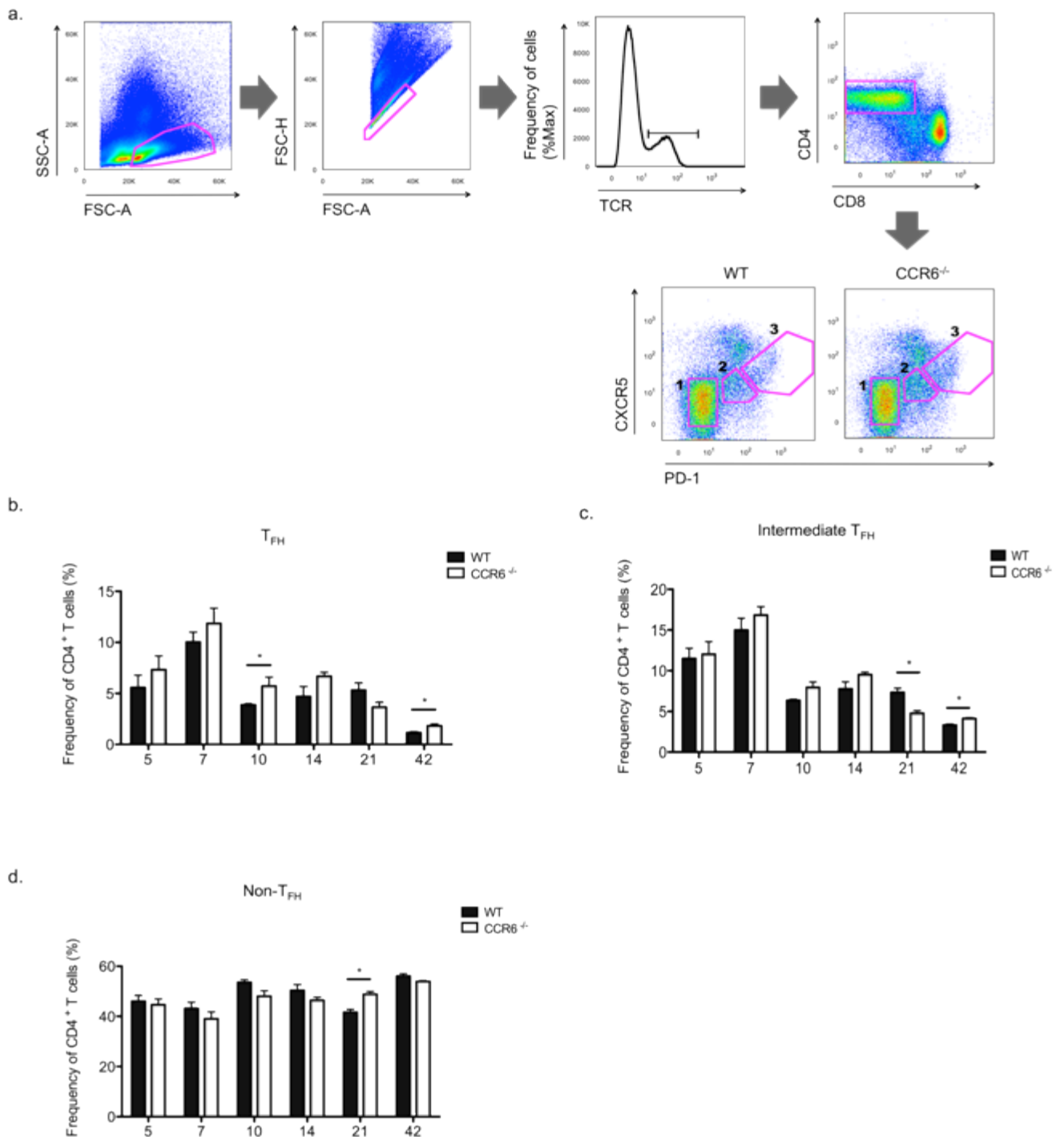


Fig. 5.1.4 The *T* Follicular Helper cell kinetics of the *T* cell-dependent humoral response in the absence of *CCR6*

WT and CCR6^{-/-} mice were i.p. immunised with 100µg of NP-KLH and the T_{FH} cell subsets were quantified by flow cytometry at days 5, 7, 10, 14, 21 and 42 after antigen challenge. The compartment was separated into three subsets based on their expression of CXCR5 and PD-1: 1) CXCR5⁺ PD-1⁻ non- T_{FH} cells, 2) CXCR5^{int} PD-1^{int} intermediate T_{FH} cells and 3) CXCR5^{hi} PD-1^{hi} T_{FH} cells, and representative images of WT and CCR6^{-/-} mice at day 10 are displayed (a). The frequency of T_{FH} cells (b), intermediate T_{FH} cells (c) and non- T_{FH} cells (d) were quantified in WT and CCR6^{-/-} mice. Figure represents data collected from 1 experiment (n=4 mice/genotype). Mann-Whitney two-tail t-test, *significant at p<0.05

Having completed the analysis of the T_{FH} cells, the FDC compartment was examined. The FDC network is essential to the GC reaction, as it provides a source of antigen for the GC B cells required for affinity selection, as well as signals that support the GC reaction^{112,283,284}. A preliminary flow cytometry study was carried out, where WT and CCR6^{-/-} mice were immunised with NP-KLH and the frequency of the FDC population was quantified 5–42 days following antigen challenge. The FDCs were identified by their forward and side scatter parameters, as well as their distinct CD21/35 expression²⁶⁸. The gating strategy used to quantify the FDC population is displayed in Fig. 5.1.5a. Although the frequency of FDCs appeared to be decreased at day 5 and increased at day 10 in the CCR6^{-/-} mice compared to the WT mice, no significant variation was detected. Similar to the cellular kinetics of the T_{FH} cells in the CCR6^{-/-} mice, the increased GC cell response and largely unchanged FDC response indicates that affinity selection may be impaired in the absence of CCR6. However, whether the impaired affinity selection of CCR6^{-/-} mice has a long-term impact on the humoral response is unknown. Consequently, the reduced T_{FH} cell and FDC populations in the CCR6^{-/-} mice may account for the increased generation of low-affinity antibody forming cells documented in the CCR6^{-/-} mice¹⁸².

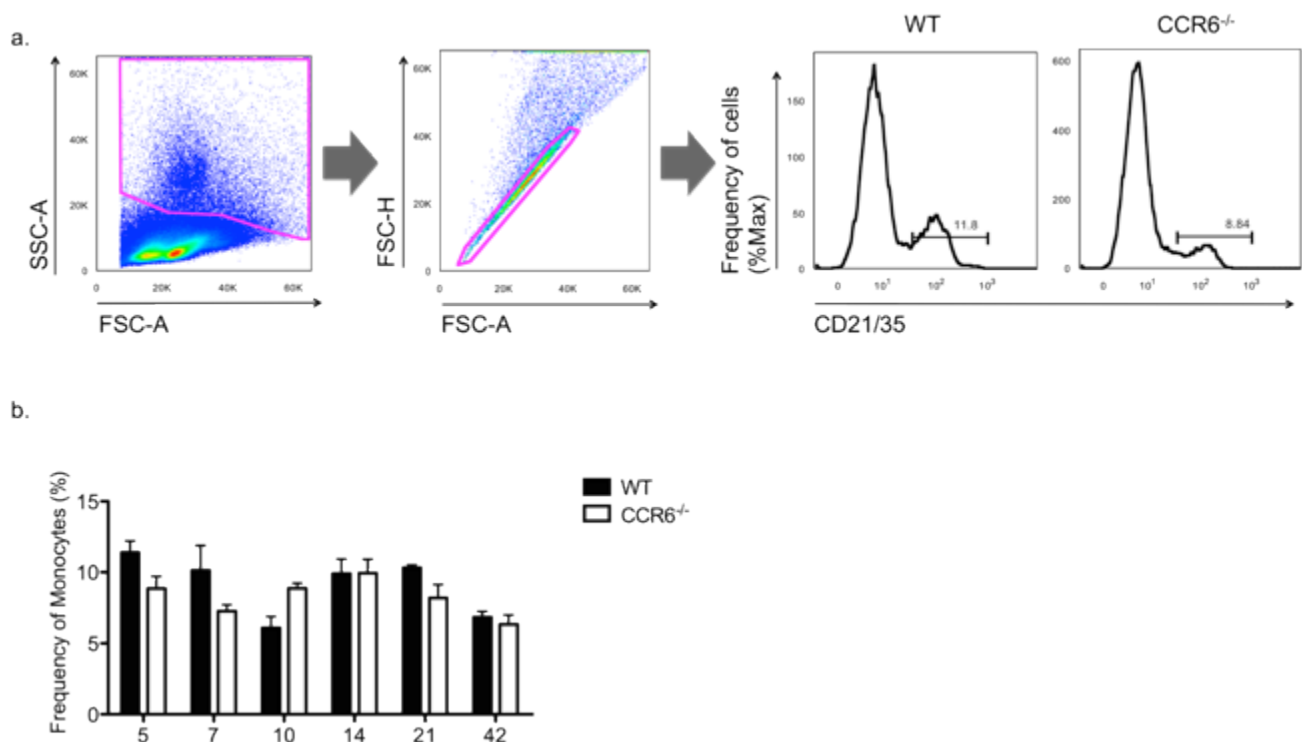


Fig. 5.1.5. The Follicular Dendritic cell kinetics of the CCR6 deficient T cell-dependent humoral response

WT and CCR6^{-/-} mice were i.p. immunised with 100µg of NP-KLH for 42 days. The FDCs were identified by flow cytometry as CD21/35⁺ monocytes (a). The frequency of FDCs was quantified in WT and CCR6^{-/-} mice at 5, 7, 10, 14, 21 and 42 days after challenge (b). Figure represents data collected from 1 experiment (n=4 mice/genotype). Mann-Whitney two-tail t-test

Having found no substantial variation in the quality of the GC response, I examined whether the end product – antibody production – was affected by the absence of CCR6. Thus far, there has been no evidence to suggest alterations in antibody generation in the CCR6^{-/-} mice. Therefore, it was hypothesised that there would be little, if any, variation in antibody production between CCR6^{-/-} and WT mice. Antibody production was quantified in the serum of CCR6^{-/-} and WT mice challenged with NP-KLH for 42 day. IgM and IgG production was analysed by ELISA, as IgM is the BCR and thus the first antibody to be produced in the response, and IgG appears later, after B cells undergo CSR. As expected, IgM production occurred first, between days 7–21 following antigen challenge (Fig. 5.1.6). This was followed by IgG2a^b production between days 7–42, and IgG1^b production between days 14–42 after antigen challenge (Fig. 5.1.6). An equivalent production of IgM was observed between CCR6^{-/-} and WT mice, following antigen challenge (Fig. 5.1.6). Similarly, IgG2a^b and IgG1^b production was similar between CCR6^{-/-} and WT mice, throughout the course of the humoral response (Fig. 5.1.6). Overall, this data suggests that antibody production is not altered in the absence of CCR6.

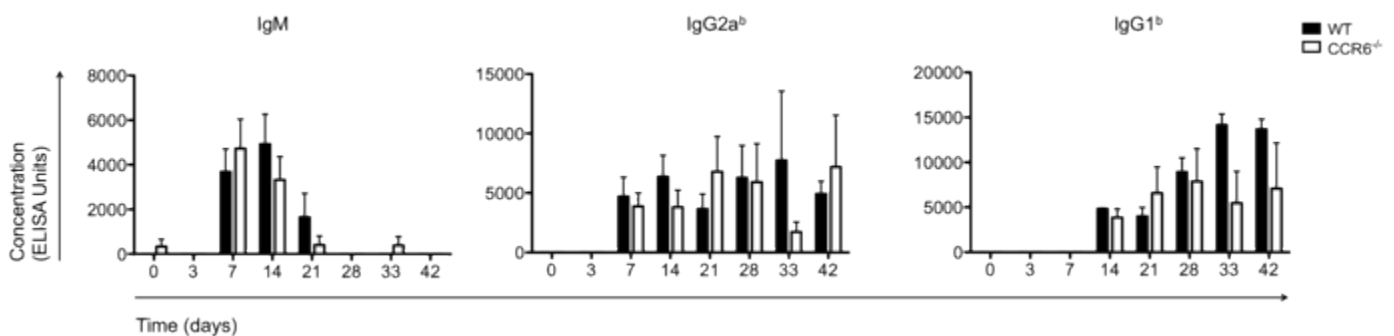


Figure 5.1.6. *T cell-dependent antibody production in the absence of CCR6*

WT and CCR6^{-/-} mice were i.p. immunised with 50–100µg of NP-KLH. Serum was isolated from blood collected from unimmunised mice (day 0) and immunised mice at days 3, 7, 14, 21, 28, 33 and 42 after antigen challenge. The concentrations of NP-specific IgM, IgG2a^b and IgG1^b levels were quantified by ELISA, using a standard curve generated from serum collected from WT mice at day 7 and 21 following immunisation. Figure represents data collected from 1 experiment (IgM, IgG1^b & IgG2a^b Day 0, 7, 14 & 21 n=5 mice/genotype; IgM, IgG1^b & IgG2a^b Day 3, and IgG1^b Day 28 n=5 WT mice, 4 CCR6^{-/-} mice; IgM & IgG2a^b Day 21 n=5 mice/genotype; IgM & IgG2a^b Day 42 n=3 WT mice, 5 CCR6^{-/-} mice; IgM Day 33 n=4 WT mice, 5 CCR6^{-/-} mice; IgG1^b Day 33 n=4 WT mice, 3 CCR6^{-/-} mice; IgG1^b Day 42 n=3 mice/genotype; IgG2a^b Day 33 n=4 mice/genotype).

Mann-Whitney two-tail t-test

To conclusively reconcile the increased frequency of low-affinity antibody forming cells previously documented in the CCR6^{-/-} mice¹⁸², which are likely to be a defect in the affinity selection due to a reduction in the T_{FH} cell and FDC compartment, I wanted to examine the frequency of mutations introduced into CCR6^{-/-} GC B cells during SHM. To achieve this, a sequencing method developed

by Professor Thomas Winkler's laboratory, was implemented. This method allows the quantification of point mutations in the variable region of NP-specific IgM and IgG genes. Specifically, the rate of mutation in IgM and IgG GC B cells from CCR6^{-/-} and WT mice challenged with NP-KLH for 5 days, could be quantified. However, this protocol first had to be optimised for the low-frequency population of GC B cells, as it had been originally designed for use with the NP-Ig specific B cell enriched transgenic B1-8 mouse strain. Briefly, this protocol involved sorting B cells, isolating RNA and synthesising cDNA for subsequent amplification and sequencing. The primers developed by the Winkler laboratory were used to specifically amplify the variable region of NP-specific IgM and IgG genes.

Initially, the primers used to isolate IgM and IgG were tested in the WT splenic B cell population using an annealing temperature gradient. The primers for both IgM and IgG appeared stable over a range of annealing temperatures and consequently, an annealing temperature of 58 °C was selected for the remaining optimisation (Fig. 5.1.7a). Next, the sensitivity of the primers was tested by serially diluting 1.4 µg of WT splenic cDNA. This was necessary, as it would only be possible to isolate 1000 GC B cells from mice, due to the low frequency of this population and the time constraints in cell sorting. This analysis revealed that the sequencing primers for IgM were quite sensitive, with the product being detected in 0.14 ng of splenic cDNA, whilst IgG was less so, as it could only be detected in samples containing 14.7 ng of splenic cDNA (Fig. 5.1.7b). Subsequently, the IgM and IgG primers were tested on 1000 splenic B cells that were isolated using anti-CD43 MicroBeads. Sample preparation of 1000 B cells was tested in two ways. RNA was either isolated using the NP-40 lysis buffer or the iScript™ RT-qPCR sample preparation reagent, prior to cDNA synthesis. The primers were also tested on samples containing RNA isolated from 10 000 splenic B cells using the iScript™ RT-qPCR sample preparation reagent. Whilst the PCR was successful, as indicated by the 18S product for all three samples, only IgM could be detected in samples containing 1000 cells (Fig. 5.1.7c). Thus, I proceeded to prepare GC samples for IgM sequencing only.

After immunising 5x CCR6^{-/-} and 5x WT mice with NP-KLH for five days, 1000 GC B cells were isolated using the MoFlo® Astrios™ cell sorter. Samples were prepared by isolating RNA using the iScript™ RT-qPCR sample preparation reagent, synthesising cDNA and amplifying the NP-specific IgM variable sequences. A representative image of WT and CCR6^{-/-} GC B cell samples prepared for sequencing is displayed in Fig. 5.1.7d. All products were then purified and precipitated, before being sent to Germany for sequencing by Dr. Andreas Hiergeist. Unfortunately, the samples could not be analysed as the reverse primer was not compatible with the sequencing machine.

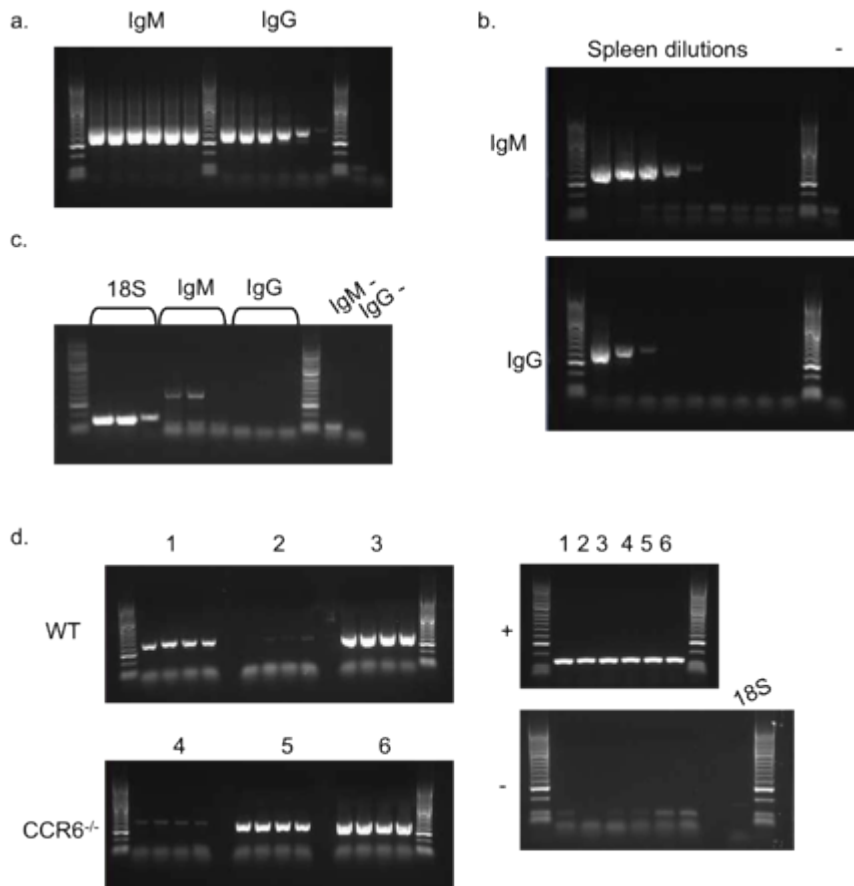


Figure 5.1.7. Preparation of antigen-specific Germinal Centre B cells for Immunoglobulin Sequencing

Naïve splenic cDNA was used to test IgM and IgG primers at annealing temperatures of 52 °C, 55 °C, 58 °C, 60 °C, 62 °C and 65 °C (left to right) (a). 1.4µg of splenic cDNA was serially diluted 8 times at a ratio of 1:10 to test the sensitivity of the IgM (top panel) and IgG (bottom panel) primers (b). The first well contains undiluted cDNA and the last well located after ladder is a no template control. The IgM and IgG primers were then tested on 3 small populations of splenic B cells: 2 wells of 1000 naïve B cells in which RNA was extracted using firstly an in-house NP-40 lysis buffer or secondly the iScriptTM RT-qPCR sample preparation reagent, followed by 1 well of 10 000 naïve B cells in which RNA was extracted using the iScriptTM RT-qPCR sample preparation reagent (left to right) (c). 18S positive controls and no template controls are indicated. WT and CCR6^{-/-} mice were i.p. immunised with 100µg of NP-KLH for 5 days. 1000 GC B cells were isolated by their λ⁺ CD38⁻ GL7⁺ expression, using the MoFlo[®] AstriosTM cell sorter. RNA was isolated using the iScriptTM RT-qPCR sample preparation reagent and cDNA synthesised. NP-specific IgM cDNA was amplified. Representative images of IgM in GC B cell samples from three WT (1–3) and three CCR6^{-/-} (4–6) mice are shown (d). The 18S positive controls (top panel), as well as IgM and 18S no template controls for each sample (bottom panel) are displayed (right panel).

In an effort to resolve this, new IgM and IgG reverse primers were designed and tested as before. Again, the primers for IgM and IgG appeared stable over various annealing temperatures and concentrations (Fig. 5.1.8a). However, both the IgM and IgG primers were even less sensitive than the previous set and as a result, IgM and IgG could not be detected in B cell samples containing 1000 cells (Fig. 5.1.8b). A final avenue to solve this issue would be to design and test nested

primers for IgM and IgG. However, considering the sensitivity of the primers, it would be more appropriate to use the B1-8 mouse model, which is enriched for NP-specific B cells, and subsequently backcross it with the CCR6^{-/-} mice. In this way, antigen-specific enriched WT and CCR6^{-/-} GC B cells could be examined.

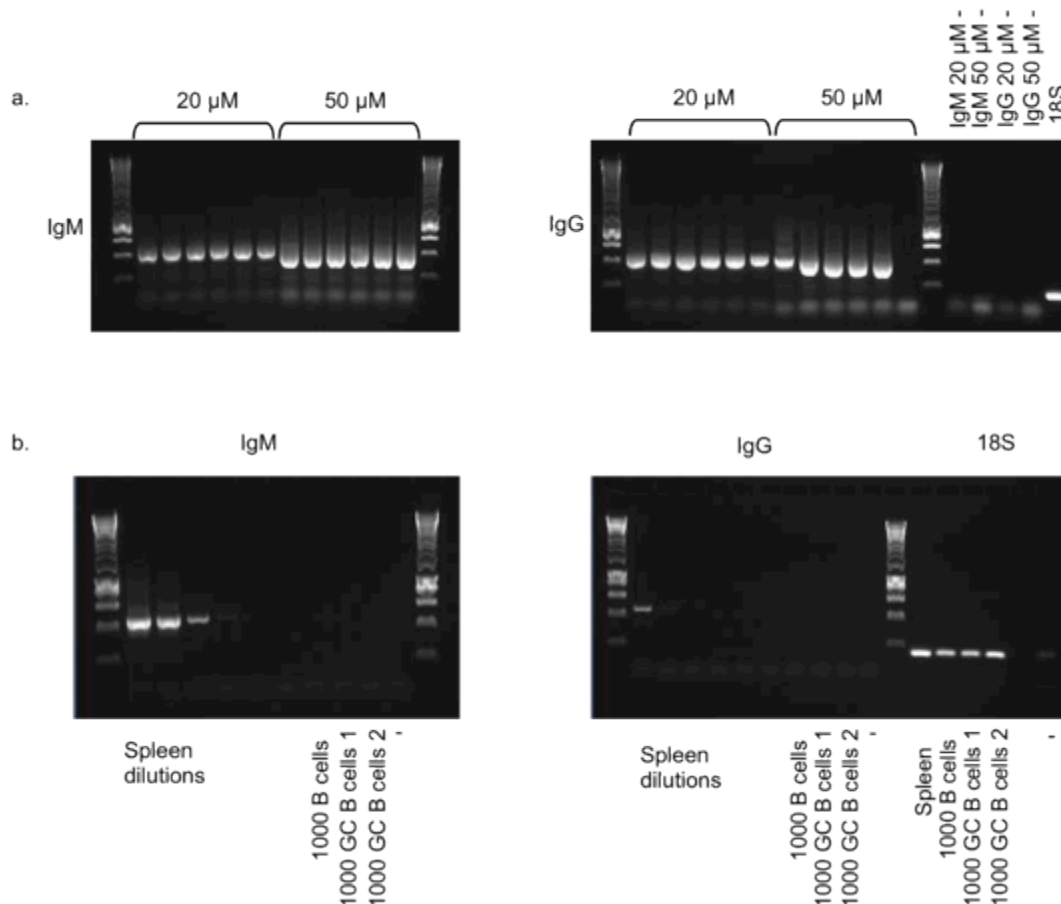


Figure 5.1.8. Optimisation of the sample preparation protocol for antigen-specific Immunoglobulin Sequencing

Naïve splenic cDNA was used to test the new IgM (left panel) and IgG (right panel) reverse primers, with the original forward primers, at annealing temperatures of 53 °C, 55 °C, 58 °C, 60 °C, 62 °C and 65 °C (left to right), at two difference concentrations; 20 μM and 50 μM (a). The no template controls and the positive 18S control are indicated. 1.4μg of splenic cDNA was then serially diluted 5 times at a ratio of 1:10 to test the sensitivity of the IgM (left panel) and IgG (right panel) primers, beginning with undiluted cDNA (b). Following the five samples of diluted cDNA, 3 samples of 1000 B cells were tested: 1000 naïve splenic B cells, and 2 samples of 1000 GC B cells (λ⁺ CD38⁻ GL7⁺) that were isolated from WT mice immunised for 5 days with 100μg of NP-KLH, using the MoFlo® Astrios™ cell sorter. The 18S positive controls for the naïve splenic B cells and the 2 samples of GC B cells are shown in right panel. The no template controls for IgM (left panel), IgG and 18S (right panel) are indicated.

5.2. The effects of CCR6 are not intrinsic to B cells

After analysing the quality of the GC reaction in the CCR6^{-/-} mice, I next examined how B cell differentiation becomes dysregulated in these mice, with an emphasis on the contribution of chemokine receptors. Whilst I have established that Bcl-6 expression is increased in CCR6^{-/-} GC B cells and is likely a result of increased T_{FH} cell-secreted IL-21, the direct cause for these alterations has yet to be determined. It is currently unclear whether the intrinsic loss of CCR6 on B cells alone is responsible for the dysregulated B cell response, or if in fact the loss of CCR6 on other leukocytes, contributes to this dysregulation. It is also unknown as to whether other chemokine receptors responsible for B cell positioning, also influence the B cell dysregulation in the CCR6^{-/-} mice.

In order to determine whether CCR6 regulates the humoral immune response exclusively via B cells, an adoptive transfer system was employed. Mixtures of naïve CCR6^{-/-} and WT splenocytes were transferred into recipient Rag mice, which lack mature B and T cell subsets. These mice were then immunised with NP-KLH. In this way, I created the precise environment that B cells underwent differentiation in, in response to antigen challenge. For instance, naïve CCR6^{-/-} B cells were transferred in combination with WT splenocytes. Thus CCR6^{-/-} B cells differentiated in a WT environment. Also, naïve WT B cells were mixed with CCR6^{-/-} splenocytes, such that WT B cells differentiated in a CCR6^{-/-} environment. It is important to note that the WT and CCR6^{-/-} B cells were always at an equivalent ratio in each recipient mouse, such that the CCR6^{-/-} and WT B cells were in direct competition with each other. For controls, recipient mice received either WT or CCR6^{-/-} splenocytes, consisting of the same total number of donor naïve B cells present in the mixtures. The splenic GC response was then analysed five days after immunisation by flow cytometry, using antibodies against CD38 and GL7. Representative images of the GC response in the reconstituted Rag mice are displayed in Fig. 5.2.1a. A significant increase in the frequency of GC B cells was detected in the recipient mice that received a mixture of WT B cells and CCR6^{-/-} splenocytes, compared to the recipient mice that received WT splenocytes (Fig. 5.2.1b). This differs to the recipient mice that received CCR6^{-/-} B cells and WT splenocytes, which generated a similar frequency of GC B cells as the recipient mice that received WT splenocytes (Fig. 5.2.1b). This result indicates that CCR6 activity is not intrinsic to B cells and in fact other cells within the spleen, such as T cells and dendritic cells, are contributing to the excessive early GC response documented in the CCR6^{-/-} mice in Chapter 4. This finding is consistent with the CCR6 expression documented in Th cells during T-B cell interactions in Chapter 3, and the altered T_{FH} cell and FDC compartments reported earlier in this chapter.

In addition to GC formation, the contribution of WT and CCR6^{-/-} B cells to the GC response was also assessed. This was accomplished by making use of the congenic markers that distinguish the WT and CCR6^{-/-} mice. For this study, the WT mice used were Ly5.1 mice, thus their splenocytes can be detected using an anti-CD45.1 antibody, whereas the CCR6^{-/-} cells can be identified using an anti-CD45.2 antibody. However, the CD45.2 antibody will also detect Rag cells, as they too express CD45.2. The expression of CD45.1 and CD45.2 on GC B cells was quantified by flow cytometry. As expected, CD45.1⁺ B cells predominated in the GC response when recipient mice received only WT cells (Fig. 5.2.1c, d). A comparably lower frequency of CD45.2⁺ B cells was also detected in these mice, however they were not transferred from CCR6^{-/-} mice, and therefore reflect the recipients' population (Fig. 5.2.1c). It is possible that these recipient cells do respond to antigen challenge and undergo differentiation into GC B cells, as they too have been provided with a donor environment. Similarly, CD45.2⁺ CCR6^{-/-} B cells predominated in the GC response when recipient mice received only CCR6^{-/-} cells (Fig. 5.2.1c, d). Upon transferring mixtures of WT and CCR6^{-/-} cells into recipient mice, CD45.2⁺ CCR6^{-/-} B cells appeared to outcompete their CD45.1⁺ WT counterparts in both mixtures, forming the majority of GC B cells (Fig. 5.2.1c). On closer inspection however, there was no difference in the contribution of WT and CCR6^{-/-} B cells, as approximately 20% of these CD45.2⁺ cells are likely recipient B cells rather than CCR6^{-/-} B cells (Fig. 5.2.1c, d). Thus, CCR6^{-/-} B cells are not preferentially recruited into the GC response and contribute equally with WT B cells. This demonstrates that the increased GC response observed in the CCR6^{-/-} mice is most likely due to the loss of CCR6 on leukocytes other than B cells.

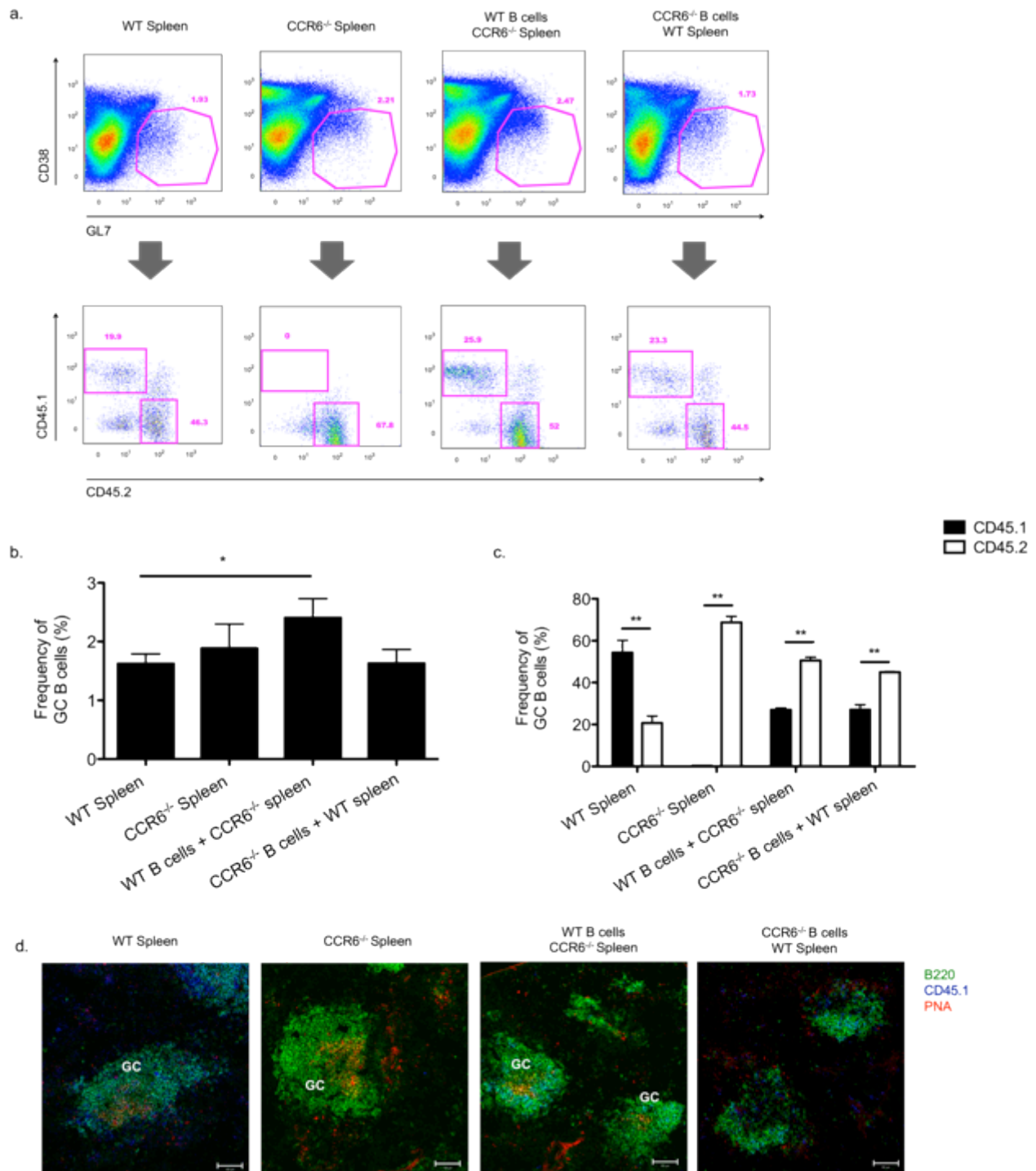


Figure 5.2.1. The contribution of CCR6 deficient leukocytes to Germinal Centre formation in the T cell-dependent humoral response

Splenic CD43⁻ naïve B cells were isolated from Ly5.1 (WT) and CCR6^{-/-} splenocytes using the AutoMACS Pro Separator. WT B cells were combined with CCR6^{-/-} splenocytes, such that each donor contributed 50% of the total number (2×10^7) of donor B cells, and were subsequently transferred into naïve recipient Rag mice. Similarly, CCR6^{-/-} B cells and WT splenocytes were transferred into Rag mice. As controls, recipient Rag mice received either WT or CCR6^{-/-} splenocytes containing a total of 2×10^7 donor B cells. All recipient mice were i.p. immunised with 50µg of NP-KLH 3 days after cell transfer. The CD38⁺ GL7⁺ GC response was analysed by flow cytometry 5 days later. Representative flow cytometry images of the GC B cell response in Rag mice are displayed (a). The frequency of GC B cells (b) and the contribution of CD45.1⁺ WT and CD45.2⁺ CCR6^{-/-} cells (c) were quantified. Spleen sections of Rag mice were stained with antibodies against B220, CD45.1 and PNA to visualise the GC response. Representative images of GC follicles are displayed (d). Data was collated from 2 independent experiments (n=6 recipient mice/transfer group, except for CCR6^{-/-} B cells + WT spleen where n=5).

Mann-Whitney two-tail t-test, *significant at $p < 0.05$; **significant at $p < 0.01$

5.3. CCR6 deficiency results in an altered chemokine receptor expression

I next investigated whether the absence of CCR6 in the CCR6^{-/-} mice affected the expression level of other chemokine receptors, perhaps influencing B cell differentiation in these mice. To determine this, I immunised WT and CCR6^{-/-} mice with NP-KLH for seven days. The expression of CXCR5, CCR7, CXCR4 and CCR6 was quantified on λ^+ B cells using flow cytometry and representative images are displayed in Fig. 5.3.1a. Initially chemokine receptor expression was quantified on all λ^+ B cells. As expected, the CCR6^{-/-} mice were negative for CCR6 (Fig. 5.3.1b). The expression of CXCR5 and CCR7 on all λ^+ B cells from CCR6^{-/-} mice was consistent with that of WT mice (Fig. 5.3.1b). However, CXCR4 expression was significantly increased on CCR6^{-/-} λ^+ B cells compared to WT λ^+ B cells (Fig. 5.3.1b). Next, precursor and GC B cells were examined for chemokine receptor expression, as alterations in chemokine receptor expression on these cells may contribute to the increased GC response observed in the CCR6^{-/-} mice in Chapter 4. A significant increase in CCR7 expression was detected on CCR6^{-/-} precursor B cells compared to WT precursor B cells (Fig. 5.3.1c). CCR7 is required for localisation to the T-B border and thus may facilitate rapid T-B cell conjugate movement and precursor localisation in the CCR6^{-/-} mice. In theory, this may facilitate the excessive early expansion of the GC response in the absence of CCR6. In terms of CXCR5 and CXCR4 expression, similar levels were detected on precursor B cells between CCR6^{-/-} and WT mice (Fig. 5.3.1c). There was also no significant variation in CXCR5, CCR7 or CXCR4 expression on GC B cells in CCR6^{-/-} mice compared to WT mice (Fig. 5.3.1d). As expected, the GC B cell population did not express CCR6 (Fig. 5.3.1d). Overall, CCR7 may play an important role in T-B cell interactions and B cell differentiation during the TD humoral response in the absence of CCR6.

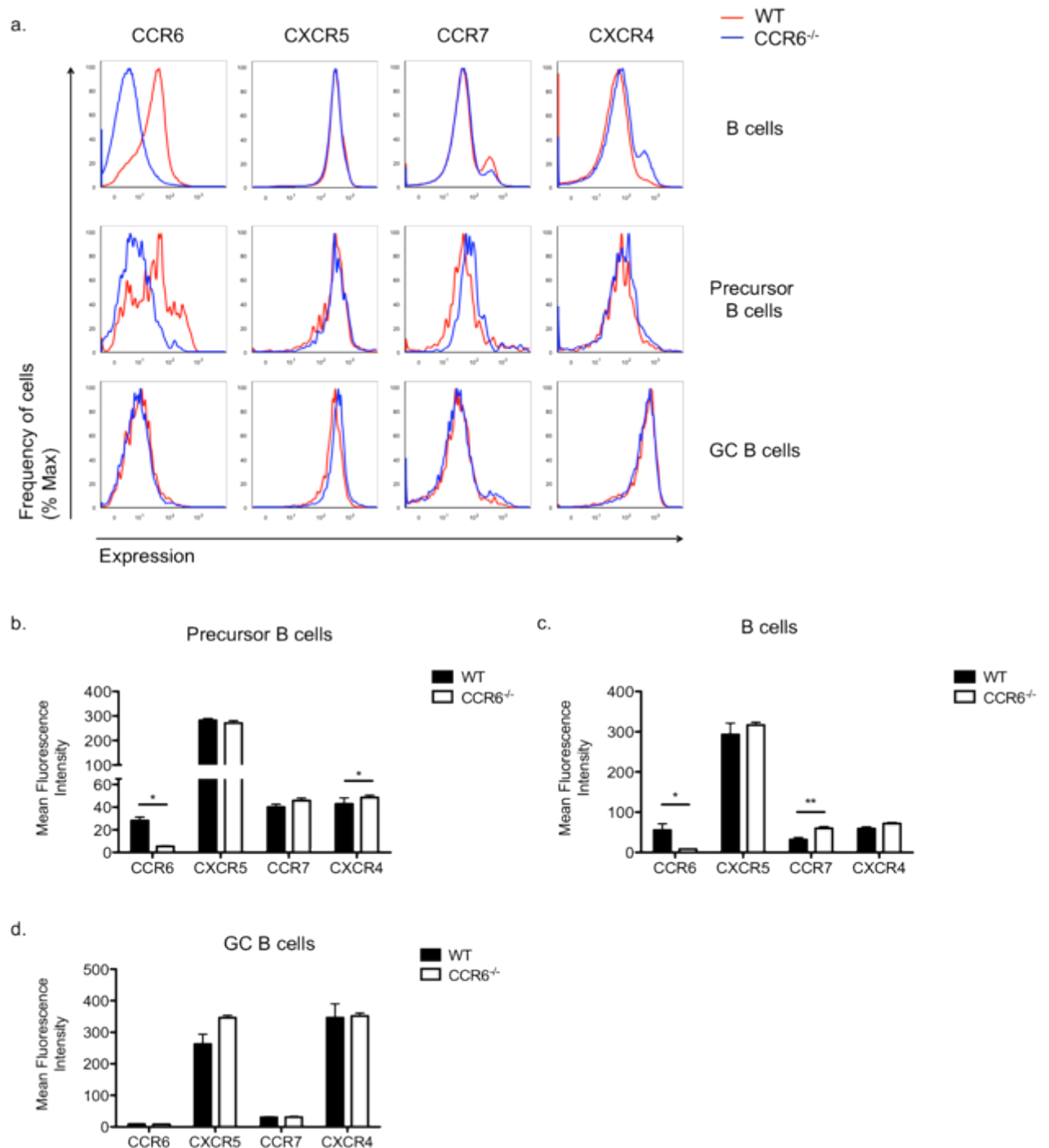


Figure 5.3.1. The chemokine receptor expression profile of CCR6 deficient B cells in the T cell-dependent humoral response

WT and CCR6^{-/-} mice were i.p. immunised with 100µg of NP-KLH for 7 days. The expression of CCR6, CCR7, CXCR5 and CXCR4 was analysed by flow cytometry on all λ^+ B cells, λ^+ CD38⁺ GL7⁺ precursor B cells and λ^+ CD38⁻ GL7⁺ GC B cells, and representative images are shown (a). The mean fluorescent intensities of each chemokine receptor was quantified on all λ^+ B cells (b), precursor B cells (c) and GC B cells (d). Data was collected from 2 independent experiments (n=8 mice/genotype, except for CCR6 & CXCR5 stains where n=4 mice/genotype). Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

To determine whether other chemokine receptors have a similar effect to CCR6 on TD B cell differentiation, chemokine receptor deficient mouse strains were immunised with NP-KLH for

seven days. B cell differentiation was quantified in CXCR5^{-/-}, CCR7^{-/-}, CCR6^{-/-} and WT mice by flow cytometry, using antibodies against CD38 and GL7. Representative images of B cell subsets in chemokine receptor deficient mouse strains are shown in Fig. 5.3.2a. As CXCR5, and CCR7 are known to play a major role during B cell differentiation and GC formation^{52,156,281}, their absence was thought to result in a reduction of this population and indeed, this was the case. A significant decrease in the GC B cell population was detected in CXCR5^{-/-} mice as expected, but also in the CCR7^{-/-} mice (Fig. 5.3.2b). This finding was surprising but not altogether unexpected, considering the vital role that CCR7 plays in TD humoral responses. Furthermore, the reduction in GC B cells observed in CCR7^{-/-} mice correlated with a significant decrease in the total number of λ^+ B cells (Fig. 5.3.2c). Consistent with the findings in Chapter 4, there was not a higher frequency of GC B cells in the CCR6^{-/-} mice compared to the WT mice, seven days after antigen challenge (Fig. 5.3.2b). Also, the frequency of all naïve and precursor B cells between CXCR5^{-/-}, CCR7^{-/-}, CCR6^{-/-} and WT mice were similar (Fig. 5.3.2d, e). This was also true for the frequency of plasma cells and memory B cells between chemokine receptor deficient mouse strains and WT mice (Fig. 5.3.2f, g). Overall, this analysis shows that alterations in GC formation do occur in other chemokine receptor deficient strains of mice, indicating just how crucial chemokine receptors are to the regulation of the humoral response.

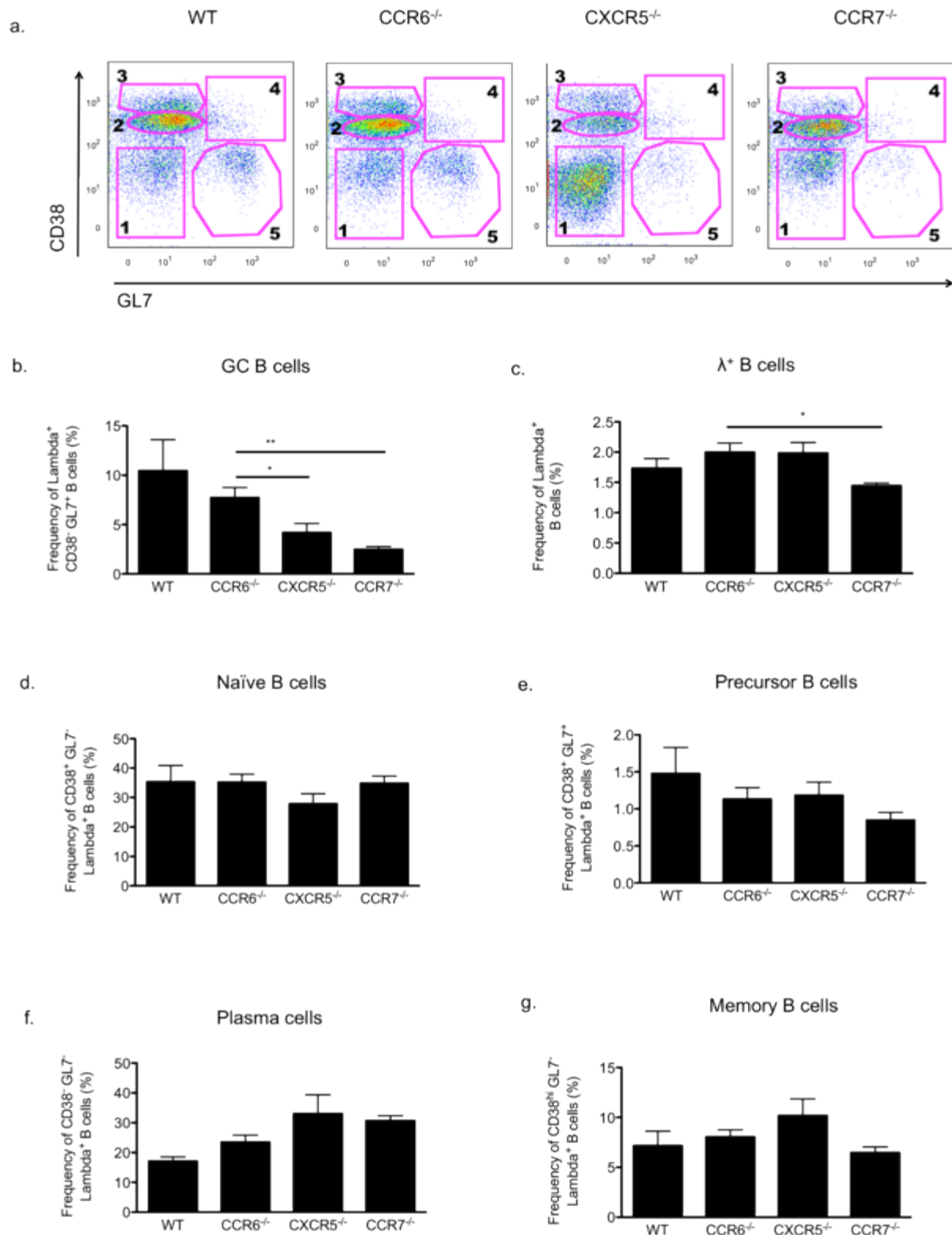


Figure 5.3.2. The influence of CXCR5 and CCR7 on T cell-dependent Germinal Centre B cell formation

The GC B cell response was analysed by flow cytometry in WT, CCR6^{-/-}, CCR7^{-/-} and CXCR5^{-/-} mice, 7 days after i.p. immunisation with 100µg of NP-KLH. B cell differentiation was examined using antibodies against CD38 and GL7: 1) CD38⁻ GL7⁻ plasma cells, 2) CD38⁺ GL7⁻ naïve B cells, 3) CD38^{hi} GL7⁻ memory B cells, 4) CD38⁺ GL7⁺ precursor B cells and 5) CD38⁻ GL7⁺ GC B cells (a). The frequency of GC B cells (b), all λ⁺ B cells (c), naïve B cells (d), precursor B cells (e), plasma cells (f), and memory B cells (g) were quantified. Data was collected from 2 independent experiments (n=8 mice/genotype).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

5.4. Concluding Remarks

This chapter has provided several insights into the mechanisms responsible for the B cell dysregulation observed in the absence of CCR6. Specifically, this chapter has examined the structural, molecular and cellular mechanisms underlying the GC response in CCR6^{-/-} mice.

As part of this investigation, antibody production was analysed by ELISA. While this analysis showed that antibody production was not affected in the CCR6^{-/-} mice, it was not possible to discriminate between low-affinity and high-affinity antibodies. This information would provide a valuable addition to the assessment of the quality of the response. It has already been shown that more low-affinity antibody forming cells are produced in CCR6^{-/-} mice compared to WT mice, seven days after antigen challenge¹⁸², though, it remains to be determined whether this translates into the production of more low-affinity antibodies. Furthermore, it will be interesting to determine not only the phenotype of these antibody forming cells, that is whether they are memory or plasma cells, but also whether these cells are derived from the GC or EF. A complete time course of the frequency of antibody forming cells is also necessary, particularly in the early stage of the humoral response, so that it can be correlated with the results reported here.

Taking into account the reduced EF response observed in the CCR6^{-/-} mice, and the fact that MZ B cell differentiation remains largely intact in these mice, it is likely that the increased low-affinity antibody response documented in the literature is derived from the GC. In an attempt to examine the quality of GC-derived antibodies or at least the frequency of mutations introduced through SHM, I set out to examine mutations in the variable region of IgM and IgG in GC B cells from CCR6^{-/-} and WT mice, using a sequencing technique developed by Professor Thomas Winkler's laboratory. Unfortunately, this was not successful due to the low-frequency of GC B cells. This approach will require a larger population of NP-specific B cells, which can be obtained when using the B1-8 mouse model. Overall, this information will provide a greater understanding as to how CCR6 ultimately affects the antibody response.

Despite examining the mechanisms of the GC response in this chapter, it is imperative that future work examines the quality of the extrafollicular response as well, so that the impairment of this population in CCR6^{-/-} mice can be adequately explained. Though, it is clear that this will be challenging until our understanding of the molecular mechanisms underlying the extrafollicular reaction is enhanced.

To determine whether the dysregulated B cell response observed in CCR6^{-/-} mice was due solely to the loss of CCR6 on B cells, or the collective loss of CCR6 on all leukocytes, an adoptive transfer model was implemented. In this model, recipient mice, which lacked mature B and T cells, were reconstituted with mixtures of CCR6^{-/-} and WT splenocytes, and challenged with NP-KLH. This investigation demonstrated that the B cell dysregulation observed in the CCR6^{-/-} mice was not intrinsic to B cells, but instead due to the loss of CCR6 on multiple leukocytes. This work indicates that CCR6 may act more globally, by directing the migration of leukocytes to specific niches in secondary lymphoid organs during the course of the humoral response. For instance, CCR6 may direct the migration of both B and T cells to the T-B border, or may also play a role in retaining B cells, T cells and dendritic cells in the GC. Thus, future work should determine precisely what other leukocytes express CCR6 and where this expression is required.

It would also be interesting to determine whether CXCR5, CCR7, CXCR4 and EBI2, chemokine receptors known to direct lymphocyte movement in the humoral response, are capable of re-establishing the humoral response in the CCR6^{-/-} mice. Such a mechanism would explain the transient nature of B cell dysregulation in the CCR6^{-/-} mice. While an increase in CCR7 expression was detected on precursor B cells deficient in CCR6, potentially enabling enhanced migration to the T-B border and as a downstream effect, selection into the GC differentiation pathway, substantial variations in chemokine receptor expression would potentially facilitate excessive GC formation. The most appropriate method to examine this would be to generate mice deficient for multiple chemokine receptors, or create further adoptive transfer chimeras using chemokine receptor deficient mice.

An unexpected finding arising from this study was the severely impaired GC B cell response observed in the CCR7^{-/-} mice. While CCR7 is involved in B cell migration during activation, particularly during the localisation of B cells to the T-B border to form conjugates with Th cells^{52,158}, a subsequent role for CCR7 in GC formation has not previously been established. Follow up studies on this work could lead to interesting insights regarding the combined expression of chemokine receptors in GC formation.

In summary, the data presented within this chapter start to uncover the mechanisms underlying CCR6 deficient B cell differentiation in the humoral response. Specifically, I have demonstrated that the increased early GC response observed in the CCR6^{-/-} mice can be attributed to the increased expression of Bcl-6, which is due to the increased production of T_{FH} cell-derived IL-21. In addition, the transiently increased GC response does not appear to affect antibody production. However,

affinity selection of GC-derived B cells appears to be impaired, as a result of a reduction in both the T_{FH} cell and FDC compartments in the GCs of CCR6^{-/-} mice. Finally, the dysregulation of B cell differentiation observed in the absence of CCR6, is not solely due to the loss of CCR6 expression on B cells, but is a result of the loss of CCR6 expression on multiple leukocytes.

Chapter 6

The expression profile of CCR6 in Systemic Lupus Erythematosus



In the past three chapters, I have established that CCR6 is upregulated on B cells upon activation during the humoral response and that CCR6 expression contributes to the efficient differentiation of GC and EF B cells. Specifically, I have demonstrated that the loss of CCR6 is associated with excessive GC formation. As such, I wanted to determine whether CCR6 expression was associated with B cell-mediated autoimmune diseases. SLE is one such disease. SLE causes widespread inflammation through the deposit of autoreactive antibodies and it is quite difficult to diagnose and subsequently treat due to its heterogeneity. Consequently, this disease model offered the opportunity to explore the potential for a diagnostic biomarker. This work would also provide a greater understanding of the human lymphocyte subsets, as well as the dysregulation of the immune system in disease.

The aim of this chapter was to correlate the GC and T_{FH} cell subsets in disease with CCR6 expression using a mouse and human model of SLE. The FasL^{gld} or generalised lymphoproliferative disease (*gld*) mouse model develops a spontaneous systemic autoimmune disease characterised by enlarged secondary lymphoid organs and increased proportions of B and T cells. The accumulation of autoreactive lymphocytes is a result of a point mutation in the FasL gene, which encodes a protein that induces apoptosis by binding the Fas receptor. This mouse exhibits similar immunological characteristics observed in SLE and is therefore a good model of autoimmunity. Given the observation that the absence of CCR6 is associated with excessive GC formation, I proposed that in a dysregulated immune response, where excessive GC proliferation occurs, CCR6 expression would be reduced. To establish this, CCR6 expression was quantified on B and T cell populations using flow cytometry. The first part of this chapter presents findings obtained from the *gld* mouse model, while the second half details the findings obtained in the study of SLE.

6.1. CCR6 expression is dysregulated in FasL^{gld} mice

As naïve *gld* mice spontaneously develop systemic autoimmunity, they were examined at 13 weeks of age when disease was evident. For controls, age-matched naïve WT mice were used. Lymphocytes were examined in the spleen, lymph nodes and blood of *gld* and WT mice.

Initially, CCR6 expression was quantified on all B cells in the spleen, lymph node and blood, between *gld* and WT mice. Representative images of CCR6 and B220 expression between *gld* and WT mice are displayed in Fig. 6.1.1a. Similar frequencies of B cells were detected between *gld* and WT mice (Fig. 6.1.1b). However, there were significant reductions in the frequencies of B cells that expressed CCR6, in the spleen, lymph node and blood of *gld* mice compared to WT mice (Fig.

6.1.1.c). Furthermore, of those B cells expressing CCR6, CCR6 expression was increased in *gld* mice, and was significantly so in the lymph node (Fig. 6.1.1d).

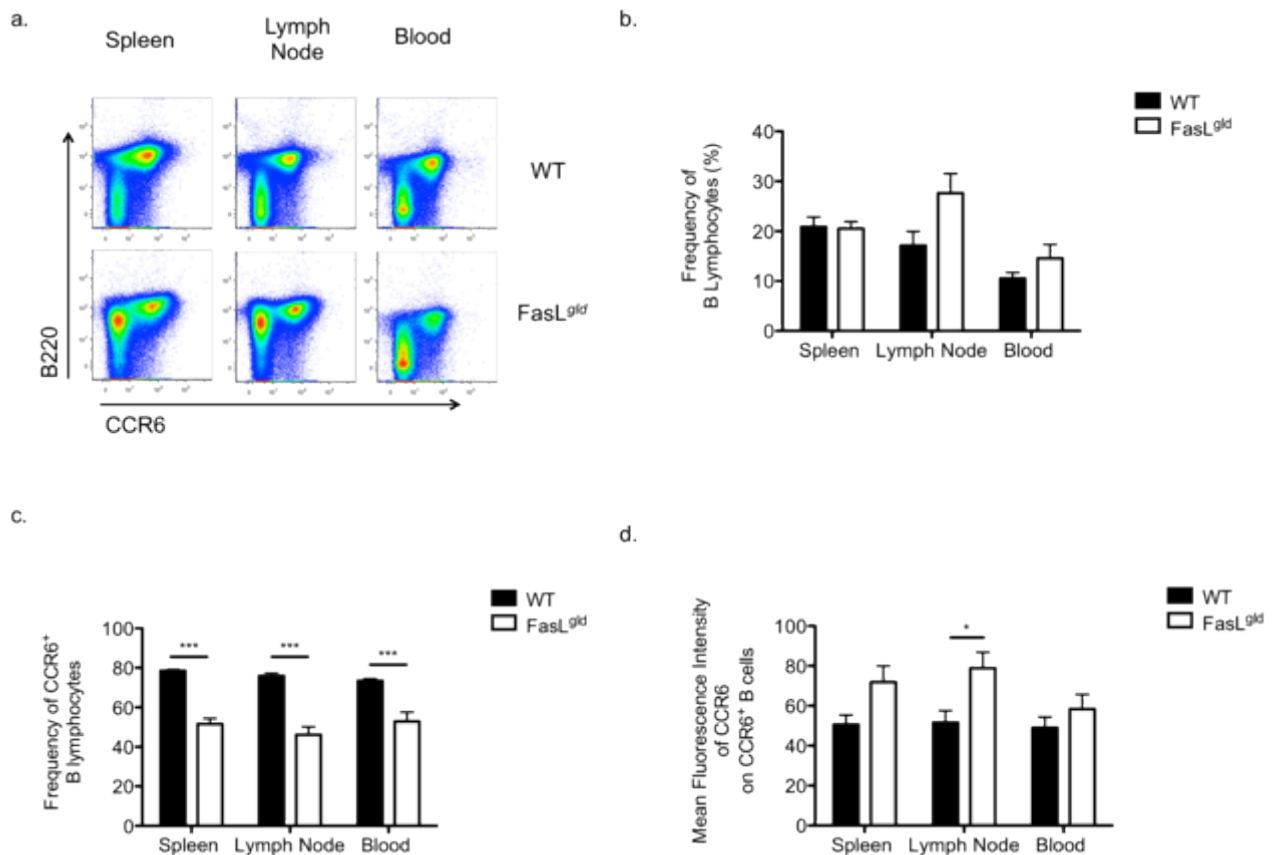


Figure 6.1.1. The CCR6⁺ B cell compartment in murine systemic autoimmunity

The B cell compartment of the spleen, inguinal lymph node and blood, in WT and *gld* mice aged 13-15 weeks, was analysed by flow cytometry. The expression of CCR6 on B cells was analysed and representative images are displayed (a). The frequency of all B cells (b) and CCR6⁺ B cells (c), along with the mean fluorescence intensity of CCR6 expression on the CCR6⁺ B cells (d) was quantified. Figure consists of data obtained from 2 independent experiments (n=8 mice/genotype).

Mann-Whitney two-tail t-test, *significant at p<0.05; ***significant at 0.001

To determine which B cells upregulated CCR6 expression in *gld* mice, B cell subsets delineated by CD38 and GL7 expression were examined in the spleen, lymph node and blood. The gating strategy used is depicted in Fig. 6.1.2a. A significantly higher frequency of GC B cells (CD38⁻ GL7⁺) was detected in the spleen, lymph node and blood of *gld* mice compared to WT mice (Fig. 6.1.2b). This correlated with a significant increase in the frequency of plasma cells (CD38⁻ GL7⁻) in the spleen, lymph node and blood, and precursor B cells (CD38⁺ GL7⁺) in the spleen and lymph node, of *gld* mice compared to WT mice (Fig. 6.1.2b). A significantly reduced frequency of naïve B cells (CD38⁺ GL7⁻) was also detected in the spleen, lymph node and blood of *gld* mice compared to WT mice (Fig. 6.1.2b). In addition, the frequency of memory B cells (CD38^{hi} GL7⁻) was significantly

increased in the lymph node and blood of *gld* mice compared to WT mice (Fig. 6.1.2b). These fluctuations in B cell frequency are consistent with our current understanding of humoral responses, whereby naïve B cells become activated, exit the naïve B cell population and enter the precursor stage, differentiating into GC B cells and subsequently, plasma and memory B cells.

Consistent with the increased CCR6 expression observed on the whole B cell population in *gld* mice, CCR6 expression was also increased on B cell subsets in *gld* mice compared to WT mice. Specifically, CCR6 expression was significantly higher on memory B cells (CD38^{hi} GL7⁻) of the spleen, lymph node and blood in *gld* mice than WT mice (Fig. 6.1.2c). CCR6 expression was also significantly increased on naïve B cells (CD38⁺ GL7⁻) in the lymph node and precursor B cells (CD38⁺ GL7⁺) in the spleen and lymph node, in *gld* mice compared to WT mice (Fig. 6.1.2c). Interestingly, CCR6 expression was significantly reduced in the plasma cells (CD38⁻ GL7⁻) of the lymph node from *gld* mice compared to WT mice (Fig. 6.1.2c). Importantly, the increased CCR6 expression observed in *gld* mice does not reflect an increase in the frequency of these populations. The upregulation of CCR6 on B cells contradicts the initial hypothesis and suggests that CCR6 expression on B cells may contribute to the pathophysiology of systemic autoimmunity.

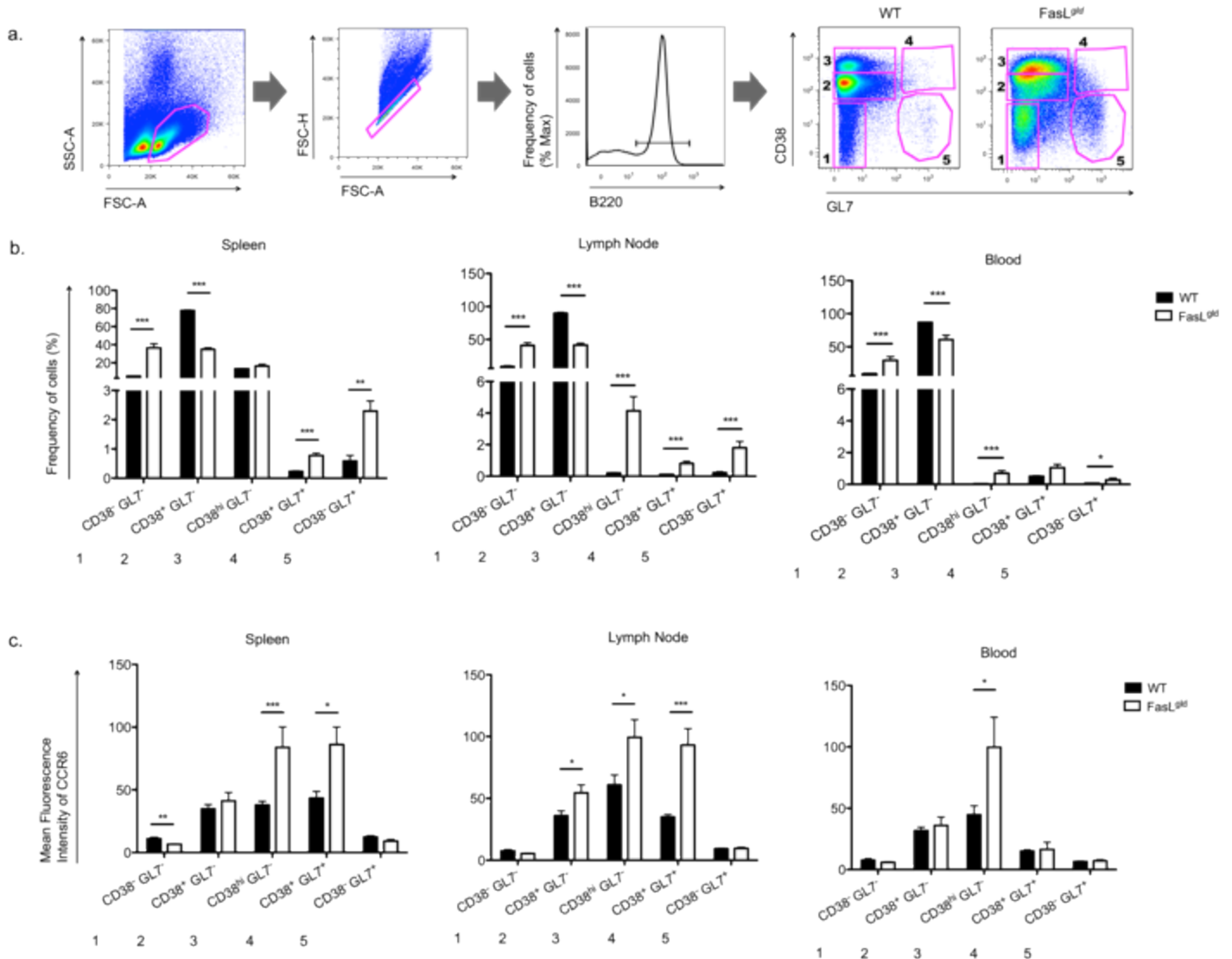


Figure 6.1.2. The characteristics of CCR6 expression during B cell differentiation in murine systemic autoimmunity

CCR6 expression was analysed by flow cytometry in naïve FasL^{gld} and WT mice aged 13-15 weeks. B cell subsets were identified by CD38 and GL7 expression: 1) CD38⁻ GL7⁻ plasma cells, 2) CD38⁺ GL7⁻ naïve B cells, 3) CD38^{hi} GL7⁻ memory B cells, 4) CD38⁺ GL7⁺ precursor B cells and 5) CD38⁻ GL7⁺ GC B cells, and representative images of WT and FasL^{gld} mice are shown (a). The frequency of B cell subsets (b) and mean fluorescence intensity of CCR6 on each B cell subset (c) was quantified in the spleen, inguinal lymph node and blood. Figure represents data obtained from 2 independent experiments (n=8 mice/genotype).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01; ***significant at 0.001

Given the B cell alterations established in the *gld* mice, I next investigated whether T_{FH} cells were also increased during disease. As mentioned previously, murine T_{FH} cells are identified by their high expression of both CXCR5 and PD-1 on CD4⁺ T cells^{274,281,285}. The gating strategy used to

identify T_{FH} cells is displayed in Fig. 6.1.3a. A significantly larger proportion of T_{FH} cells ($CXCR5^{hi}$ $PD-1^{hi}$) was detected in the spleen, lymph node and blood of *gld* mice compared to WT mice (Fig. 6.1.3b). This coincided with a significantly larger proportion of intermediate T_{FH} cells ($CXCR5^{int}$ $PD-1^{int}$) in the *gld* mice compared to the WT mice (Fig. 6.1.3b). In addition, there was a significant reduction in the frequency of non- T_{FH} cells ($CXCR5^{-}$ $PD-1^{-}$) in *gld* mice compared to WT mice (Fig. 6.1.3b). A significant reduction in the frequency of lymph node $CD4^{+}$ T cells was also observed in the *gld* mice compared to WT mice (Fig. 6.1.3b). Therefore, it appears that more non- T_{FH} cells are recruited into T_{FH} cell differentiation, resulting in the large intermediate and genuine T_{FH} cell populations observed in the *gld* mice.

Following the analysis of T cell frequency in *gld* mice, each subset was then examined for CCR6 expression. The expression of CCR6 was significantly upregulated on all $CD4^{+}$ T cells and non- T_{FH} cells from the spleen, lymph node and blood of *gld* mice compared to WT mice (Fig. 6.1.3c, d). However, CCR6 expression was significantly reduced on intermediate T_{FH} cells in the *gld* mice compared to the WT mice (Fig. 6.1.3c, d). Also, there was no significant variation in the levels of CCR6 expression on T_{FH} cells in the spleen, lymph node and blood between *gld* and WT mice (Fig. 6.1.3c, d). Again, alterations in CCR6 expression were not a result of alterations in T cell frequency. Overall, this data provides further evidence that CCR6 contributes to T_{FH} cell differentiation in humoral responses and may contribute to the pathophysiology of systemic autoimmunity.

Consequently, excessive GC and T_{FH} cell differentiation in the $FasL^{gld}$ mouse model is associated with increased CCR6 expression on naïve, memory and precursor B cells, and reduced CCR6 expression on intermediate T_{FH} cells. These results indicate that CCR6 could potentially be used as a biomarker to detect lymphocyte dysregulation, and therefore autoimmune disease.

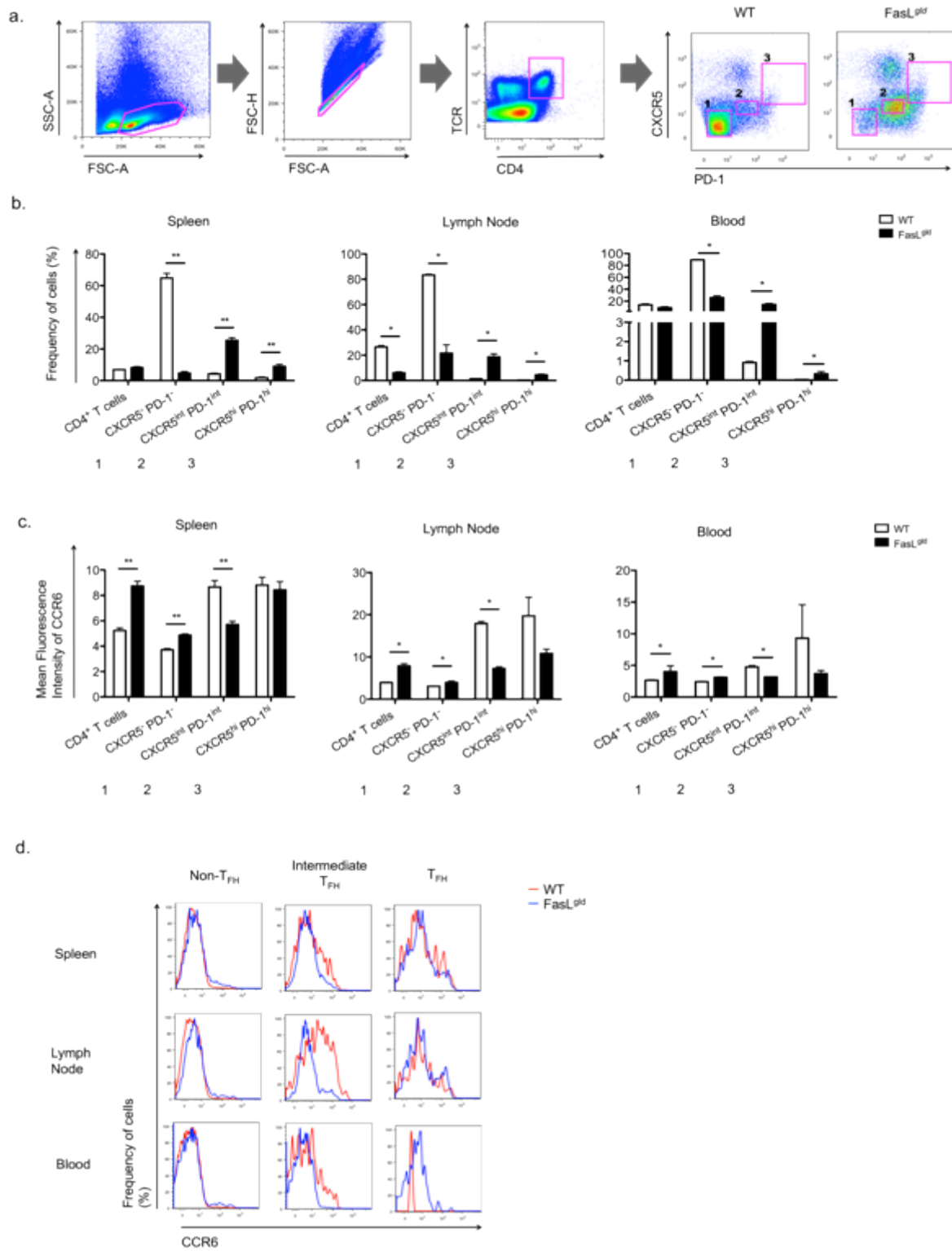


Figure 6.1.3. The expression of CCR6 on T Follicular Helper cells in murine systemic autoimmunity

The T_{FH} cell compartment was analysed by flow cytometry in naïve WT and FasL^{gld} mice, aged 13-15 weeks. Subsets of T_{FH} cells were distinguished by their expression of CXCR5 and PD-1: 1) CXCR5^{int} PD-1^{int} non-T_{FH} cells, 2) CXCR5^{int} PD-1^{int} intermediate T_{FH} cells and 3) CXCR5^{hi} PD-1^{hi} T_{FH} cells, and representative images of WT and FasL^{gld} mice are displayed (a). The frequencies of T cell subsets (b) and mean fluorescence intensity of CCR6 expression on each T cell subset (c) was quantified in the spleen, inguinal lymph node and blood. Representative images of CCR6 expression in T_{FH} cell subsets of WT and *gld* mice are displayed (d). Figure represents data collected from 1 experiment (Spleen n=8 WT mice, 4 FasL^{gld} mice; Lymph node & Blood n= 4mice/genotype).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

6.2. Prominent CCR6 expression identifies SLE

Having gained promising results from the *gld* mouse model, I examined whether CCR6 expression was also altered in humans during SLE. While a previous study has broadly examined the frequency of CCR6⁺ B cells in SLE²²¹, it was limited as the authors did not discriminate between B cell subsets and it had few participants. Therefore, I conducted a small preliminary study in Tasmania, in collaboration with Dr. Murray Adams. Participants were recruited into the study based on whether or not they had a diagnosis of SLE and were between the ages of 18–80 years.

Participants provided a sample of blood, from which leukocytes were isolated for flow cytometry analysis. The frequency and mean fluorescence intensity of CCR6 expression was quantified on B and T cell populations in participants diagnosed with SLE and healthy controls. The CD19⁺ B cell population in humans can be delineated into 6 subsets using antibodies against CD38 and IgD: CD38^{low/int} IgD⁺ naïve B cells, CD38⁺ IgD⁺ Pre-GC B cells, CD38^{low/int} IgD⁺ memory B cells and CD38⁺ IgD⁺ B cells represent both GC and plasma cells²⁸⁶. GC B cells were separated from plasma cells based on their expression of CD27²⁸⁶. The gating strategy employed to identify these subsets is displayed in Fig. 6.2.1a. CCR6 expression was quantified on each of these subsets and representative images are shown in Fig. 6.2.1b. The frequency of all CD19⁺ and CCR6⁺ CD19⁺ cells in SLE participants, appeared slightly elevated, but were not significantly different to control participants (Fig. 6.2.1c). Interestingly, a significant reduction in the frequency of Pre-GC B cells was detected in SLE participants compared to controls (Fig. 6.2.1c). The reduction in Pre-GC B cells during SLE could be attributed to increased GC seeding in secondary lymphoid organs, resulting in fewer circulatory B cells. The GC population also appeared reduced in participants diagnosed with SLE, although this was not significant (Fig. 6.2.1c). There was also no significant alterations in the plasma and memory B cell populations between SLE and control participants (Fig. 6.2.1c).

It is perplexing that pre-GC and GC B cells can be detected in the blood, given that they inherently reside within secondary lymphoid organs. It is possible that these circulatory populations represent distinct populations with migratory capabilities that share similar characteristics to genuine pre-GC and GC B cells. Such populations would allow the expansion of the B cell response to multiple secondary lymphoid organs. To clarify this, analysis of secondary lymphoid organs would provide a better indication of the B cell compartment, however these are less accessible in humans.

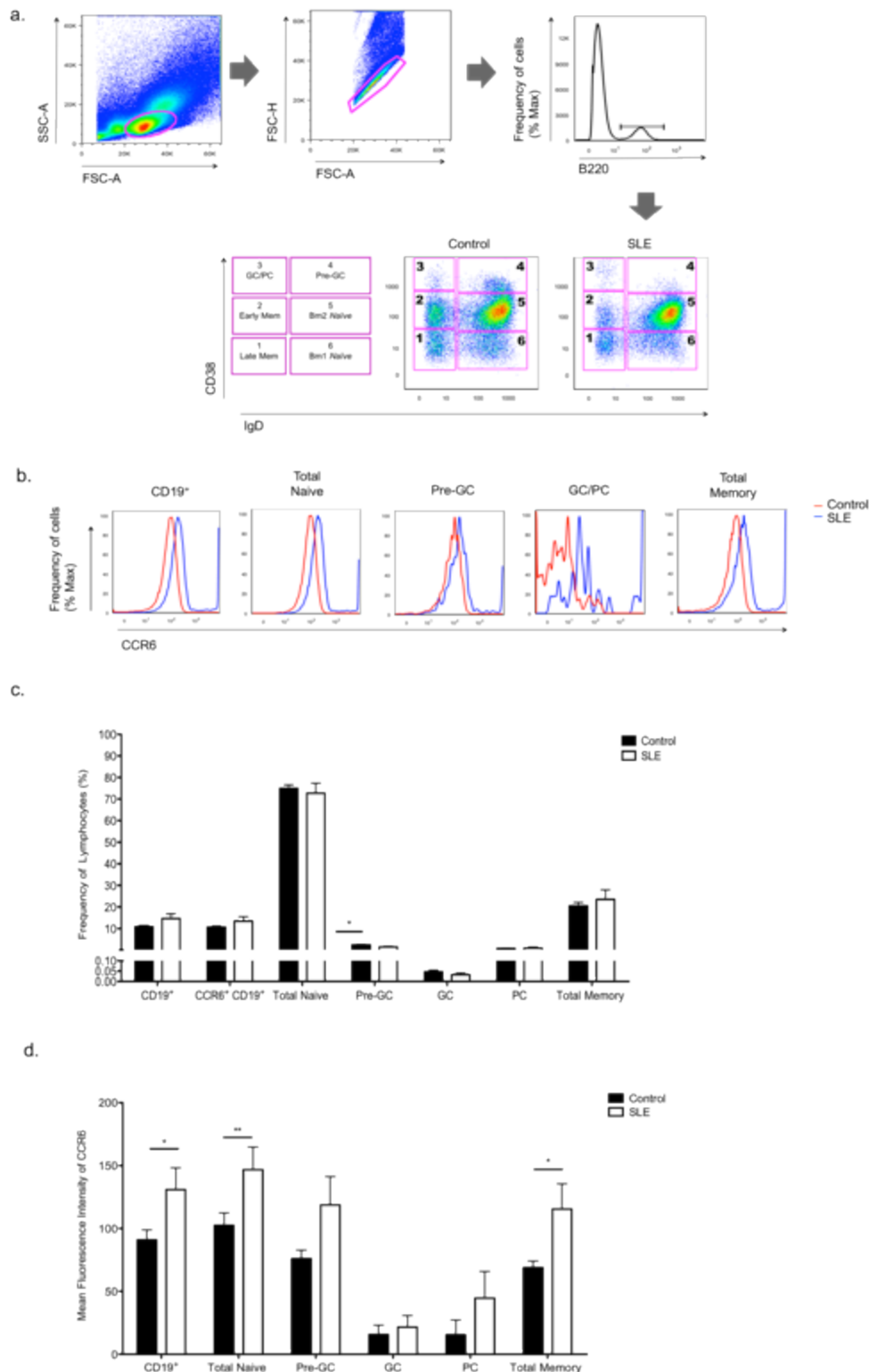


Figure 6.2.1. The expression of CCR6 in the circulating B cell population during Systemic Lupus Erythematosus

Human peripheral CD19⁺ B cell subsets: memory B cells (1&2), GC/plasma cell (PC) (3), Pre-GC cells (4) and memory B cells (5&6), were identified by flow cytometry according to their expression of CD38 and IgD (a). The GC subset was distinguished from PCs based on their expression of CD27. Representative images of CCR6 expression on CD19⁺, naïve, Pre-GC, GC/PC and memory B cells, in SLE and control participants are shown (b). The frequency of all CD19⁺, CCR6⁺ CD19⁺ naïve, Pre-GC, GC, PC and memory B cells were quantified in SLE and control participants (c). The mean fluorescence intensity of CCR6 expression was quantified in each B cell subset between SLE and control participants (d). (n= 19 SLE, 14 Controls).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

Following the quantification of B cell frequency, CCR6 expression was examined. A significant increase in CCR6 expression was detected on all CD19⁺ B cells in SLE participants compared to control participants (Fig. 6.2.1d). When individual B cell subsets were examined, the naïve and memory B cells were found to have a significantly higher expression of CCR6 in SLE participants than control participants (Fig. 6.2.1d). In addition, the Pre-GC, GC and plasma B cells appeared to have a higher expression of CCR6 in SLE participants compared to control participants (Fig. 6.2.1d). The increase in CCR6 expression on these B cells during SLE reflects the overall increase in CCR6 expression observed on the CD19⁺ B cell population. Although contrary to initial expectations, the findings from the mouse and human model of SLE concur with each other and demonstrate that CCR6 expression is increased on naïve and memory circulatory B cells during SLE. This finding shows that CCR6 can be used as a biomarker of disease, due to its pronounced expression during SLE.

Having analysed the B cell compartment in SLE, I next examined the T_{FH} cell population. Human T_{FH} cells are more difficult to identify than mouse, due to the lack of consistency in defining T_{FH} cells in the clinical field. While initial studies defined T_{FH} cells by high CXCR5 expression on CD4⁺ T cells^{119,273}, others have reported on T_{FH} cells as CXCR5⁺ ICOS⁺ CD4⁺ T cells^{287,288}, CXCR5⁺ PD-1⁺ CD4⁺ T cells²⁸⁹, CXCR5⁺ CCR7⁻ CD4⁺ T cells²⁹⁰ and CXCR5⁺ PD-1⁺ CCR7⁻ CD4⁺ T cells^{276,291}. Again, it has been questioned whether these circulatory T_{FH} cells are in fact genuine. For instance, while the circulatory T_{FH} cells represent the T_{FH} cells in secondary lymphoid organs, it has been suggested that they act as memory cells, descendants of T_{FH} cells that are capable of differentiating into fully-fledged T_{FH}^{224,276,292}. Consequently, I chose to define the human circulatory T_{FH} cell population as CXCR5⁺ PD-1⁺ CCR7⁻ CD4⁺ T cells and this gating strategy is presented in Fig. 6.2.2a. This classification allows the accurate detection of circulatory T_{FH} cells and is comparable to the literature. When the T cell population was examined, a significant increase in the frequency of circulating T_{FH} cells was detected in SLE participants compared to control participants (Fig. 6.2.2b). However, the frequency of all lymphocytes, CD4⁺ T cells and CXCR5⁺ CD4⁺ T cells, were similar between SLE and control participants (Fig. 6.2.2b).

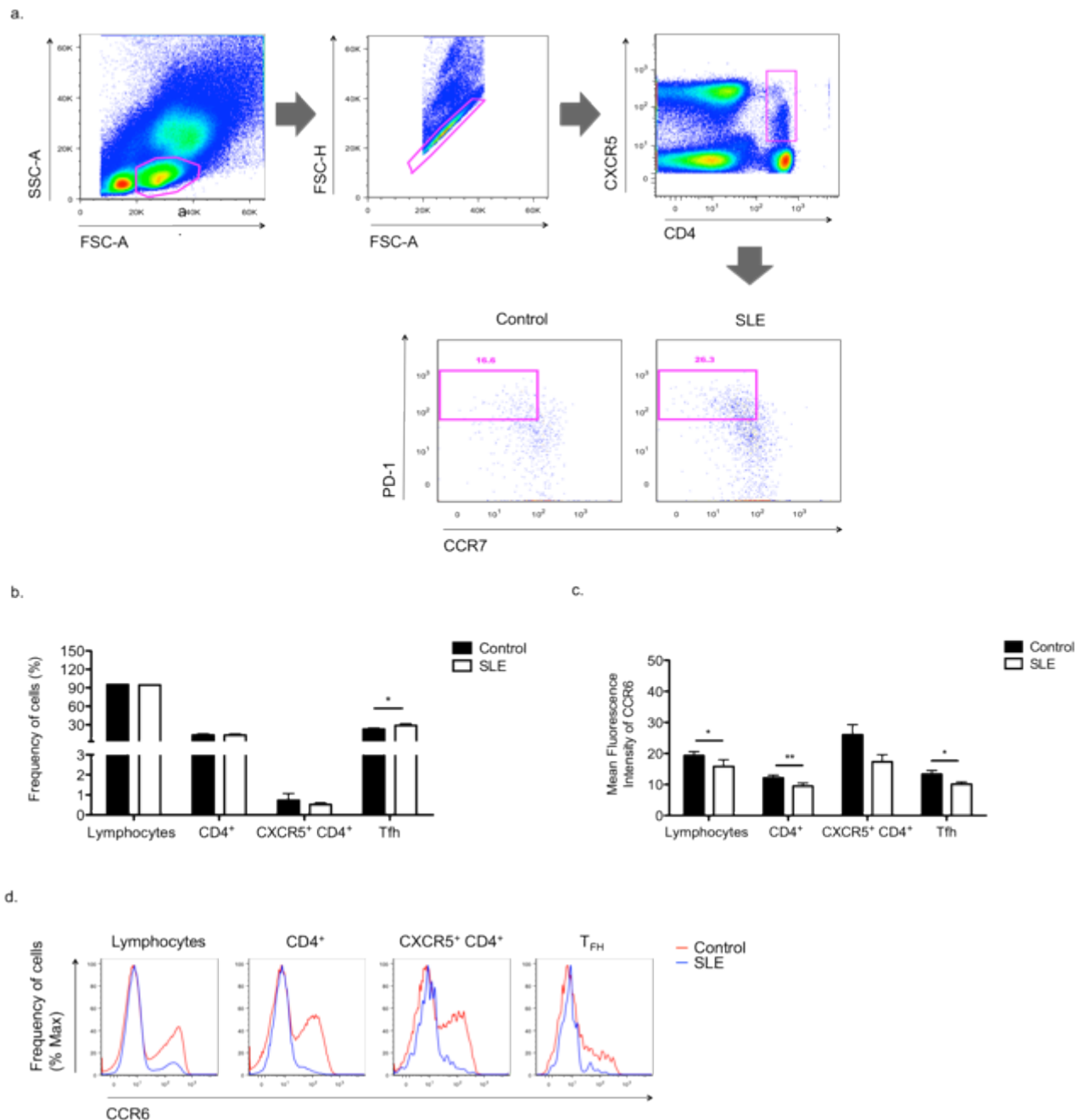


Figure 6.2.2. The expression of CCR6 on the circulating T Follicular Helper cell population in Systemic Lupus Erythematosus

Human peripheral T_{FH} cells were identified by flow cytometry as CXCR5⁺ CD4⁺ PD-1⁺ CCR7⁻ cells and representative images of SLE and control participants are shown (a). The frequency of all lymphocytes, CD4⁺ cells, CXCR5⁺ CD4⁺ cells and T_{FH} cells were quantified in SLE and control participants (b). The mean fluorescence intensity of CCR6 expression was quantified on each T cell subset in participants diagnosed with SLE and controls (c). Representative images of CCR6 expression in the T cell subsets are displayed (d). (n= 13 SLE, 16 Controls).

Mann-Whitney two-tail t-test, *significant at p<0.05; **significant at p<0.01

Next, CCR6 expression was quantified on T cell subsets during SLE. Interestingly, CCR6 expression was significantly reduced on all lymphocytes, CD4⁺ T cells and T_{FH} cells in SLE participants compared to control participants (Fig. 6.2.2c, d). There also appeared to be a reduction

in CCR6 expression on intermediate T_{FH} cells in SLE participants compared to control participants (Fig. 6.2.2c, d). This decrease in CCR6 expression on human T_{FH} cells, is similar to that of the intermediate T_{FH} cells observed in *gld* mice and also highlights the potential of CCR6 to be used as a marker of systemic autoimmune diseases.

While the results obtained from this clinical study of SLE are original, significant and interesting, it is important to consider their relationship to a normal heterogeneous population. Participants from this study were sampled from the local state population and the data generated is not normally distributed, as was determined by graphical inspection. Hence, the Mann-Whitney t-test was selected for statistical analysis. Due to the small samples size in this study, it is possible that the data does not display a normal distribution, whereas a large population would accurately show whether the population is normally distributed or not. Thus, a retrospective power analysis was carried out based on the effect sizes (mean and standard deviation) observed here for CCR6 expression on B and T cell subsets. The exposure rate for B and T cell analysis was 42.42 % and 44.82 % respectively. This revealed that the current study had approximately 20% power for CCR6 expression on B cells and approximately 50% power for CCR6 expression on T cells (Table 6.2.1). In clinical studies, it is widely agreed that a power of 80% is an acceptable probability of detecting statistically significant effects, that is, variations with a p-value <0.05. It is clear that the current study is underpowered, and such a small population would be more likely to produce both type-I (false positive) and type-II (false negative) errors, thereby placing the reliability of the observed effects into question. To rectify this, this study should be repeated with a larger cohort so that greater sensitivity can be provided to detect effects of the magnitude depicted here. It is also necessary to consider the age and gender of participants in this study. While age and gender are associated with SLE²⁹³⁻²⁹⁶, preliminary analysis in this study indicates that age and gender is not associated with chemokine receptor expression, or lymphocyte frequency in SLE. It has also been documented that chemokine receptor expression is not associated with age or gender in SLE²²¹. However, it is possible that chemokine receptor expression and lymphocyte frequency is associated with disease severity. This concurs with published literature showing that chemokine expression and circulatory B cell frequency is correlated with SLE disease activity²⁹⁷⁻³⁰⁰. The current study did not have ethics approval to document a detailed medical history, including disease activity or severity, secondary inflammations and all treatments in participants diagnosed with SLE. As such, the possibility that these factors have contributed to chemokine receptor expression and lymphocyte frequency cannot be excluded and would need to be investigated in future studies. While this study was restricted, its purpose was as a preliminary investigation into CCR6 expression during

autoimmune disease, the results of which justify the need for a further large-scale study in the future.

Table 6.2.1. Statistical power of Systemic Lupus Erythematosus study

CCR6 Expression	Current Power	Sample size required for 80% Power
CD19 ⁺ B cells	20 %	210 (121 Controls & 89 SLE)
Pre-GC B cells	19 %	216 (124 Controls & 92 SLE)
GC/PC B cells	19 %	220 (127 Controls & 93 SLE)
Total Memory B cells	37 %	98 (56 Controls & 42 SLE)
Total Naïve B cells	20 %	204 (117 Controls & 87 SLE)
Lymphocytes	29 %	113 (62 Controls & 51 SLE)
CD4 ⁺ T cells	53 %	54 (30 Controls & 24 SLE)
CXCR5 ⁺ CD4 ⁺ T cells	59 %	48 (26 Controls & 22 SLE)
CXCR5 ⁺ PD-1 ⁺ CCR7 ^{lo} CD4 ⁺ T cells	66 %	41 (23 Controls & 18 SLE)

6.3. Concluding Remarks

Throughout this chapter, I have examined CCR6 expression on B and T cell subsets in a mouse and human model of systemic autoimmunity. One important aspect to consider in this study is the validity of the mouse model of autoimmunity. Mouse models are extremely useful tools to investigate disease susceptibility, mechanisms of disease, and therapeutic strategies, because they offer greater sample accessibility than humans. Currently, several different mouse models are used in SLE research³⁰¹. I have made use of the FasL^{gld} strain, which has a mutation in the FasL gene, causing the spontaneous development of systemic autoimmune disease. It is similar to that of the MRL^{lpr} mouse model, which has a mutation in the lpr gene. The *gld* mouse and other such strains, are considered SLE-like models as they present with similar immunological attributes that characterise SLE. Specifically, the *gld* mouse model exhibits lymphadenopathy and autoantibody production.

To correlate the results obtained between mice and humans, I analysed lymphocytes in the blood and secondary lymphoid organs during systemic autoimmune disease. In the *gld* mice, significant increases in the precursor, GC, plasma cell and memory B cells, along with a significant decrease in naïve B cells, were detected when compared to WT mice. Also, CCR6 expression was significantly increased on naïve and memory B cells in *gld* mice compared to WT mice. These alterations in the B cell compartment of *gld* mice correlated with a significant reduction in non-T_{FH} cells and significant increases in the intermediate T_{FH} and T_{FH} cell populations. Furthermore, CCR6 expression was significantly increased on all CD4⁺ T cells and non-T_{FH} cells, and it was

significantly reduced on intermediate T_{FH} cells. Thus, the effector B and T cell populations of *gld* mice were generally increased during systemic autoimmunity, and while CCR6 expression trends were different between B and T cells, significant variations were detected during systemic autoimmunity. Finally, the systemic lymphoproliferation reported here in the *gld* mice, is consistent with the literature³⁰².

In the study of SLE, I identified human B cell subsets as CD38^{lo/int} IgD⁺ naïve cells, CD38^{hi} IgD⁺ pre-GC B cells, CD38^{hi} IgD⁻ CD27⁺ GC B cells, CD38^{hi} IgD⁻ CD27⁺ plasma cells and CD38^{low/int} IgD⁻ memory B cells. This is consistent with previously published work²⁸⁶. In addition, human T cells were detected based on CXCR5, CCR7, PD-1 and CD3 expression, and this profile was also consistent with previously published reports³⁰³. During SLE, the increased CCR6 expression on naïve and memory B cells, and the reduced CCR6 expression on all CD4⁺ T cells and T_{FH} cells, reflected the trends of CCR6 expression observed in the murine model of systemic autoimmunity. In terms of population frequency, a significant increase in T_{FH} cells was observed during SLE, correlating with the murine model, however, a significant decrease in Pre-GC B cells was detected during SLE, differing to the data collected in *gld* mice. Whilst there is variability in the data between the human and murine models of systemic autoimmunity, attributed to both species and disease differences, it is reassuring to observe similar alterations in the population frequency and CCR6 expression between both models.

This study provided invaluable insights into autoimmunity, however, it was restricted by the low number of participants. Statistical significance was achieved as the characteristics examined were not normally distributed, allowing a non-parametric t-test to be used for analysis. Furthermore, the number of participants in this study is similar to other published reports in this field, including the publication by Henneken and colleagues (2005), which examined the frequency of CCR6⁺ B cells during SLE. Together, these studies provide strong evidence demonstrating a role for CCR6 in the pathophysiology of SLE.

In summary, the findings established in the FasL^{gld} mouse model were applied to a clinical study of SLE, which demonstrated that SLE participants can be distinguished from healthy controls by 1) the increased CCR6 expression on the total circulatory B cell population, reflecting the upregulated CCR6 expression on naïve and memory B cells and 2) the reduced expression of CCR6 on the circulatory T_{FH} cell population. These prominent alterations highlight the potential for the development of a biomarker that targets CCR6 expression. Such a marker would be beneficial, as it would identify SLE, and in theory other autoimmune diseases that feature B cell dysregulation.

However, a larger follow up study with a more clinically defined cohort is needed before this application can be advocated. Overall, these findings are important for not only autoimmune disease, but they also provide insight into the mechanisms of protective antibody responses generated during disease and vaccination.

Chapter 7

Discussion and Conclusion

Given the distinct expression of CCR6 during TD B cell differentiation documented in the literature, it was surprising that very little was known regarding the function of this chemokine receptor. Thus, in considering the fundamental role that chemokine receptors, more broadly, have in positioning lymphocytes, I set out to determine this unknown. I hypothesised that CCR6 contributes to B cell differentiation in the humoral immune response. In this thesis, I have provided a direct quantitative assessment of the role of CCR6 during B cell differentiation. As briefly discussed in the previous four results chapters, this work has revealed a number of previously unreported features of TD humoral responses. Furthermore, three findings, the principal findings of this thesis, have emerged:

- 1) CCR6 is upregulated upon follicular B cell activation
- 2) CCR6 contributes to efficient GC and EF B cell differentiation
- 3) CCR6 is associated with SLE

These findings and their implications are discussed below in the context of the current literature in the field.

7.1. CCR6 is upregulated upon follicular B cell activation

B cell positioning in secondary lymphoid organs is largely determined by CXCR5 and CCR7^{52,158}. Though CCR6 has been implicated as an additional receptor in this process, its expression and migratory capacity has not been fully defined. In this study, I investigated the ability of antigen-specific B cells to induce CCR6 expression during activation. HEL activation of MD4 B cells *in vitro* for 24 hrs, significantly increased the intensity of CCR6 expression on the cell surface. CCR6 expression was maximal 8 hrs after stimulation. However, the cell surface expression of CCR6 after B cell activation did not correlate with the mRNA expression of CCR6. In fact, CCR6 mRNA levels were significantly reduced following antigen-specific activation. It was possible that an early transient increase in CCR6 mRNA could have occurred, such that 8 hrs post activation was too late to observe an increase in mRNA. Thus, transcripts were examined early after activation. Again, CCR6 mRNA was significantly reduced 2 hrs after B cell activation. These results differ from studies using human peripheral blood and tonsillar B cells activated *ex vivo*¹⁸⁴⁻¹⁸⁶. For instance, Liao and colleagues (2002) activated human B cells through IgM cross-linking for 3 days and reported no change in either surface expression or mRNA levels of CCR6. This discrepancy is likely due to the difference in the method used to activate B cells and of course, species variation between studies. It is generally accepted though, that cell surface expression does not always reflect transcript levels and in this case, it is possible that upon activation, B cells may export their intracellular supply of CCR6 to the cell surface and simultaneously downregulate CCR6 gene

expression, offering an explanation for the increased surface expression of CCR6 and decreased levels of CCR6 mRNA.

When CCR6 expression was examined on antigen-specific B cell subsets defined by CD38 and GL7 expression *in vivo*, a distinct pattern was observed. Naïve and memory B cells were found to have a moderate expression of CCR6 and this expression was reduced on GC B cells and to a lesser extent plasma cells. This expression profile is consistent with published studies in both mice^{188,190} and humans¹⁸⁵⁻¹⁸⁷. In accordance with the previous *in vitro* findings of this study, CCR6 expression was significantly increased on precursor B cells *in vivo*, which appear following activation, prior to GC differentiation. This confirms the physiological relevance of the *in vitro* model of B cell activation and is in agreement with a recent publication, documenting the appearance of CCR6^{hi} CD95⁺ GL7⁺ IgD⁺ B cells, prior to GC formation⁷³. Additionally, this study has revealed that CCR6 is expressed on T-B cell conjugate pairs *ex vivo*. However, it is clear that CCR6 is not an absolute requirement for T-B cell interactions, as CCR6^{-/-} mice are still capable of efficiently forming T-B cell conjugates. Overall, these results suggest that CCR6 is involved in the early B cell events of the humoral response.

The question that remains is how does CCR6 affect B cells? Taking into account that CCR6 belongs to a family of G-protein coupled chemokine receptors, well known for guiding migration, and that CCR6-dependent migration of follicular B cells has been previously reported^{188,189}, it is likely that CCR6 directs activated B cell movement. Specifically, CCR6 expression may be required for the migration of naïve, memory and precursor B cells around secondary lymphoid organs, but must be downregulated on B cells for entry into the GC. Furthermore, this study has demonstrated that CCL20 is produced by T_{FH} cell precursors, which are known to differentiate at the T-B border. The intermediate T_{FH} cell-secreted CCL20 may provide a signal that retains CCR6⁺ B and T cells at the T-B border. In line with this, CCL20 has been shown as chemotactic for human lymphocytes^{164,170}. However, studies documenting CCL20 expression are lacking. Furthermore, the CCR6-CCL20 dependent migration of antigen-specific activated B cells has not been investigated and will need to be directly established. Alternatively, other cells within secondary lymphoid could also express CCL20.

Interestingly, CCR6 shares a similar expression profile on B cells, to another chemokine receptor, EBI2, which is known to contribute to B cell movement. EBI2 is expressed at high levels in naïve B cells and is transiently upregulated following B cell activation and during T cell help^{304,305}. EBI2 expression is downregulated on GC B cells, but maintained on plasma cells³⁰⁴. In fact, it has been

demonstrated that EBI2 expression on activated B cells is essential for B cells to migrate to extrafollicular sites to establish EF, whereas its downregulation allows B cells to migrate inside the B cell follicle to establish GC follicles^{157,158}. Furthermore, EBI2 contributes to the CXCR5- and CCR7-dependent B cell organisation at the T-B cell border³⁰⁵. Thus, it is plausible to speculate a similar role for CCR6. CCR6 expression may aid B cell localisation to the T-B cell border and the migration of precursor B cells at the edge of B cell follicles, prior to B cell fate decisions. This role would be consistent with a model of balanced responsiveness by B cells, originally proposed by Reif and colleagues (2002). In this model, B cells alter their chemokine receptor expression in response to antigen, by integrating the various chemokine signals that spatially define secondary lymphoid organs. In this way, B cells are able to constantly re-position themselves as necessary. It is likely that this model extends further than the migration effects of CXCR5 and CCR7, such that B cells are also influenced by CXCR4 and EBI2. Given the distinct expression of CCR6 on B cells throughout the TD humoral response and its prominent increase on activated B cells, it is reasonable to propose that CCR6 also contributes to this process. In support of this, evidence from this study shows that CXCR5, CCR7 and CXCR4 surface expression is significantly reduced in CCR6^{-/-} B cells compared to WT, after activation *in vitro*. Furthermore, CCR7 expression was significantly increased on CCR6^{-/-} precursor B cell compared to WT B cells *in vivo*. This indicates that CCR6 may work with several chemokine receptors to correctly position B cells. This would be ideally confirmed using chemokine receptor deficient double knockouts or mixed adoptive transfer models.

7.2. CCR6 contributes to efficient B cell differentiation

The upregulation of CCR6 during B cell activation, in combination with its distinct expression on mature B cell subsets in response to TD antigens, indicates a critical role for CCR6 in B cell positioning. The extent of this role was examined in CCR6^{-/-} mice immunised with NP-KLH. This study has demonstrated that in the absence of CCR6 there is a significant increase in the GC B cell response. The fact that this increase was observed only at day 3 and 5 post immunisation emphasises that CCR6 primarily acts early in the humoral response. There are several possible explanations for the increased GC response documented in the CCR6^{-/-} mice. This alteration could be a result of either an increased number of B cells being activated, enhanced B cell proliferation, or alternatively, a defect in apoptosis resulting in the accumulation of GC B cells.

A significant increase in total frequency of antigen-specific B cells was also observed in CCR6^{-/-} mice 5 days after immunisation, correlating with the increase in GC B cells. This suggests that more

B cells undergo proliferation or expansion, rather than an inherent increase in the follicular B cell compartment in CCR6^{-/-} mice. Furthermore, the significant reduction in the frequency of naïve B cells in CCR6^{-/-} mice at days 3 and 5 post antigen challenge, suggests that more antigen-specific B cells are activated and recruited into B cell differentiation. This does not exclude the possibility that B cells are preferentially selected to expand into GCs. To determine if CCR6^{-/-} B cells are preferentially recruited to undergo GC differentiation, the transcriptional profile of GC B cells was examined. This analysis demonstrated that CCR6 deficient GC B cells have an inherent increase in Bcl-6 expression – the master regulator of the GC reaction, indicating that activated B cells are encouraged to proliferate in the GC. Furthermore, this increase in Bcl-6 expression in GC B cells is likely caused by the increase in IL-21 production by T_{FH} cells, as IL-21 is known to directly act on Bcl-6 in GC B cells^{121,122,279}. In fact, it has been demonstrated that IL-21 mediated interactions between T_{FH} cells and GC B cells, enable the selection of high-affinity B cells clones³⁰⁶.

Given that CCR6 is expressed on naïve and activated B cells and is then downregulated on GC B cells, it is possible that Bcl-6 represses CCR6 expression during GC B cell differentiation. Bcl-6 is already known to repress the expression of genes associated with B cell differentiation¹⁴², and the reduction of CCR6 expression on GC B cells may enable B cells to migrate into the follicle to establish GC follicles. In this way, increased Bcl-6 levels would cause a reduction in CCR6 expression, thereby enabling increased GC formation in a normal response. This theory is supported by the significant reduction in CCR6 gene expression detected during *in vitro* B cell activation. Indeed, this is also the case for EBI2. Whilst EBI2 expression is upregulated upon B cell activation, Bcl-6 represses EBI2 expression, allowing B cells to migrate into the follicle to form GCs^{142,304}. Considering the analogous expression between CCR6 and EBI2, it is possible that both chemokine receptors have similar roles, or in fact, may work together to position B cells.

Although the GC response was altered, there was, remarkably, no major effect on the quality or efficiency of the GC reaction. Notably, the light and dark zone structure of the GC was maintained in the absence of CCR6, indicating firstly that CCR6 is not required for the organisation of B cells within the GC and more importantly, that the GC reaction occurs as per normal. In support of this observation, there was no significant difference in the mRNA expression of AID, the enzyme responsible for initiating SHM and isotype switching, demonstrating that the expression of CCR6 does not influence the genetic events of the GC reaction. While significantly higher levels of Id2 were detected in CCR6^{-/-} GC B cells, this did not seem to suppress AID expression or inhibit subsequent B cell differentiation. Furthermore, the increased production of IL-21 from T_{FH} cells did not appear to induce plasma cell nor long term memory differentiation in this study, as would be

expected^{121,122,307}. In agreement with these findings, plasma cell differentiation did not appear to be dysregulated, as indicated by the similar levels of Blimp-1 and IRF4 observed between WT and CCR6^{-/-} GC B cells. This was substantiated by the similar frequency of plasma cells generated between CCR6^{-/-} and WT mice. However, this was not the case for memory B cells. Brief, yet significant increases in the frequency of early memory B cells were observed in CCR6^{-/-} mice, approximately correlating with the increased GC responses detected previously. Despite the altered GC response, there did not appear to be an alteration in the quantity of antibodies secreted. Antigen-specific serum IgM and IgG levels were similar between CCR6^{-/-} and WT mice. This did not though exclude the possibility that antibody affinity is altered in the absence of CCR6. With this in mind, the Körner laboratory has previously shown that more low-affinity than high-affinity antibody forming cells are present in CCR6^{-/-} mice compared to WT mice, seven days after antigen challenge¹⁸². This result contradicts the expectation that an increased GC response would generate more high-affinity than low-affinity antibodies. Unfortunately, attempts in this study to conclusively demonstrate the quality of SHM in CCR6^{-/-} GC B cells, which would have provided insights into the affinity of terminally differentiated B cells derived from the GC, were unsuccessful, due to the low frequency of the GC population. Given that AID expression was not altered in CCR6^{-/-} GC B cells, it is likely that SHM is not impaired, but rather a defect in affinity selection is responsible for the altered antibody affinity in the absence of CCR6.

Surprisingly, despite an increased GC response in the CCR6^{-/-} mice, there was not a corresponding increase in the frequency of T_{FH} cells nor FDCs, rather both populations occurred at a similar frequency between CCR6^{-/-} and WT mice. There are two possible consequences of this finding. Firstly, the T_{FH} cells and FDCs may be distributed equally between follicles, providing a greater selection pressure for GC B cells and theoretically favouring the production of high-affinity B cells. However, this is not the case, as evidenced by the increased frequency of low-affinity antibody forming cells in CCR6^{-/-} mice¹⁸². Therefore it is more appropriate to speculate that the selection of B cells in CCR6^{-/-} mice is less efficient, as the same number of T_{FH} cells and FDCs are expected to serve an increased GC response in the CCR6^{-/-} mice 5 days after antigen challenge. This would therefore offer an explanation for the increased number of low-affinity antibody forming cells in the CCR6^{-/-} mice. Alternatively, the EF response could have accounted for the increased population of low-affinity antibody forming cells: however, this possibility was eliminated when it was demonstrated that the peak of the EF response was significantly reduced in CCR6^{-/-} mice.

Interestingly, the reduction of EF B cells observed in CCR6^{-/-} mice indicates that EF B cells normally express CCR6. Of course, this needs to be substantiated in a WT model. Also, the

decrease in the EF population in the absence of CCR6 indicates that activated B cells fail to migrate to extrafollicular sites. Furthermore, the increased GC B cell response observed in the CCR6^{-/-} mice, theoretically coincides with the rapid production of EF-derived antibody-secreting. It is possible that the increased GC response has actually compensated for the loss of the EF response, such that activated B cells deficient for CCR6 fail to migrate to extrafollicular sites, preferentially migrating into the B cell follicle. This can be reconciled when considering the expression of CCR6 on EF and GC B cells. The GC B cells normally have a reduced expression of CCR6, suggesting that after activation, B cells selected to differentiate into GCs must downregulate CCR6 expression to enter the B cell follicle and establish GC follicles. Whereas B cells destined to form EF, must maintain CCR6 expression to migrate to extrafollicular sites that is, if EF B cells do indeed express CCR6. In this way, antibody-secreting cells of low-affinity may be quickly generated in the EF during a normal response.

By the same token, EBI2 seems to perform a similar function to the one proposed here for CCR6. In the absence of EBI2, the EF response is diminished, while the GC response continues efficiently³⁰⁴. It will be necessary to determine whether the expression of EBI2 and CXCR4, the chemokine receptors that direct B cell migration to extrafollicular sites and thus EF formation, is altered in CCR6^{-/-} mice. This will indicate whether CCR6 contributes to the migration of extrafollicular B cells. The fact that the extrafollicular response is not completely abolished in the CCR6^{-/-} mice indicates that other chemokine receptors, such as EBI2 and CXCR4, are still able to direct B cell migration and the formation of EF. To clearly demonstrate that B cell migration is altered in the CCR6^{-/-} mice, specifically that B cells are directed to the GC rather than the EF, will require the visualisation of B cells during TD humoral responses.

As this study was initially focused on determining the impact of CCR6 on the GC reaction, the EF response was less well investigated. Granted the EF B cell population is more challenging to investigate due to the lack of definitive surface markers and lack of access to specialist mouse models, the EF response does need to be quantified in its entirety, such that a picture of both early and late B cell kinetics is collected. Such data would show whether the diminished EF response is sustained or transient, much in the same way that the GC response is transiently increased.

Overall, no detrimental effects were observed on the outcome of the humoral response. CCR6 appears to be solely involved in B cell positioning, which is crucial in establishing the humoral response and efficiently generating antibody-secreting cells to combat infection. Specifically, the

modulation of CCR6 surface expression on B cells appears to contribute to directing their migration to EF or GC sites, and consequently, their subsequent differentiation.

7.3. CCR6 is associated with SLE

The cause of SLE currently remains elusive. The hallmark production of autoreactive antibodies indicates a critical collapse in B cell tolerance, facilitating the expansion of autoreactive antibody-secreting B cells. It is clear that early B cell interactions with antigen and Th cells are imperative to initiate and drive B cell differentiation, and thus would be prime targets of tolerance breakdowns. Indeed, it has emerged that breakdowns in SLE likely occur early, for instance during B cell development in the bone marrow³⁰⁸, T-B cell interactions³⁰⁹, and the precursor²⁴¹ or GC stages³¹⁰. Whatever the case may be, whether SLE is caused by a single error or multiple breakdown events, this dysfunction creates an environment fostering the survival of autoreactive B cells. The key aim of the present study was to identify whether CCR6 contributes to the pathophysiology of SLE. Although the results from this study are derived from small numbers of participants, they highlight the importance of CCR6 and represent the first investigation to correlate CCR6 expression with B cell dysregulation in SLE.

The initial finding of a significant reduction in the frequency of circulatory pre-GC B cells in SLE participants, concurs with the general circulatory lymphopenia known to be associated with SLE^{228,311}. However it has not yet been specifically described for this subset. The decrease in circulatory pre-GC and GC B cells may reflect increased recruitment of these cells to secondary lymphoid organs, where they can seed and differentiate into antibody-secreting cells. This theory is consistent with the increased populations of GC and memory B cells that have been reported in the tonsils of SLE participants compared to controls²⁴¹. While pre-GC B cells have actually been documented to increase in children diagnosed with SLE³¹², the discrepancy in this study can be attributed to the variation in B cell populations between children and adults.

Interestingly, the increased frequency of circulatory T_{FH} cells observed in SLE participants in this study, could indicate that their absence from secondary lymphoid organs may cause a defect in B cell help and differentiation. Alternatively, it may reflect an increased GC response, whereby T_{FH} cells migrate between multiple secondary lymphoid organs to seed and support other GC reactions. Regardless, this observation concurs with published data that suggests the increased circulating T_{FH} cell population contributes to the pathogenesis of disease^{247,288}. Consequently, this study suggests

that alterations in the pre-GC, GC and T_{FH} cell populations contribute to the pathophysiology of SLE.

Previously, the frequency of CCR6⁺ B cells, but not the intensity of CCR6 expression on B cells had been investigated in SLE. Specifically, Henneken and colleagues (2005) found that SLE participants tended to have fewer CCR6⁺ B cells, though this was not significant²²¹. Likewise, in the current study, which recruited only a few more SLE participants (n=14 vs. n=11), the frequency of CCR6⁺ B cells during SLE was not significantly different from controls. This variation probably reflects the heterogeneous nature or different disease states of SLE. However, when the intensity of CCR6 expression was examined, a significant increase was observed on all B cells from SLE participants, which correlated with significant increases in the naïve and memory B cell populations. The expression of CCR6 also appeared to increase on pre-GC and GC B cells, however this was not significant. Nevertheless, the substantial upregulation of CCR6 on naïve and memory B cells could aid the migration of these B cells to activation sites in secondary lymphoid organs including the T-B border, resulting in a larger number of B cells being selected to differentiate into GCs. In addition, it is likely that the increased CCR6 expression observed on B cells in SLE is a consequence of the breakdown in tolerance, rather than a causative factor. Overall, this work suggests that CCR6 may contribute to B cell dysregulation and therefore is relevant to the pathophysiology of SLE. This is not the first time that CCR6 has been implicated in the pathophysiology of autoimmune disease. In fact, a similar role for CCR6 has been proposed in RA, whereby the CCR6-CCL20 axis mediates the recruitment of B cells²¹⁸ and other mononuclear cells to the joints²¹⁵, thereby contributing to inflammation.

It is generally accepted that the lymphocyte populations of the blood reflect those in secondary lymphoid organs and by extension, disease state. The results presented in this study have demonstrated that CCR6 is an excellent candidate for use as a disease biomarker. Blocking of CCR6, or indeed other chemokine receptors imperative for B cell migration may prove valuable in preventing disease severity. However, for now, the clinical usefulness of CCR6 is proposed to mainly aid diagnosis and monitor disease severity. The most convenient method to analyse disease status in humans is through blood tests. Thus, it would be ideal to develop a flow cytometry panel that can be used to quantify the population frequency and chemokine receptor expression of B cells in the blood. Such a tool, similar to the one used in this study, would provide a clear and conclusive indication of disease, and could be used to tailor personalised medicine in the future. Accordingly, future research should focus on examining an array of chemokine receptor expression in SLE, to develop a more comprehensive profile of disease. Perhaps the best example of this novel idea is the

expression of CCR6, CCR7, CXCR4 CXCR5, CCR5 and CXCR3, that was recently documented in the blood and joints of RA patients²¹⁸.

To summarise, this work has provided insights into the pathophysiology of SLE and the usefulness of CCR6 in therapeutic applications. As this study was restricted to a small SLE population, it would be worthwhile to substantiate these findings in a larger cohort with well-defined disease activity. Finally, this work has paved the way for further investigations into the use of chemokine receptors as biomarkers of disease and the development of medications.

7.4. Conclusion

Chemokine receptors are crucial for the correct positioning of B cells in the humoral response, which is essential for the efficient differentiation of antibody-secreting cells. In this thesis, I have focused on uncovering the previously undefined role of CCR6 on B cells in the humoral response. I hypothesised that CCR6 expression contributes to the efficient differentiation of B cells during TD humoral immune responses. As a result of this investigation, this thesis has presented evidence supporting a role for CCR6 in the humoral response. Specifically, I have characterised the expression of CCR6 during B cell activation, the differentiation of B cells in the absence of CCR6, the mechanisms that underlie CCR6 deficient B cell differentiation and the relevance of CCR6 in B cell-mediated autoimmunity.

This is the first study to examine the temporal kinetics of CCR6 expression, how this expression affects B cell fate, and the dynamics of CCR6 expression on circulating B cells in SLE. In doing so, this study has extended our previous understanding of the chemokine receptor driven spatial organisation of B cells in secondary lymphoid organs and provided great insight into the B cell dysregulation that occurs during autoimmune disease. As a result of this work, I propose that CCR6 expression contributes to the organisation of B cells during activation, and that the subsequent modulation of CCR6 expression guides B cells to either follicular or extrafollicular sites, where they continue their terminal differentiation. This proposal complements the current model of B cell movement in the humoral response, whereby CCR6 would work in conjunction with CXCR5, CCR7, EBI2 and CXCR4, to correctly position B cells for differentiation in response to TD antigens.

Overall, this study has conclusively demonstrated that CCR6 contributes to the efficient differentiation of B cells during the TD humoral response. While this thesis has made substantial

progress in defining the role of CCR6, it is imperative that further research be undertaken to visualise B cell localisation during the humoral response in the absence of CCR6. This will definitively show the role of CCR6 in B cell movement. In addition, research efforts should now focus on clearly defining the EF reaction and subsequently, the role of CCR6 in the EF differentiation pathway. Finally, it will be invaluable to compile profiles of chemokine receptor expression during autoimmune disease, as this information will be useful to direct the development of therapeutics and to diagnose disease. In conclusion, this thesis has demonstrated a critical role for CCR6 in the regulation of B cell differentiation in the humoral immune response.

Chapter 8

References

1. Dempsey, P.W., Vaidya, S.A. & Cheng, G. The art of war: Innate and adaptive immune responses. *Cellular and Molecular Life Sciences* **60**, 2604–2621 (2003).
2. Medzhitov, R. & Janeway Jr, C.A. Innate immune recognition and control of adaptive immune responses. *Seminars in Immunology* **10**, 351–353 (1998).
3. Iwasaki, A. & Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *Science* **327**, 291–295 (2010).
4. Clark, R. & Kupper, T. Old meets new: The interaction between innate and adaptive immunity. *The Journal of Investigative Dermatology* **125**, 629–637 (2005).
5. Chaplin, D.D. Overview of the immune response. *The Journal of Allergy and Clinical Immunology* **125**, 1–41 (2010).
6. Ruddie, N.H. & Akirav, E.M. Secondary lymphoid organs: Responding to genetic and environmental cues in ontogeny and immune response. *The Journal of Immunology* **183**, 2205–2212 (2009).
7. Mebius, R.E. & Kraal, G. Structure and function of the spleen. *Nature Reviews Immunology* **5**, 606–616 (2005).
8. Batista, F.D. & Harwood, N.E. The who, how and where of antigen presentation to B cells. *Nature Reviews Immunology* **9**, 15–27 (2009).
9. Schroeder Jr, H.W. & Cavacini, L. Structure and function of immunoglobulins. *The Journal of Allergy and Clinical Immunology* **152**, 1–24 (2010).
10. Dudley, D.D., Chaudhuri, J., Bassing, C.H. & Alt, F.W. Mechanism and control of V(D)J recombination versus class switch recombination: Similarities and differences. *Advances in Immunology* **86**, 43–112 (2005).
11. Jung, D., Giallourakis, C., Mostoslavsky, R. & Alt, F.W. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annual Review of Immunology* **24**, 541–570 (2006).
12. Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* **381**, 751–758 (1996).
13. Hardy, R.R. & Hayakawa, K. B cell developmental pathways. *Annual Review of Immunology* **19**, 595–621 (2001).
14. Osmond, D.G. Proliferation kinetics and the lifespan of B cells in central and peripheral lymphoid organs. *Current Biology* **3**, 179–185 (1991).
15. Melchers, F., ten Boekel, E., Seidl, T., Kong, X.C., Yamagami, T., Onishi, K., Shimizu, T., Rolink, A.G. & Andersson, J. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. *Immunological Reviews* **175**, 33–46 (2000).
16. Nutt, S.L. & Kee, B.L. The transcriptional regulation of B cell lineage commitment. *Immunity Reviews* **26**, 715–725 (2007).
17. Hardy, R.R., Kincade, P.W. & Dorshkind, K. The protean nature of cells in the B lymphocyte lineage. *Immunity Reviews* **26**, 703–714 (2007).
18. Barneda-Zahonero, B., Roman-Gonzalez, L., Collazo, O., Mahmoudi, T. & Parra, M. Epigenetic regulation of B lymphocyte differentiation, transdifferentiation, and reprogramming. *Comparative and Functional Genomics*, 1–10 (2012).
19. Rolink, A.G., Andersson, J. & Melchers, F. Molecular mechanisms guiding late stages of B-cell development. *Immunological Reviews* **197**, 41–50 (2004).
20. Su, T.T., Gui, B., Wei, B., Braun, J. & Rawlings, D.J. Signaling in transitional type 2 B cells is critical for peripheral B-cell development. *Immunological Reviews* **197**, 161–178 (2004).
21. Thomas, M.D., Srivastava, B. & Allman, D. Regulation of peripheral B cell maturation. *Cellular Immunology* **239**, 92–102 (2006).
22. Cancro, M.P. Peripheral B-cell maturation: the intersection of selection and homeostasis. *Immunological Reviews* **197**, 89–101 (2004).

23. Shinall, M.S., Gonzalez-Fernandez, M., Noelle, R.J. & Waldschmidt, T.J. Identification of murine germinal center B cell subsets defined by the expression of surface isotypes and differentiation antigens. *The Journal of Immunology* **164**, 5729–5738 (2000).
24. Ridderstad, A. & Tarlinton, D. Kinetics of establishing the memory B cell population as revealed by CD38 expression. *The Journal of Immunology* **160**, 4688–4695 (1998).
25. Sanderson, R.D., Lalor, P. & Bernfield, M. B lymphocytes express and lose syndecan at specific stages of differentiation. *Cell Regulation* **1**, 27–35 (1989).
26. Smith, K.G.C., Hewitson, T.D., Nossal, G.J.V. & Tarlinton, D.M. The phenotype and fate of the antibody-forming cells of the splenic foci. *The European Journal of Immunology* **26**, 444–448 (1996).
27. McHeyzer-Williams, M.G., McLean, M.J., Lalor, P.A. & Nossal, G.J.V. Antigen-driven B cell differentiation *in vivo*. *The Journal of Experimental Medicine* **178**, 295–307 (1993).
28. Taylor, J.J., Pape, K.A. & Jenkins, M.K. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *The Journal of Experimental Medicine* **209**, 597–606 (2012).
29. Maul, R.W. & Gearhart, P.J. AID and Somatic Hypermutation. *Advances in Immunology* **105**, 159–191 (2010).
30. Neuberger, M.S. & Rada, C. Somatic hypermutation: Activation-induced deaminase for C/G followed by polymerase η for A/T. *The Journal of Experimental Medicine* **204**, 7–10 (2007).
31. Xu, Z., Zan, H., Pone, E.J., Mai, T. & Casali, P. Immunoglobulin class switch DNA recombination: Induction, targeting and beyond. *Nature Reviews Immunology* **12**, 517–531 (2013).
32. Stavnezer, J., Guikema, J.E.J. & Schrader, C.E. Mechanism and Regulation of Class Switch Recombination. *Annual Review of Immunology* **26**, 261–292 (2008).
33. Cattoretti, G., Büttner, M., Shakhovich, R., Kremmer, E., Alobeid, B. & Niedobitek, G. Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. *Blood* **107**, 3967–3975 (2006).
34. Zaheen, A., Boulianne, B., Parsa, J.-Y., Ramachandran, S., Gommeman, J.L. & Martin, A. AID constrains germinal center size by rendering B cells susceptible to apoptosis. *Blood* **114**, 547–554 (2009).
35. Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. & Honjo, T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553–563 (2000).
36. Gearhart, P.J. & Bogenhagen, D.F. Clusters of point mutations are found exclusively around rearranged antibody variable genes. *Immunology* **80**, 3439–3443 (1983).
37. Muramatsu, M., Sankaranand, V.S., Anant, S., Sugai, M., Kinoshita, K., Davidson, N.O. & Honjo, T. Specific expression of Activation-induced Cytidine Deaminase (AID), a novel member of the RNA-editing deaminase family in germinal centre B cells. *The Journal of Biological Chemistry* **274**, 18470–18476 (1999).
38. Pape, K.A., Catron, D.M., Itano, A.A. & Jenkins, M.K. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity* **26**, 491–502 (2007).
39. Roozendaal, R., Mempel, T.R., Pitcher, L.A., Gonzalez, S.F., Verschoor, A., Mebius, R.E., von Andrian, U.H. & Carroll, M. Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity* **30**, 264–276 (2009).
40. Catron, D.M., Pape, K.A., Fife, B.T., van Rooijen, N. & Jenkins, M.K. A protease-dependent mechanism for initiating T-dependent B cell responses to large particulate antigens. *The Journal of Immunology* **184**, 3609–3617 (2010).

41. Carrasco, Y.R. & Batista, F.D. B cells acquire particulate antigen in a macrophage rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* **27**, 160–171 (2007).
42. Phan, T.G., Grigorova, I., Okada, T. & Cyster, J.G. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nature Immunology* **8**, 992–1000 (2007).
43. Qi, H., Egen, J.G., Huang, A.Y.C. & Germain, R.N. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science* **312**, 1672–1676 (2006).
44. Harwood, N.E. & Batista, F.D. Early events in B cell activation. *Annual Review of Immunology* **28**, 185–210 (2010).
45. Dal Porto, J., Gauld, S.B., Merrell, K.T., Mills, D., Pugh-Bernard, A.E. & Cambier, J. B cell antigen receptor signalling 101. *Molecular Immunology* **41**, 599–613 (2004).
46. Kurosaki, T. Regulation of B-cell signal transduction by adaptor proteins. *Nature Reviews Immunology* **2**, 354–363 (2002).
47. Packard, T.A. & Cambier, J.C. B lymphocyte antigen receptor signalling: Initiation, amplification, and regulation. *F1000Prime Reports* **5**, 1–8 (2013).
48. Avalos, A.M. & Ploegh, H.L. Early BCR events and antigen capture, processing, and loading on MHC class II on B cells. *Frontiers in Immunology* **5**, 1–5 (2014).
49. Garside, P., Ingulli, E., Merica, R.R., Johnson, J.G., Noelle, R.J. & Jenkins, M.K. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science* **281**, 96–99 (1998).
50. Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D. & Cyster, J.G. Antigen - engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLOS Biology* **3**, 1047–1061 (2005).
51. Kerfoot, S.M., Yaari, G., Patel, J.R., Johnson, K.L., Gonzalez, D.G., Kleinstein, S.H. & Habermann, A.M. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* **34**, 947–960 (2011).
52. Reif, K., Ekland, E.H., Nakano, H., Lipp, M., Förster, R. & Cyster, J.G. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* **416**, 94–99 (2002).
53. Jacob, J., Kassir, R. & Kelsoe, G. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of the responding cell populations. *The Journal of Experimental Medicine* **173**, 1165–1175 (1991).
54. Kearse, K.P., Cassatt, D.R., Kaplan, A.M. & Cohen, D.A. The requirement for surface Ig signalling as a prerequisite for T cell:B cell interactions. *The Journal of Immunology* **140**, 1770–1778 (1988).
55. Lanzavecchia, A. Antigen-specific interactions between T and B cells. *Nature* **314**, 537–539 (1985).
56. Sanders, V.M., Synder, J.M., Uhr, J.W. & Vitetta, E.S. Characterization of the physical interaction between antigen-specific B and T cells. *The Journal of Immunology* **137**, 2395–2404 (1986).
57. Kupfer, A., Swain, S.L., Janeway Jr, C.A. & Singer, S.J. The specific direct interaction of helper T cells and antigen-presenting B cells. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 6080–6083 (1986).
58. Noelle, R.J., McCann, J., Marshall, L. & Bartlett, W.C. Cognate interactions between helper T cells and B cells III. Contact-dependent, lymphokine-independent induction of B cell cycle entry by activated helper T cells. *The Journal of Immunology* **143**, 1807–1814 (1989).
59. Lane, P.J.L., McConnell, F.M., Clark, E.A. & Mellins, E. Rapid signalling to B cells by antigen-specific T cells requires CD18/CD54 interaction. *The Journal of Immunology* **147**, 4103–4108 (1991).

60. Qi, H., Cannons, J.L., Klauschen, F., Schwartzberg, P.L. & Germain, R.N. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* **455**, 764–769 (2008).
61. Cannons, J.L., Qi, H., Lu, K.T., Dutta, M., Gomez-Rodriguez, J., Cheng, J., Wakeland, E.K., Germain, R.N. & Schwartzberg, P.L. Optimal germinal center responses require a multistage T cell: B cell adhesion process involving integrins, SLAM-associated protein and CD84. *Immunity* **32**, 253–265 (2010).
62. Van den Eertwegh, A.J.M., Noelle, R.J., Roy, M., Shepherd, D.M., Aruffi, A., Ledbetter, J.A., Boersma, W.J.A. & Claassen, E. *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I. *In vivo* expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T-B cell interactions. *The Journal of Experimental Medicine* **178**, 1555–1565 (1993).
63. Armitage, R.J., Fanslow, W.C., Strockbine, L., Sato, T.A., Clifford, K.N., Macduff, B.M., Anderson, D.M., Gimpel, S.D., Davis-Smith, T. & Maliszewski, C.R. Molecular and biological characterisation of a murine ligand for CD40. *Nature* **357**, 80–82 (1992).
64. Noelle, R.J., Roy, M., Shepherd, D.M., Stamenkovic, I., Ledbetter, J.A. & Aruffo, A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6550–6554 (1992).
65. Linsley, P.S., Clark, E.A. & Ledbetter, J.A. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 5031–5035 (1990).
66. Clark, E.A. & Ledbetter, J.A. How B and T cells talk to each other. *Nature* **367**, 425–428 (1994).
67. Kupfer, H., Monks, C.R.F. & Kupfer, A. Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: Immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. *The Journal of Experimental Medicine* **179**, 1507–1515 (1994).
68. Ducheze, S., Rodrigues, M., Bertrand, F. & Valitutti, S. Reciprocal polarization of T and B cells at the immunological synapse. *The Journal of Immunology* **187**, 1–10 (2011).
69. Bartlett, W.C., McCann, J., Shepherd, D.M., Roy, M. & Noelle, R.J. Cognate interactions between helper T cells and B cells IV. Requirements for the expression of effector phase activity by helper T cells. *The Journal of Immunology* **145**, 3956–3962 (1990).
70. Bartlett, W.C., Michael, A., McCann, J., Yuan, D., Claassen, E. & Noelle, R.J. Cognate interactions between helper T cells and B cells II. Dissection of cognate help by using a class-II restricted, antigen-specific, IL-2 dependent helper T cell clone. *The Journal of Immunology* **143**, 1745–1754 (1989).
71. Toellner, K.-M., Luther, S.A., Sze, D.M.-Y., Choy, R.K.-W., Taylor, D.R., MacLennan, I.C. & Acha-Orbea, H. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *The Journal of Experimental Medicine* **187**, 1193–1204 (1998).
72. Toellner, K.-M., Gulbranson-Judge, A., Taylor, D.R., Sze, D.M.-Y. & MacLennan, I.C. Immunoglobulin switch transcript production *in vivo* related to the site and time of antigen-specific B cell activation. *The Journal of Experimental Medicine* **183**, 2303–2312 (1996).
73. Schwickert, T., Victoria, G.D., Fooksman, D.R., Kamphorst, A.O., Mugnier, M.R., Gitlin, A.D., Dustin, M.L. & Nussenzweig, M.C. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *The Journal of Experimental Medicine* **208**, 1243–1252 (2011).
74. Coffey, F., Alabyev, B. & Manser, T. Initial clonal expansion of germinal center B cells takes place at the perimeter of follicles. *Immunity* **30**, 599–609 (2009).
75. Chan, T.D., Gatto, D., Wood, K., Camidge, T., Basten, A. & Brink, R. Antigen affinity controls rapid T-dependent antibody production by driving the expansion rather than the

- differentiation of extrafollicular migration of early plasmablasts. *The Journal of Immunology* **183**, 3139–3149 (2009).
76. Pape, K.A., Kouskoff, V., Nemazee, D., Tang, H.L., Cyster, J.G., Tze, L.E., Hippen, K.L., Behrens, T.W. & Jenkins, M.K. Visualization of the genesis and fate of isotype-switched B cells during a primary immune response. *The Journal of Experimental Medicine* **197**, 1677–1687 (2003).
77. Jacob, J. & Kelsoe, G. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *The Journal of Experimental Medicine* **176**, 679–687 (1992).
78. Paus, D., Phan, T.G., Chan, T.D., Gardram, S., Basten, A. & Brink, R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal centre B cell differentiation. *The Journal of Experimental Medicine* **203**, 1081–1091 (2006).
79. O'Connor, B.P., Vogel, L.A., Zhang, W., Loo, W., Shnider, D., Lind, E.F., Ratliff, M., Noelle, R.J. & Erickson, L.D. Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. *The Journal of Immunology* **177**, 7723–7732 (2006).
80. Dal Porto, J.M., Harberman, A.M., Shlomchik, M.J. & Kelsoe, G. Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. *The Journal of Immunology* **161**, 5373–5381 (1998).
81. Kaji, T., Ishige, A., Hikida, M., Taka, J., Hijikata, A., Kubo, M., Nagashima, T., Takahashi, Y., Kurosaki, T., Okada, M., Ohara, O., Rajewsky, K. & Takemori, T. Distinct cellular pathways select germline-encoded and somatically mutated antibodies into immunological memory. *The Journal of Experimental Medicine* **209**, 2079–2097 (2012).
82. Vinuesa, C.G., Gulbranson-Judge, A., Khan, M., O'Leary, P., Cascalho, M., Wabl, M., Klaus, G.G.B., Owen, M.J. & MacLennan, I.C.M. Dendritic cells associated with plasmablast survival. *The European Journal of Immunology* **29**, 3712–3721 (1999).
83. Odegard, J.M., Marks, B.R., DiPlacido, L.D., Poholek, A.C., Kono, D.H., Dong, C., Flavell, R.A. & Craft, J. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *The Journal of Experimental Medicine* **205**, 2873–2886 (2008).
84. Jacob, J., Miller, C. & Kelsoe, G. *In situ* studies of the antigen-driven somatic hypermutation of immunoglobulin genes. *Immunology and Cell Biology* **70**, 145–152 (1992).
85. Takahashi, Y., Dutta, P.R., Cerasoli, D.M. & Kelsoe, G. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *The Journal of Experimental Medicine* **187**, 885–895 (1998).
86. Smith, K.G.S., Light, A., Nossal, G.J.V. & Tarlinton, D. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *The EMBO Journal* **16**, 2996–3006 (1997).
87. Relmold, A.M., Iwakoshi, N.N., Manis, J., Vallabhajosyula, P., Szomolanyi-Tsuda, E., Gravalles, E.M., Friend, D., Grusby, M.J., Alt, F. & Glimcher, L.H. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412**, 300–307 (2001).
88. Shaffer, A.L., Lin, K.-I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltner, J.M., Yang, L., Zhao, H., Calame, K. & Staudt, L.M. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* **17**, 51–62 (2002).
89. Shapiro-Shelef, M., Lin, K.-I., McHeyzer-Williams, L.J., Liao, J., McHeyzer-Williams, M.G. & Calame, K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* **19**, 607–620 (2003).
90. MacLennan, I.C.M., Toellner, K.-M., Cunningham, A.F., Serre, K., Sze, D.M.-Y., Zúñiga, E., Cook, M.C. & Vinuesa, C.G. Extrafollicular antibody responses. *Immunological Reviews* **194**, 8–18 (2003).

91. Butcher, E.C., Rouse, R.V., Coffman, R.L., Nottenburg, C.N., Hardy, R.R. & Weissman, I.L. Surface phenotype of peyer's patch germinal center cells: Implications for the role of germinal centres in B cell differentiation. *The Journal of Immunology* **129**, 2698–2707 (1982).
92. Coico, R.F., Bhogal, B.S. & Thorbecke, G.J. Relationship of germinal centers in lymphoid tissue to immunological memory VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. *The Journal of Immunology* **131**, 2254–2257 (1983).
93. Schwickert, T.A., Alabyev, B., Manser, T. & Nussenzweig, M.C. Germinal center reutilization by newly activated B cells. *The Journal of Experimental Medicine* **206**, 2907–2914 (2009).
94. Kimoto, H., Nagaoka, H., Adachi, Y., Mizuochi, T., Azuma, T., Yagi, T., Sata, T., Yonehara, S., Tsunetsugu-Yokota, Y., Taniguchi, M. & Takemori, T. Accumulation of somatic hypermutation and antigen-driven selection in rapidly cycling surface Ig⁺ germinal centre (GC) B cells which occupy GC at high frequency during the primary anti-hapten immune response in mice. *The European Journal of Immunology* **27**, 268–279 (1997).
95. Hauser, A.E., Junt, T., Mempel, T.R., Sneddon, M.W., Kleinstein, S.H., Henrickson, S.E., von Andrian, U.H., Shlomchik, M.J. & Haberman, A.M. Definition of germinal-center B cell migration *in vivo* reveals predominant intrazonal circulation patterns. *Immunity* **26**, 655–667 (2007).
96. Allen, C.D.C., Okada, T., Tang, H.L. & Cyster, J.G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528–531 (2007).
97. Victora, G.D., Schwickert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L. & Nussenzweig, M.C. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* **143**, 592–605 (2010).
98. Blink, E.J., Light, A., Kallies, A., Nutt, S.L., Hodgkin, P.D. & Tarlinton, D. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *The Journal of Experimental Medicine* **201**, 545–554 (2005).
99. Thorbecke, G.J., Amin, A.R. & Tsiagbe, V.K. Biology of germinal centers in lymphoid tissue. *The FASEB Journal* **8**, 832–840 (1994).
100. Allen, C.D.C., Okada, T. & Cyster, J.G. Germinal-center organization and cellular dynamics. *Immunity* **27**, 190–202 (2007).
101. Onizuka, T., Moriyama, M., Yamochi, T., Kuroda, T., Kazama, A., Kanazawa, N., Sato, K., Kato, T., Ota, H. & Mori, S. Bcl-6 gene product, a 92- to 98-kD nuclear phosphoprotein, is highly expressed in germinal center B cells and their neoplastic counterparts. *Blood* **86**, 28–37 (1995).
102. Dent, A.L., Shaffer, A.L., Yu, X., Allman, D. & Staudt, L.M. Control of inflammation, cytokine expression and germinal center formation by Bcl-6. *Science* **276**, 589–592 (1997).
103. Kitano, M., Moriyama, S., Ando, Y., Hikida, M., Mori, Y., Kurosaki, T. & Okada, T. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* **34**, 961–972 (2011).
104. Willis, S.N., Good-Jacobson, K.L., Curtis, J., Light, A., Tellier, J., Shi, W., Smyth, M.J., Tarlinton, D.M., Belz, G.T., Corcoran, L.M., Kallies, A. & Nutt, S.L. Transcription factor IRF4 regulates germinal center cell formation through a B cell-intrinsic mechanism. *The Journal of Immunology* **192**, 3200–3206 (2014).
105. Mittrücker, H.-W., Matsuyama, T., Grossman, A., Kündig, T.M., Potter, J., Shahinian, A., Wakeham, A., Patterson, B., Ohashi, P.S. & Mak, T.W. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science* **275**, 540–543 (1997).
106. Ochiai, K., Maienschein-Cline, M., Simonetti, G., Chen, J., Rosenthal, R., Brink, R., Chong, A.S., Klein, U., Dinner, A.R., Singh, H. & Sciammas, R. Transcriptional regulation of

- germinal center B and plasma cell fates by dynamical control of IRF4. *Immunity* **38**, 918–929 (2013).
107. Allen, C.D.C. & Cyster, J.G. Follicular dendritic cell networks of primary follicles and germinal centers: Phenotype and function. *Seminars in Immunology* **20**, 14–25 (2008).
108. Vinuesa, C., Linterman, M.A., Goodnow, C.C. & Randall, K.L. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunological Reviews* **237**, 72–89 (2010).
109. Fazilleau, N., Mark, L., McHeyzer-Williams, L.J. & McHeyzer-Williams, M.G. Follicular Helper T cells: Lineage and location. *Immunity* **30**, 324–335 (2009).
110. Ma, C.S., Deenick, E.K., Batten, M. & Tangye, S.G. The origins, function and regulation of T follicular helper cells. *The Journal of Experimental Medicine* **209**, 1241–1253 (2012).
111. Ramiscal, R.R. & Vinuesa, C.G. T-cell subsets in the germinal center. *Immunological Reviews* **252**, 146–155 (2013).
112. Suzuki, K., Grigorova, I., Phan, T.G., Kelly, L.M. & Cyster, J.G. Visualizing B cell capture of cognate antigen from follicular dendritic cells. *The Journal of Experimental Medicine* **206**, 1485–1493 (2009).
113. Aydar, Y., Sukumar, S., Szakal, A.K. & Tew, J.G. The influence of immune complex-bearing follicular dendritic cells on the IgM response, Ig class switching, and production of high affinity IgG. *The Journal of Immunology* **174**, 5358–5366 (2005).
114. Wu, Y., Sukumar, S., Shikh, M.E.E., Best, A.M., Szakal, A.K. & Tew, J.G. Immune complex-bearing follicular dendritic cells deliver a late antigen signal that promotes somatic hypermutation. *The Journal of Immunology* **180**, 281–290 (2008).
115. Choe, J., Kim, H.-S., Zhang, X., Armitage, R.J. & Choi, Y.S. Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. *The Journal of Immunology* **157**, 1006–1016 (1996).
116. Koopman, G., Keehnen, R.M.J., Lindhout, E., Zhou, D.F.H., de Groot, C. & Pals, S.T. Germinal center B cells rescued from apoptosis by CD40 ligation or attachment to follicular dendritic cells, but not by engagement of surface immunoglobulin or adhesion receptors, become resistant to CD95-induced apoptosis. *The European Journal of Immunology* **27**, 1–7 (1997).
117. Keşmir, C. & De Boer, R.J. A mathematical model on germinal center kinetics and termination. *The Journal of Immunology* **163**, 2463–2469 (1999).
118. Randall, T.D., Heath, A.W., Santos-Argumedo, L., Howard, M.C., Weissman, I.L. & Lund, F.E. Arrest of B lymphocyte terminal differentiation by CD40 signalling: Mechanisms for lack of antibody-secreting cells in germinal centers. *Immunity* **8**, 733–742 (1998).
119. Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M. & Förster, R. Follicular helper T cells express CXC chemokine receptor 5, localise to B cell follicles, and support immunoglobulin production. *The Journal of Experimental Medicine* **192**, 1545–1551 (2000).
120. Kawabe, T., Naka, T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T. & Kikutani, H. The immune response in CD40-deficient mice: Impaired immunoglobulin class switching and germinal center formation. *Immunity* **1**, 167–178 (1994).
121. Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J. & Vinuesa, C. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *The Journal of Experimental Medicine* **207**, 353–363 (2010).
122. Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.-M., Smyth, M.J., Nutt, S.L. & Tarlinton, D. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *The Journal of Experimental Medicine* **207**, 365–378 (2010).

123. Bryant, V.L., Ma, C.S., Avery, D.T., Li, Y., Good, K.L., Corcoran, L.M., de Waal Malefyt, R. & Tangye, S.G. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5⁺ T follicular helper cells. *The Journal of Immunology* **179**, 8180–8190 (2007).
124. Reinhardt, R.L., Liang, H.-E. & Locksley, R.M. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nature Immunology* **10**, 385–393 (2009).
125. Yusuf, I., Kageyama, R., Monticelli, L., Johnston, R.J., DiToro, D., Hansen, K., Barnett, B. & Crotty, S. Germinal center T follicular helper cell IL-4 production is dependent on signalling lymphocytic activation molecule receptor (CD150). *The Journal of Immunology* **185**, 190–202 (2010).
126. Zhang, Y., Meyer - Hermann, M., Geroche, L.A., Figge, M.T., Khan, M., Goodall, M., Young, S.P., Reynolds, A., Falciani, F., Waisman, A., Notley, C.A., Ehrenstein, M.R., Kosco - Vilbois, M. & Toellner, K.-M. Germinal centre B cells govern their own fate via antibody feedback. *The Journal of Experimental Medicine* **210**, 1–8 (2013).
127. Phan, T.G., Paus, D., Chan, T.D., Turner, M.L., Nutt, S.L., Basten, A. & Brink, R. High affinity germinal center B cells are actively selected into the plasma cell compartment. *The Journal of Experimental Medicine* **203**, 2419–2424 (2006).
128. Han, S., Zheng, B., Dal Porto, J. & Kelsoe, G. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self tolerance. *The Journal of Experimental Medicine* **182**, 1635–1644 (1995).
129. Ekland, E.H., Forster, R., Lipp, M. & G, C.J. Requirements for follicular exclusion and competitive elimination of autoantigen-binding B cells. *The Journal of Immunology* **172**, 4700–4708 (2004).
130. Shlomchik, M.J. & Weisel, F. Germinal center selection and the development of memory B and plasma cells. *Immunological Reviews* **247**, 52–63 (2012).
131. Good-Jacobson, K.L. & Shlomchik, M.J. Plasticity and Heterogeneity in the generation of memory B cells and long-lived plasma cells: The influence of germinal center interactions and dynamics. *The Journal of Immunology* **185**, 3117–3125 (2010).
132. Meyer-Hermann, M., Mohr, E., Pelletier, N., Zhang, Y., Victora, G.D. & Toellner, K.-M. A theory of germinal center B cell selection, division and exit. *Cell Reports* **2**, 162–174 (2012).
133. Manz, R.A., Thiel, A. & Radbruch, A. Lifetime of plasma cells in the bone marrow. *Nature* **388**, 133–134 (1997).
134. Silfka, M.K., Antia, R., Whitmire, J.K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363–372 (1998).
135. Driver, D.J., McHeyzer-Williams, L.J., Cool, M., Stetson, D.B. & McHeyzer-Williams, M.G. Development and maintenance of a B220⁺ Memory B cell compartment. *The Journal of Immunology* **167**, 1393–1405 (2001).
136. McHeyzer-Williams, L.J., Cool, M. & McHeyzer-Williams, M.G. Antigen-specific B cell Memory: Expression and replenishment of a novel B220⁺ Memory B cell compartment. *The Journal of Experimental Medicine* **191**, 1149–1165 (2000).
137. Dogan, I., Bertocchi, B., Vilmont, V., Delbos, F., Mégret, J., Storck, S., Reynaud, C.-A. & Weill, J.-C. Multiple layers of B cell memory with different effector functions. *Nature Immunology* **10**, 1292–1299 (2009).
138. Pape, K.A., Taylor, J.J., Maul, R.W., Gearhart, P.J. & Jenkins, M.K. Different B cell populations mediate early and late memory during an endogenous immune response. *Science* **331**, 1203–1207 (2011).
139. Angelin-Duclos, C., Cattoretti, G., Lin, K.-I. & Calame, K. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression *in vivo*. *The Journal of Immunology* **165**, 5462–5471 (2000).

140. Klein, U., Casola, S., Cattoretti, G., Shen, Q., Lia, M., Mo, T., Ludwig, T., Rajewsky, K. & Dalla-Favera, R. Transcription factor IRF4 controls plasma cell differentiation and class switch recombination. *Nature Immunology* **7**, 773–782 (2006).
141. Sciammas, R., Shaffer, A.L., Schatz, J.H., Zhao, H., Staudt, L.M. & Singh, H. Graded expression of Interferon Regulatory Factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* **25**, 225–236 (2006).
142. Shaffer, A.L., Yu, X., He, Y., Boldrick, J., Chan, E.P. & Staudt, L.M. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity* **13**, 199–212 (2000).
143. Shaffer, A.L., Shapiro-Shelef, M., Iwakoshi, N.N., Lee, A.-H., Qian, S.-B., Zhao, H., Yu, X., Yang, L., Tan, B.K., Rosenwald, A., Hurt, E.M., Petroulakis, E., Sonenberg, N., Yewdell, J.W., Calame, K., Glimcher, L.H. & Staudt, L.M. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* **21**, 81–93 (2004).
144. Todd, D.J., McHeyzer-Williams, L.J., Kowal, C., Lee, A.-H., Volpe, B.T., Diamond, B., McHeyzer-Williams, M.G. & Glimcher, L.H. XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. *The Journal of Experimental Medicine* **206**, 2151–2159 (2009).
145. Barberis, A., Widehorn, K., Vitelli, L. & Busslinger, M. A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes & Development* **4**, 849–859 (1990).
146. Lin, K.-I., Angelin-Duclos, C., Kuo, T.C. & Calame, K. Blimp-1- dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Molecular and Cellular Biology* **22**, 4771–4780 (2002).
147. Nera, K.-P., Kohonen, P., Narvi, E., Peippo, A., Mustonen, L., Terho, P., Koskela, K., Buerstedde, J.-M. & Lassila, O. Loss of Pax5 promotes plasma cell differentiation. *Immunity* **24**, 283–293 (2006).
148. Kim, C.H. & Broxmeyer, H.E. Chemokines: Signal lamps for trafficking of T and B cells for development and effector function. *The Journal of Leukocyte Biology* **65**, 6–15 (1999).
149. Stein, J.V. & Nombela-Arrieta, C. Chemokine control of lymphocyte trafficking: A general overview. *Immunology* **116**, 1–12 (2005).
150. Murdoch, C. & Finn, A. Chemokine receptors and their role in inflammation and infectious disease. *Blood* **95**, 3032–3043 (2000).
151. Zlotnik, A. & Yoshie, O. Chemokines: A new classification system and their role in immunity. *Immunity* **12**, 121–127 (2000).
152. Allen, S.J., Crown, S.E. & Handel, T.M. Chemokine:Receptor structure, interactions and antagonism. *Annual Review of Immunology* **25**, 787–820 (2007).
153. Soriano, S.F., Serrano, A., Hernanz-Falcón, P., Martín de Ana, A., Monterrubio, M., Martínez-A, C., Rodríguez-Frade, M. & Mellado, M. Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *The European Journal of Immunology* **33**, 1328–1333 (2003).
154. Moser, B. & Willmann, K. Chemokines: Role in inflammation and immune surveillance. *The Annals of the Rheumatic Diseases* **63**, ii84–ii89 (2004).
155. Esche, C., Stellato, C. & Beck, L.A. Chemokines: Key players in Innate and Adaptive Immunity. *The Journal of Investigative Dermatology* **125**, 615–628 (2005).
156. Förster, R., Mattis, A.E., Kremmer, E., Wolf, E., Brem, G. & Lipp, M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87**, 1037–1047 (1996).
157. Gatto, D., Paus, D., Basten, A., Mackay, C. & Brink, R. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune response. *Immunity* **31**, 259–269 (2009).

158. Gatto, D., Wood, K. & Brink, R. EBI2 operates independently of but in cooperation with CXCR5 and CCR7 to direct B cell migration and organization in follicles and the germinal center. *The Journal of Immunology* **187**, 4621–4628 (2011).
159. Allen, C.C.C., Ansel, K.M., Low, C., Lesley, R., Tamamura, H., Fujii, N. & Cyster, J.G. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nature Immunology* **5**, 943–952 (2004).
160. Wehrli, N., Legler, D.F., Finke, D., Toellner, K.-M., Loetscher, P., Baggiolini, M., MacLennan, I.C.M. & Acha-Orbea, H. Changing responsiveness to chemokines allows medullary plasmablasts to leave lymph nodes. *The European Journal of Immunology* **31**, 609–616 (2001).
161. Hargreaves, D.C., Hyman, P.L., Lu, T.T., Ngo, V.N., Bidgol, A., Suzuki, G., Zou, Y.R., Littman, D.R. & Cyster, J.G. A coordinated change in chemokine responsiveness guides plasma cell movements. *The Journal of Experimental Medicine* **194**, 45–56 (2001).
162. Liao, F., Lee, H.H. & Farber, J.M. Cloning of STRL22, a new human gene encoding a G-protein-coupled receptor related to chemokine receptors and located on chromosome 6q27. *Genomics* **15**, 175–180 (1997).
163. Baba, M., Imai, T., Nishimura, M., Kakizaki, M., Takagi, S., Heishima, K., Nomiyama, H. & Yoshie, O. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. *The Journal of Biological Chemistry* **272**, 14893–14898 (1997).
164. Liao, F., Alderson, R., Su, J., Ullrich, S.J., Kreider, B.L. & Farber, J.M. STRL22 is a receptor for the CC chemokine MIP-3 α . *Biochemical and Biophysical Research Communications* **236**, 212–217 (1997).
165. Greaves, D.R., Wang, W., Dairaghi, D.J., Dieu, M.C., de Saint-Vis, B., Franz-Bacon, K., Rossi, D., Caux, C., McClanahan, T., Gordon, S., Zlotnik, A. & Schall, T.J. CCR6, a chemokine receptor that interacts with Macrophage Inflammatory Protein 3 ∞ and is highly expressed in human dendritic cells. *The Journal of Experimental Medicine* **186**, 837–844 (1997).
166. Yang, D., Chertov, O., Bykovskaia, N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schröder, J.M., Wang, J.M., Howard, M.Z. & Oppenheim, J.J. β -Defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**, 525–528 (1999).
167. Oppenheim, J.J., Biragyn, A., Kwak, L.W. & Yang, D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *The Annals of the Rheumatic Diseases* **62**, ii17–ii21 (2003).
168. Harder, J., Gläser, R. & Schröder, J.M. Human antimicrobial proteins—effectors of innate immunity. *The Journal of Endotoxin Research* **13**, 317–338 (2007).
169. Nakatsuji, T. & Gallo, R.L. Antimicrobial peptides: Old molecules with new ideas. *The Journal of Investigative Dermatology* **132**, 887–895 (2012).
170. Hieshima, K., Imai, T., Opdenakker, G., Damme Van, J., Kusuda, J., Tei, H., Sakaki, Y., Takatsuki, K., Miura, R., Yoshie, O. & Nomiyama, H. Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. *The Journal of Biological Chemistry* **272**, 5846–5853 (1997).
171. Power, C.A., Church, D.J., Meyer, A., Alouani, S., Proudfoot, A.E.I., Clark-Lewis, I., Sozzani, S., Mantovani, A. & Wells, T.N.C. Cloning and characterisation of a specific receptor for the novel CC chemokine MIP-3 ∞ from lung dendritic cells. *The Journal of Experimental Medicine* **186**, 825–835 (1997).
172. Varona, R., Zaballo, A., Gutiérrez, J., Martín, P., Roncal, F., Albar, J.P., Ardavin, C. & Márquez, G. Molecular cloning, functional characterisation and mRNA expression analysis of the murine chemokine receptor CCR6 and its specific ligand MIP-3 ∞ . *Federation of European Biochemical Societies Letters* **440**, 188–194 (1998).
173. Varona, R., Villares, R., Carramolino, L., Goya, Í., Zaballo, Á., Gutiérrez, J., Torres, M., Martínez-A, C. & Márquez, G. CCR6-deficient mice have impaired leukocyte homeostasis

- and altered delayed-type hypersensitivity responses. *The Journal of Clinical Investigation* **107**, R37–R45 (2001).
174. Cook, D.N., Prosser, D.M., Forster, R., Zhang, J., Kuklin, N.A., Abbondanzo, S.J., Niu, X., Chen, S., Manfra, D.J., Wiekowski, M.T., Sullivan, L.M., Smith, S.R., Greenberg, H.B., Narula, S.K., Lipp, M. & Lira, S.A. CCR6 mediates dendritic cell localisation, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* **12**, 495–503 (2000).
175. Zhao, X., Sato, A., Dela Cruz, C.S., Linehan, M., Luegering, A., Kucharzik, T., Shirakawa, A.-K., Marquez, G., Farber, J.M., Williams, I. & Iwasaki, A. CCL9 is secreted by the follicle-associated epithelium and recruits dome region peyer's patch CD11b⁺ dendritic cells. *The Journal of Immunology* **171**, 2797–2803 (2003).
176. McDonald, K.G., McDonough, J.S., Wang, C., Kucharzik, T., Williams, I.R. & Newberry, R.D. CC chemokine receptor 6 expression by B lymphocytes is essential for the development of isolated lymphoid follicles. *Gastrointestinal, Hepatobiliary and Pancreatic Pathology* **170**, 1229–1240 (2007).
177. Luegering, A., Floer, M., Westphal, S., Masser, C., Spahn, T.W., Schmidt, M.A., Domschke, W., Williams, I.R. & Kucharzik, T. Absence of CCR6 inhibits CD4⁺ regulatory T-cell development and M-cell formation inside peyer's patches. *The American Journal of Pathology* **166**, 1647–1654 (2005).
178. Ebisawa, M., Hase, K., Takahashi, D., Kitamura, H., Knoop, K.A., Williams, I.R. & Ohno, H. CCR6^{hi}CD11c^{int} B cells promote M-cell differentiation in Peyer's patch. *International Immunology* **23**, 261–269 (2011).
179. Varona, R., Cadenas, V., Gómez, L., Martínez-A, C. & Márquez, G. CCR6 regulates CD4⁺ T-cell-mediated acute graft-versus-host disease responses. *Blood* **106**, 18–26 (2005).
180. Geherin, S.A., Fintushel, S.R., Lee, M.H., Wilson, R.P., Patel, R.T., Alt, C., Young, A.J., Hay, J.B. & Debes, G.F. The skin, a novel niche for recirculating B cells. *The Journal of Immunology* **188**, 6027–6035 (2012).
181. Kucharzik, T., Hudson III, J.T., Waikel, R.L., Martin, W.D. & Williams, I.R. CCR6 expression distinguishes mouse myeloid and lymphoid dendritic cell subsets: demonstration using a CCR6 EGFP knock-in mouse. *The European Journal of Immunology* **32**, 104–112 (2002).
182. Wiede, F., Fromm, P.D., Comerford, I., Kara, E., Bannan, J., Schub, W., Ranasinghe, C., Tarlinton, D., Winkler, T., McColl, S. & Körner, H. CCR6 is transiently upregulated on B cells after activation and modulates the germinal center reaction in the mouse. *Immunology and Cell Biology* **91**, 335–339 (2013).
183. Liao, F., Rabin, R.L., Smith, C.S., Sharma, G., Nutman, T.B. & Farber, J.M. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 γ . *The Journal of Immunology* **162**, 186–194 (1999).
184. Brandes, M., Legler, D.F., Spoerri, B., Schaerli, P. & Moser, B. Activation-dependent modulation of B lymphocyte migration to chemokines. *International Immunology* **12**, 1285–1292 (2000).
185. Krzysiek, R., Lefevre, E.A., Bernard, J., Foussat, A., Galanaud, P., Louache, F. & Richard, Y. Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3 γ /CCL20 in human B cells. *Blood* **96**, 2338–2345 (2000).
186. Liao, F., Shirakawa, A.-K., Foley, J.F., Rabin, R.L. & Farber, J.M. Human B cells become highly responsive to Macrophage-Inflammatory Protein-3 γ /CC Chemokine Ligand-20 after cellular activation without changes in CCR6 expression or ligand binding. *The Journal of Immunology* **168**, 4871–4880 (2002).
187. Corcione, A., Tortolina, G., Bonecchi, R., Battilana, N., Taborelli, G., Malavasi, F., Sozzani, S., Ottonello, L., Dallegri, F. & Pistoia, V. Chemotaxis of human tonsil B lymphocytes to CC chemokine receptor (CCR) 1, CCR2 and CCR4 ligands is restricted to non-germinal center cells. *International Immunology* **14**, 883–892 (2002).

188. Bowman, E.P., Campbell, J.J., Soler, D., Dong, Z., Manlongat, N., Picarella, D., Hardy, R.R. & Buthcer, E.C. Developmental switches in chemokine response profiles during B cell differentiation and maturation. *The Journal of Experimental Medicine* **191**, 1303–1317 (2000).
189. Meissner, A., Zilles, O., Varona, R., Jozefowski, K., Ritter, U., Marquez, G., Hallmann, R. & Körner, H. CC chemokine ligand 20 partially controls adhesion of naïve B cells to activated endothelial cells under shear stress. *Blood* **102**, 2724–2727 (2003).
190. Bhattacharya, D., Cheah, M.T., Franco, C.B., Hosen, N., Pin, C.L., Sha, W.C. & Weissman, I.L. Transcriptional profiling of antigen-dependent murine B cell differentiation and memory formation. *The Journal of Immunology* **179**, 6808–6819 (2007).
191. Goodnow, C.C., Vinuesa, C., Randall, K.L., Mackay, F. & Brink, R. Control systems and decision making for antibody production. *Nature Immunology* **11**, 681–688 (2010).
192. Shlomchik, M.J. Sites and stages of autoreactive B cell activation and regulation. *Immunity* **28**, 18–28 (2008).
193. Wu, Y., Ren, M., Yang, R., Liang, X., Ma, Y., Tang, Y., Huang, L., Ye, J., Chen, K., Wang, P. & Shen, H. Reduced immunomodulation potential of bone marrow-derived mesenchymal stem cells induced CCR4⁺ CCR6⁺ Th/Treg subset imbalance in Ankylosing Spondylitis. *Arthritis Research and Therapy* **13**, 1–15 (2011).
194. Aoki, N., Kido, M., Iwamoto, S., Nishiura, H., Maruoka, R., Tanaka, J., Watanabe, T., Tanaka, Y., Okazaki, T., Chiba, T. & Watanabe, N. Dysregulated generation of Follicular helper T cells in the spleen triggers Fatal Autoimmune Hepatitis in mice. *Gastroenterology* **140**, 1322–1333 (2011).
195. Méndez-Reguera, A., Pérez-Montesinos, G., Alcántara-Hernández, M., Martínez-Estrada, V., Cazarin-Barrientos, J.R., Rojas-Espinosa, O., Jurado-Santacruz, F., Huerta-Yepe, S. & Bonifaz-Alfonzo, L. Pathogenic CCR6⁺ dendritic cells in the skin lesions of discoid lupus patients: A role for damage-associated molecular patterns. *The European Journal of Dermatology* **23**, 169–182 (2013).
196. Fagin, U., OPitann, S., Gross, W.L. & Lamprecht, P. Increased frequency of CCR4⁺ and CCR6⁺ memory T-cells including CCR7⁺ CD45RA^{med} very early memory cells in Granulomatosis with Polyangitis (Wegener's). *Arthritis Research and Therapy* **14**, 1–9 (2012).
197. Kleinewietfeld, M., Puentes, F., Borsellino, G., Battistini, L., Röttschke, O. & Falk, K. CCR6 expression defines regulatory effector/memory-like cells within the CD25⁺ CD4⁺ T-cell subset. *Blood* **105**, 2877–2886 (2005).
198. Elhofy, A., DePaolo, R.W., Lira, S.A., Lukacs, N.W. & Karpus, W.J. Mice deficient for CCR6 fail to control chronic Experimental Autoimmune Encephalomyelitis. *The Journal of Neuroimmunology* **213**, 91–99 (2009).
199. Villares, R., Cadenas, V., Lozano, M., Almonacid, L., Zaballos, A., Martínez-A, C. & Varona, R. CCR6 regulates EAE pathogenesis by controlling regulatory CD4⁺ T-cell recruitment to target tissues. *The European Journal of Immunology* **39**, 1671–1681 (2009).
200. Reboldi, A., Coisne, C., Baumjohann, D., Benvenuto, F., Bottinelli, D., Lira, S., Uccelli, A., Lanzavecchia, A., Engelhardt, B. & Sallusto, F. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nature Immunology* **10**, 514–523 (2009).
201. Moriguchi, K., Miyamoto, K., Tanaka, N., Yoshie, O. & Kusunoki, S. The importance of CCR4 and CCR5 in Experimental Autoimmune Encephalomyelitis. *The Journal of Neuroimmunology* **257**, 53–58 (2013).
202. Serafini, B., Columba-Cabezas, S., Di Rosa, F. & Aloisi, F. Intracerebral recruitment and maturation of Dendritic cells in the onsets and progression of Experimental Autoimmune Encephalomyelitis. *The American Journal of Pathology* **157**, 1991–2002 (2000).

203. Liston, A., Kohler, R.E., Townley, S., Haylock-Jacobs, S., Comerford, I., Caon, A.C., Webster, J., Harrison, J.M., Swann, J., Clark-Lewis, I., Korner, H. & McColl, S.R. Inhibition of CCR6 function reduces the severity of Experimental Autoimmune Encephalomyelitis via effects on the priming phase of the immune response. *The Journal of Immunology* **182**, 3121–3130 (2009).
204. Kohler, R.E., Caon, A.C., Willenborg, D.O., Clark-Lewis, I. & McColl, S. A role for Macrophage Inflammatory Protein-3 α / CC Chemokine Ligand 20 in immune priming during T cell-mediated inflammation of the central nervous system. *The Journal of Immunology* **170**, 6298–6306 (2003).
205. Ambrosini, E., Columba-Cabezas, S., Serafini, B., Muscella, A. & Aloisi, F. Astrocytes are the major intracerebral source of Macrophage Inflammatory Protein-3 α /CCL20 in relapsing Experimental Autoimmune Encephalomyelitis and *in vitro*. *Glia* **41**, 290–300 (2003).
206. Hansen, A., Reiter, K., Ziprian, T., HJacobi, A., Hoffmann, A., Gosemann, M., Scholze, J., Lipsky, P.E. & Dörner, T. Dysregulation of chemokine receptor expression and function by B cells of patients with Primary Sjögren's Syndrome. *Arthritis and Rheumatism* **21**, 2109–2119 (2005).
207. Li, X.-y., Wu, Z.-b., Zheng, Z.-h., Li, X.-y., Chen, L.-n. & Zhu, P. Role of the frequency of blood CD4⁺ CXCR5⁺ CCR6⁺ T cells in autoimmunity in patients with Sjögren's Syndrome. *Biochemical and Biophysical Research Communications* **422**, 238–244 (2012).
208. Li, D., Li, J., Duan, Y. & Zhou, X. Expression of LL-37, human beta defensin-2, and CCR6 mRNA in patients with Psoriasis Vulgaris. *The Journal of Huazhong University of Science and Technology* **24**, 404–406 (2004).
209. Homey, B., Dieu-Nosjean, M.-C., Wiesenborn, A., Massacrier, C., Pin, J.-J., Oldham, E., Catron, D.M., Buchanan, M.E., Müller, A., Malefyt, R.d.W., Deng, G., Orozco, R., Ruzicka, T., Lehmann, P., Lebecque, S., Caux, C. & Zlotnik, A. Up-regulation of Macrophage Inflammatory Protein-3 α /CCL20 and CC Chemokine Receptor 6 in Psoriasis. *The Journal of Immunology* **164**, 6621–6632 (2000).
210. Teraki, Y., Takebayashi, M.R. & Shiohara, T. Homing receptor and chemokine receptor on intraepidermal T cells in Psoriasis Vulgaris. *Experimental Dermatology* **29**, 658–663 (2004).
211. Kochi, Y., Okada, Y., Suzuki, A., Ikari, K., Terao, C., Takahashi, A., Yamazaki, K., Hosono, N., Myouzen, K., Tsunoda, T., Kamatani, N., Furuichi, T., Ikegawa, S., Ohmura, K., Mimori, T., Matsuda, F., Iwamoto, T., Momohara, S., Yamanaka, H., Yamada, R., Kubo, M., Nakamura, Y. & Yamamoto, K. A regulatory variant in CCR6 is associated with Rheumatoid Arthritis susceptibility. *Nature Genetics* **42**, 515–519 (2010).
212. Eyre, S., Bowes, J., Diogo, D., Lee, A.-H., Barton, A., Martin, P., Zhernakova, A., Stahl, E., Viatte, S., McAllister, K., Amos, C.I., Padyukov, L., Toes, R.E.M., Huizinga, T.W.J., Wijmenga, C., Trynka, G., Franke, L., Westra, H.-J., Alfredsson, L., Hu, X., Sandor, C., de Bakker, P.I.W., Davila, S., Khor, C.C., Heng, K.K., Andrews, R., Edkins, S., Hunt, S.E., Langford, C., Symmons, D., Syndicate, B.i.R.A.G.a.G.S., Consortium, W.T.C.C., Concannon, P., Onengut-Gumuscu, S., Rich, S.S., Deloukas, P., Gonzalez-Gay, M.A., Rodriguez-Rodriguez, L., Ärletig, L., Martin, J., Rantapää-Dahlqvist, S., Plenge, R., Raychaudhuri, S., Klareskog, L., Gregersen, P.K. & Worthington, J. High density genetic mapping identifies new susceptibility loci for Rheumatoid Arthritis. *Nature Genetics* **44**, 1336–1340 (2012).
213. Okada, Y., Terao, C., Ikari, K., Kochi, Y., Ohmura, K., Suzuki, A., Kawaguchi, T., Stahl, E.A., Kurreeman, F.A.S., Nishida, N., Ohmiya, H., Myouzen, K., Takahashi, M., Sawada, T., Nishioka, Y., Yukioka, M., Matsubara, T., Wakitani, S., Teshima, R., Tohma, S., Takasugi, K., Shimada, K., Murasawa, A., Honjo, S., Matsuo, K., Tanaka, H., Tajima, K., Suzuki, T., Iwamoto, T., Kawamura, Y., Tani, H., Okazaki, Y., Sasaki, T., Gregersen, P.K., Padyukov, L., Worthington, J., Siminovitch, K.A., Lathrop, M., Taniguchi, A., Takahashi,

- A., Tokunaga, K., Kubo, M., Nakamura, Y., Kamatani, N., Mimori, T., Plenge, R.M., Yamanaka, H., Momohara, S., Yamada, R., Matsuda, F. & Yamamoto, K. Meta-analysis identifies nine new loci associated with Rheumatoid Arthritis in the Japanese population. *Nature Genetics* **44**, 511–516 (2012).
214. Stahl, E.A., Raychaudhuri, S., Remmers, E.F., Xie, G., Eyre, S., Thomson, B.P., Li, Y., Kurzeeman, F.A.S., Zhernakova, A., Hinks, A., Guiducci, C., Chen, R., Alfredsson, L., Amos, C.I., Ardlie, K.G., Consortium, B., Barton, A., Bowes, J., Brouwer, E., Burt, N.P., Catanese, J.J., Coblyn, J., Coenen, M.J.H., Costenbader, K.H., Criswell, L.A., Crusius, J.B.A., Cui, J., de Bakker, P.I.W., De Jager, P.L., Ding, B., Emery, P., Flynn, E., Harrison, P., Hocking, L.J., Huizinga, T.W.J., Kastner, D.L., Ke, X., Lee, A.T., Liu, X., Martin, P., Morgan, A.W., Padyukov, L., Posthumus, M.D., Radstake, T.R.D.J., Reid, D.M., Seielstad, M., Seldin, M.F., Shadick, N.A., Steer, S., Tak, P.P., Thomson, W., van der Helm-van Mil, A.H.M., van der Horst-Bruinsma, I.E., van der Schoot, C.E., van Riel, P.L.C.M., Weinblatt, M.E., Wilson, A.G., Wolbink, G.J., Wordsworth, B.P., Consortium, Y., Wijmenga, C., Karlson, E.W., Toes, R.E.M., de Vries, N., Begovich, A.B., Worthington, J., Siminovitch, K.A., Gregersen, P.K., Klareskog, L. & Plenge, R.M. Genome-wide association study meta-analysis identifies seven new Rheumatoid Arthritis risk loci. *Nature Genetics* **42**, 508–583 (2010).
215. Matsui, T., Akahoshi, T., Namai, R., Hasimoto, A., Kurihara, Y., Rana, M., Nishimura, A., Endo, H., Kawai, S., Takagishi, K. & Kondo, H. Selective recruitment of CCR6-expressing cells by increased production of MIP-3 α in Rheumatoid Arthritis. *Clinical and Experimental Immunology* **125**, 155–161 (2001).
216. Tanida, S., Yoshitomi, H., Nishitani, K., Ishikawa, M., Kitaori, T., Ito, H. & Nakamura, T. CCL20 produced in the cytokine network of Rheumatoid Arthritis recruits CCR6⁺ mononuclear cells and enhances the production of IL-6. *Cytokine* **47**, 112–118 (2009).
217. Hirota, K., Yoshitomi, H., Hashimoto, M., Maeda, S., Teradaira, S., Sugimoto, N., Yamaguchi, T., Nomura, T., Ito, H., Nakamura, T., Sakaguchi, N. & Sakaguchi, S. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in Rheumatoid Arthritis and its animal model. *The Journal of Experimental Medicine* **204**, 2803–2812 (2007).
218. Nanki, T., Takada, K., Komano, Y., Morio, T., Kanegane, H., Lipsky, P.E. & Miyasaka, N. Chemokine receptor expression and functional effects of chemokines on B cells: Implications in the pathogenesis of Rheumatoid Arthritis. *Arthritis, Research and Therapy* **11**, 1–11 (2009).
219. Lisignoli, G., Piacentini, A., Cristino, S., CGrassi, F., Cavallo, C., Cattini, L., Tonnarelli, B., Manferdini, C. & Facchini, A. CCL20 chemokine induces both osteoblast proliferation and osteoclast differentiation: Increased levels of CCL20 are expressed in subchondral bone tissue of Rheumatoid Arthritis patients. *The Journal of Cellular Physiology* **210**, 798–806 (2007).
220. Ruth, J.H., Shahrara, S., Park, C.C., Morel, J.C., Kumar, P., Qin, S. & Koch, A.E. Role of macrophage inflammatory protein-3 α and its ligand CCR6 in Rheumatoid Arthritis. *Laboratory Investigation* **83**, 579–588 (2003).
221. Henneken, M., Dörner, T., Burmester, G.-R. & Berek, C. Differential expression of chemokine receptors on peripheral blood B cells from patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus. *Arthritis Research and Therapy* **7**, 1–13 (2005).
222. Ruth, J.H., Rottman, B., Katschke, K.J.J., Qin, S., Wu, L., LaRosa, G., Ponath, P., Pope, R.M. & Koch, A.E. Selective lymphocyte chemokine receptor expression in the Rheumatoid joint. *Arthritis and Rheumatism* **44**, 2750–2760 (2001).
223. Li, Y.M., Chen, Z.Q., Yao, X., Yang, A.Z., Li, A.S., Liu, D.M. & Gong, J.Q. mRNA expression of chemokine receptors on peripheral blood mononuclear cells and correlation

- with clinical features in Systemic Lupus Erythematosus patients. *Chinese Medical Sciences Journal* **25**, 162–168 (2010).
224. Coz, C.L., Joublin, A., Pasquali, J.-L., Korganow, A.-S., Dumortier, H. & Monneaux, F. Circulating Tfh subset distribution is strongly affected in lupus patients with an active disease. *PLoS ONE* **8**, 1–12 (2013).
225. Brkic, Z., Corneth, O.B., van Helden-Meeuwsen, C.G., Dolhain, R.J.E.M., Maria, N.I., Paulissen, S.M.J., Davelaar, N., van Hamburg, J.P., van Daele, P.L., Dalm, V.A., van Hagen, P.M., Hazes, J.M.W., Versnel, M.A. & Lubberts, E. T-helper 17 cell cytokines and interferon type 1: Partners in crime in Systemic Lupus Erythematosus? *Arthritis Research and Therapy* **16**, 1–9 (2014).
226. Eriksson, C., Eneslätt, K., Ivanoff, J., Rantapää-Dahlqvist, S. & Sundqvist, K.G. Abnormal expression of chemokine receptors on T-cells from patients with Systemic Lupus Erythematosus. *Lupus* **12**, 766–774 (2003).
227. Koumakis, E., Bouaziz, M., Dieudé, P., Ruiz, B., Riemekasten, G., Airo, P., Müller-Nurasyid, M., Cusi, D., Matucci-Cerinic, M., Melchers, I., Salvi, E., Strauch, K., Peters, A., Cuomo, G., Hachulla, E., Diot, E., Hunzelmann, N., Caramaschi, P., Ricciari, V., Distler, J.H.W., Tarner, I., Avouac, J., Letenneur, L., Amouyel, P., Lambert, J.-C., Chicchia, G., Boileau, C. & Allanore, Y. A regulatory variant in CCR6 is associated with susceptibility to Antibiotopoisomerase-Positive Systemic Sclerosis. *Arthritis and Rheumatism* **65**, 3202–3208 (2013).
228. Petri, M., Orbai, A.-M., Alarcón, G.S., Gordon, C., Merrill, J.T., Fortin, P.R., Bruce, I.N., Isenberg, D., Wallace, D.J., Nived, O., Sturfelt, G., Ramsey-Gordon, R., Bae, S.-C., Hanly, J.G., Sánchez-Guerrero, J., Clarke, A., Aranow, C., Manzi, S., Urowitz, M., Gladman, D., Kalunian, K., Costner, M., Werth, V.P., Zoma, A., Bernatsky, S., Ruiz-Irastorza, G., Khamashta, M.A., Jacobsen, S., Buyon, J.P., Maddison, P., Dooley, M.A., van Vollenhoven, R.F., Ginzler, E., Stoll, T., Peschken, C., Jorizzo, J.L., Callen, J.P., Lim, S., Fessler, B.J., Inanc, M., Kamen, D.L., Rahman, A., Steinsson, K., Franks Jr, A.G., Sigler, L., Hameed, S., Fang, H., Pham, N., Brey, R., Weisman, M.H., McGwin Jr, G. & Magder, L.S. Derivation and validation of the Systemic Lupus International Collaborating Clinics Classification Criteria for Systemic Lupus Erythematosus. *Arthritis and Rheumatism* **64**, 2677–2686 (2012).
229. Harbeck, R.J., Bardana, E.J., Kohler, P.F. & Carr, R.I. DNA: Anti-DNA Complexes: Their detection in Systemic Lupus Erythematosus sera. *The Journal of Clinical Investigation* **52**, 789–795 (1973).
230. Tron, F., Letarte, J., Barreira, M.C.R.-A. & Inserm, P.L. Specific detection of circulating DNA:anti-DNA immune complexes in human Systemic Lupus Erythematosus sera using murine monoclonal anti-DNA antibody. *Clinical and Experimental Immunology* **49**, 481–487 (1982).
231. Koffler, D., Schur, P.H. & Kunkel, H.G. Immunological studies concerning the nephritis of Systemic Lupus Erythematosus. *The Journal of Experimental Medicine* **126**, 607–624 (1967).
232. Suci-Foca, N., Buda, J.A., Thiem, T. & Reemtsma, K. Impaired responsiveness of lymphocytes in patients with Systemic Lupus Erythematosus. *Clinical and Experimental Immunology* **18**, 295–301 (1974).
233. Hughes, P., Holt, S. & Rowell, N.R. Leukocyte migration-inhibition in Systemic Lupus Erythematosus. *Annals of the Rheumatic Diseases* **33**, 48–52 (1974).
234. Chan, O.T.M., Hannum, L.G., Haberman, A.M., Madaio, M.P. & Shlomchik, M.J. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *The Journal of Experimental Medicine* **189**, 1639–1647 (1999).

235. Shlomchik, M.J., Madaio, M.P., Ni, D., Tounstein, M. & Huszar, D. The role of B cells in lpr/lpr-induced autoimmunity. *The Journal of Experimental Medicine* **180**, 1295–1306 (1994).
236. Chan, O. & Shlomchik, M.J. A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice. *The Journal of Immunology* **160**, 51–59 (1998).
237. Nijnik, A., Ferry, H., Lewis, G., Rapsomaniki, E., Leung, J.C.H., Daser, A., Lambe, T., Goodnow, C.C. & Cornall, R.J. Spontaneous B cell hyperactivity in autoimmune-prone MRL mice. *International Immunology* **18**, 1127–1137 (2006).
238. Luzina, I.G., Atamas, S.P., Storrer, C.E., daSilva, L.C., Kelsoe, G., Papadimitriou, J.C. & Handwerger, B.S. Spontaneous formation of germinal centres in autoimmune mice. *The Journal of Leukocyte Biology* **70**, 578–584 (2001).
239. Liossi, S.-N.C., Kovacs, B., Dennis, G., Kammer, G.M. & Tsokos, G.C. B cells from patients with Systemic Lupus Erythematosus display abnormal antigen receptor-mediated early signal transduction events. *The Journal of Clinical Investigation* **98**, 2549–2557 (1996).
240. Odendahl, M., Jacobi, A., Hansen, A., Feist, E., Hiepe, F., Burmester, G.R., Lipsky, P.E., Rabdbruch, A. & Dörner, T. Disturbed peripheral B lymphocyte homeostasis in Systemic Lupus Erythematosus. *The Journal of Immunology* **165**, 5970–5979 (2000).
241. Cappione, A.I., Anolik, J.H., Pugh-Bernard, A.E., Barnard, J., Dutcher, P., Silvermann, G. & Sanz, I. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *The Journal of Clinical Investigation* **115**, 3205–3216 (2005).
242. Yurasov, S., Wardemann, H., Hammersen, J., Tsuiji, M., Meffre, E., Pascual, V. & Nussenzweig, M.C. Defective B cell tolerance checkpoints in Systemic Lupus Erythematosus. *The Journal of Experimental Medicine* **201**, 703–711 (2005).
243. Tobón, G.J., Izquierdo, J.H. & Cañas, C.A. B lymphocytes: Development, tolerance, and their role in autoimmunity—Focus on Systemic Lupus Erythematosus. *Autoimmune Diseases* **2013**, 1–17 (2013).
244. Dörner, T., Giesecke, C. & Lipsky, P.E. Mechanisms of B cell autoimmunity in SLE. *Arthritis Research and Therapy* **13**, 243–255 (2011).
245. Chan, V.S.-F., Tsang, H.H.-L., Tam, R.C.-Y., Lu, L. & Lau, C.-S. B-cell-targeted therapies in Systemic Lupus Erythematosus. *Cellular and Molecular Immunology* **10**, 133–142 (2013).
246. Yu, B., Guan, M., Peng, Y., Shao, Y., Zhang, C., Yue, X., Zhang, J., Yang, H., Zou, H., Ye, W., Wan, J. & Zhang, W. Copy number variations of interleukin-17F, interleukin-21 and interleukin-22 are associated with Systemic Lupus Erythematosus. *Arthritis and Rheumatism* **63**, 3487–3492 (2011).
247. Feng, X., Wang, S., Chen, J., Lu, L., Hua, B., Li, X., Tsao, B.P. & Sun, L. Inhibition of aberrant circulating Tfh cell proportions by corticosteroids in patients with Systemic Lupus Erythematosus. *PLoS ONE* **7**, 1–9 (2012).
248. Shah, K., Lee, W.-W., Lee, S.-H., Kim, S.H., Kang, S.W., Craft, J. & Kang, I. Dysregulated balance of Th17 and Th1 cells in Systemic Lupus Erythematosus. *Arthritis Research and Therapy* **12**, 1–10 (2010).
249. Dolff, S., Quandt, D., Wilde, B., Feldkamp, T., Hua, F., Cai, X., Specker, C., Kribben, A., Kallenberg, C.G.M. & Witzke, O. Increased expression of costimulatory markers CD134 and CD80 on interleukin-17 producing T cells in patients with Systemic Lupus Erythematosus. *Arthritis Research and Therapy* **12**, 1–9 (2010).
250. Jack, R.S., Imanishi-Kari, T. & Rajewsky, K. Idiotypic analysis of the response of C57BL/6 mice to the (4-hydroxy-3-nitrophenyl)acetyl group. *The European Journal of Immunology* **7**, 559–565 (1977).

251. Reth, M., Hämmerling, G.J. & Rajewsky, K. Analysis of the repertoire of anti-NP antibodies in C57BL/6 mice by cell fusion I. Characterisation of antibody families in the primary and hyperimmune response. *The European Journal of Immunology* **8**, 393–400 (1978).
252. Jacob, J., Przylepa, J., Miller, C. & Kelsoe, G. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *The Journal of Experimental Medicine* **178**, 1293–1307 (1993).
253. Rose, M.L., Birbeck, M.S.C., Wallis, V.J., Forrester, J.A. & Davies, A.J.S. Peanut lectin binding properties of germinal centers of mouse lymphoid tissue. *Nature* **284**, 364–366 (1980).
254. Guinamar, R., Okigaki, M., Schlessinger, J. & Ravetch, J.V. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nature Immunology* **1**, 31–36 (2000).
255. Martin, F., Oliver, A.M. & Kearney, J.F. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* **14**, 617–629 (2001).
256. Attanavanich, K. & Kearney, J.F. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *The Journal of Immunology* **172**, 803–811 (2004).
257. Gray, D., McConnell, I., Kumararatne, D.S., MacLennan, I.C., Humphrey, J.H. & Bazin, H. Marginal zone B cells express CR1 and CR2 receptors. *The European Journal of Immunology* **14**, 47–52 (1984).
258. Waldschmidt, T.J., Kroese, F.G., Tygrett, L.T., Conrad, D.H. & Lynch, R.G. The expression of B cell surface receptors. III. The murine low-affinity IgE Fc receptor is not expressed on Ly 1 or 'Ly 1-like' B cells. *International Immunology* **3**, 305–315 (1991).
259. Laszlo, G., Hathcock, K.S., Dickler, H.B. & Hodes, R.J. Characterization of a novel cell-surface molecule expressed on subpopulations of activated T and B cells. *The Journal of Immunology* **150**, 5252–5262 (1993).
260. Cervenak, L., Magyar, A., Boja, R. & Lászlo, G. Differential expression of GL7 activation antigen on bone marrow B cell subpopulations and peripheral B cells. *Immunology Letters* **78**, 89–96 (2001).
261. Oliver, A.M., Martin, F. & Kearney, J.F. Mouse CD38 is down-regulated on germinal center B cells and mature plasma cells. *The Journal of Immunology* **158**, 1108–1115 (1997).
262. Donís-Hernández, E.R., Parkhouse, R.M. & Santos-Argumedo, L. Ontogeny, distribution and function of CD38-expressing B lymphocytes in mice. *The European Journal of Immunology* **31**, 1261–1267 (2001).
263. Phan, T.G., Amesbury, M., Gardam, S., Crosbie, J., Hasbold, J., Hodgkin, P.D., Basten, A. & Brink, R. B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. *The Journal of Experimental Medicine* **197**, 845–860 (2003).
264. Song, H. & Cerny, J. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *The Journal of Experimental Medicine* **198**, 1923–1935 (2003).
265. Cinamon, G., Zachariah, M.A., Lam, O.M., Foss Jr, F.W. & Cyster, J.G. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nature Immunology* **9**, 54–62 (2008).
266. Arnon, T.I., Horton, R.M., Grigorova, I.L. & Cyster, J.G. Visualization of splenic marginal zone B cell shuttling and follicular B cell egress. *Nature* **493**, 684–688 (2013).
267. Ferguson, A.R., Youd, M.E. & Corley, R.B. Marginal Zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *International Immunology* **16**, 1411–1422 (2004).

268. Fang, Y., Xu, C., Fu, Y.-X., Holers, M. & Molina, H. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *The Journal of Immunology* **160**, 5273–5279 (1998).
269. Gonda, H., Sugai, M., Nambu, Y., Katakai, T., Agata, Y., Mori, J.K., Yokota, Y. & Shimizu, A. The balance between Pax5 and Id2 activities is the key to AID gene expression. *The Journal of Experimental Medicine* **198**, 1427–1437 (2003).
270. Becker-Herman, S., Lanter, F. & Shachar, I. Id2 negatively regulates B cell differentiation in the spleen. *The Journal of Immunology* **168**, 5507–5513 (2002).
271. Schebesta, A., McManus, S., Salvaggio, G., Delogu, A., Busslinger, G.A. & Busslinger, M. Transcription factor Pax5 activates the chromatin of key genes involved in B cell signalling, adhesion, migration and immune function. *Immunity* **27**, 49–63 (2007).
272. Cattoretti, G., Shakhovich, R., Smith, P.M., Jäck, H.-M., Murty, V.V. & Alobeid, B. Stages of germinal center transit are defined by B cell transcription factor coexpression and relative abundance. *The Journal of Immunology* **177**, 6930–6939 (2006).
273. Schaerli, P., Willmann, K., Lang, A.B., Lipp, M., Loetscher, P. & Moser, B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *The Journal of Experimental Medicine* **192**, 1553–1562 (2000).
274. Haynes, N.M., Allen, C.D.C., Lesley, R., Ansel, K.M., Killeen, N. & Cyster, J.G. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1^{high} germinal center-associated population. *The Journal of Immunology* **179**, 5099–5108 (2007).
275. Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., Ellyard, J.I., Parish, I.A., Ma, C.S., Li, Q.-J., Parish, C.R., Mackay, C.R. & Vinuesa, C. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* **31**, 457–468 (2009).
276. He, J., Tsai, L.M., Leong, Y.A., Hu, X., Ma, S.C., Chevalier, N., Sun, X., Vandenberg, K., Rockman, S., Ding, Y., Zhu, L., Wei, W., Wang, C., Karnowski, A., Belz, G.T., Ghali, J.R., Cook, M.C., Riminton, D.S., Veillette, A., Schwartzberg, P.L., Mackay, F., Brink, R., Tangye, S.G., Vinuesa, C.G., Mackay, C.R., Li, Z. & Yu, D. Circulating precursor CCR7^{lo} PD-1^{hi} CXCR5⁺ CD4⁺ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* **39**, 770–781 (2013).
277. Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R. & King, C. A fundamental role for Interleukin-21 in the generation of T follicular helper cells. *Immunity* **29**, 127–137 (2008).
278. Ozaki, K., Spolski, R., Feng, C.G., Qi, C.-F., Cheng, J., Sher, A., Morse III, H.C., Liu, C., Schwartzberg, P.L. & Leonard, W.J. A critical role for IL-21 in regulating immunoglobulin production. *Science* **298**, 1630–1634 (2002).
279. Ozaki, K., Spolski, R., Ettinger, R., Kim, H.-P., Wang, G., Qi, C.-F., Hwu, P., Shaffer, D.J., Akilesh, S., Roopenian, D.C., Morse III, H.C., Lipsky, P.E. & Leonard, W.J. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *The Journal of Immunology* **173**, 5361–5371 (2004).
280. Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.-H. & Dong, C. Bcl-6 mediates the development of T follicular helper cells. *Science* **325**, 1001–1005 (2009).
281. Hardtke, S., Ohl, L. & Förster, R. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B cell help. *Blood* **106**, 1924–1931 (2005).
282. Chung, Y., Tanaka, S., Chu, F., Nurieva, R.I., Martinez, G.J., Rawak, S., Wang, Y.-H., Lim, H., Reynolds, J.M., Zhou, X.-H., Fan, H.-M., Liu, Z.-M., Neelapu, S.S. & Dong, C. Follicular regulatory T cells expressing FoxP3 and Bcl-6 suppress germinal center reactions. *Nature Medicine* **17**, 983–988 (2011).

283. Wang, X., Cho, B., Suzuki, K., Xu, Y., Green, J.A., An, J. & Cyster, J.G. Follicular dendritic cells help establish follicle identity and promote B cell retention in germinal centers. *The Journal of Experimental Medicine* **208**, 2497–2510 (2011).
284. Victoratos, P., Lagnel, J., Tzima, S., Alimzhanov, M.B., Rajewsky, K., Pasparakis, M. & Kollias, G. FDC-specific functions of p55TNFR and IKK2 in the development of FDC networks and of antibody responses. *Immunity* **24**, 65–77 (2006).
285. Ansel, M.K., McHeyzer-Williams, L.J., Ngo, V.N., McHeyzer-Williams, M.G. & Cyster, J.G. *In vivo*-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *The Journal of Experimental Medicine* **190**, 1123–1134 (1999).
286. Kaminski, D.A., Wei, C., Qian, Y., Rosenberg, A.F. & Sanz, I. Advances in human B cell phenotypic profiling. *Frontiers in Immunology* **3**, 1–15 (2012).
287. Ma, J., Zhu, C., Ma, B., Tian, J., Baidgon, S.E., Mao, C., Wu, W., Chen, J., Tong, J., Yang, M., Jiao, Z., Xu, H., Lu, L. & Wang, S. Increased frequency of circulating Follicular Helper T cells in patients with Rheumatoid Arthritis. *Clinical and Developmental Immunology* **2012**, 1–7 (2012).
288. Simpson, N., Gatenby, P.A., Wilson, A., Malik, S., Fulcher, D.A., Tangye, S.G., Manku, H., Vyse, T.J., Roncador, G., Huttley, G.A., Goodnow, C.C., Vinuesa, C.G. & Cook, M.C. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe Systemic Lupus Erythematosus. *Arthritis and Rheumatism* **62**, 234–244 (2010).
289. Yang, X., Yang, J., Chu, Y., Xue, Y., Xuan, D., Zheng, S. & Zou, H. T follicular helper cells and regulatory B cell dynamics in Systemic Lupus Erythematosus. *PLoS ONE* **9**, 1–9 (2014).
290. Watanabe, T., Suzuki, J., Mitsuo, A., Tamayama, Y., Katagiri, A., Amaon, H., Morimoto, S., Tokano, Y. & Takasaki, Y. Striking alteration of some populations of T/B cells in Systemic Lupus Erythematosus: relationship to expression of CD62L or some chemokine receptors. *Lupus* **17**, 26–33 (2008).
291. Wang, C., Hillsamer, P. & Kim, C.H. Phenotype, effector function, and tissue localization of PD-1 expressing human follicular helper T cell subsets. *BioMed Central Immunology* **12**, 1–15 (2011).
292. Morita, R., Schmitt, N., Bentebibel, S.-E., Ranganathan, R., Bourdey, L., Zurawski, G., Foucat, E., Duallers, M., Oh, S., Sabzghabaei, N., Lavecchio, E.M., Punaro, M., Pascual, V., Banchereau, J. & Ueno, H. Human blood CXCR5⁺ CD4⁺ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* **34**, 108–121 (2011).
293. Murphy, G. & Isenberg, D. Effect of gender on clinical presentation in Systemic Lupus Erythematosus. *Rheumatology* **52**, 2108–2115 (2013).
294. Merola, J.F., Berma, B., Lu, B., Karlson, E.W., Massarotti, E., Schur, P.H. & Costenbader, K.H. Clinical manifestations and survival among adults with (SLE) according to age at diagnosis. *Lupus* **23**, 778–784 (2014).
295. Webb, R., Kelly, J.A., Somers, E.C., Hughes, T., Kaufman, K.M., Sanchez, E., Nath, S.K., Bruner, G., Alarcón-Riquelme, M.E., Gilkeson, G.S., Kamen, D.L., Richardson, B.C., Harley, J.B. & Sawalha, A.H. Early disease onset is predicted by a higher genetic risk for lupus and is associated with a more severe phenotype in lupus patients. *Annals of the Rheumatic Diseases* **70**, 151–156 (2011).
296. Brunner, H.I., Gladman, D.D., Ibañez, D., Urowitz, M.D. & Silverman, E.D. Difference in disease features between childhood-onset and adult-onset Systemic Lupus Erythematosus. *Arthritis and Rheumatism* **58**, 556–562 (2008).

297. Wong, C.K., Wong, P.T.Y., Tam, L.S., Li, E.K., Chen, D.P. & Lam, C.W.K. Elevated production of B cell chemokine CXCL13 is correlated with Systemic Lupus Erythematosus disease activity. *The Journal of Clinical Immunology* **30**, 45–52 (2010).
298. Ezzat, M.H.M., EL-Gammasy, T.M.A., Shaheen, K.Y.A. & Shokr, E.S.M. Elevated production of serum B-cell-attracting chemokine-1 (BCA-1/CXCL13) is correlated with childhood-onset lupus disease activity, severity, and renal involvement. *Lupus* **20**, 845–854 (2011).
299. Jacobi, A.M., Odendahl, M., Reiter, K., Bruns, A., Burmester, G.R., Radbruch, A., Va;et, G., Lipsky, P.E. & Dörner, T. Correlation between circulating CD27^{high} plasma cells and disease activity in patients with Systemic Lupus Erythematosus. *Arthritis and Rheumatism* **48**, 1332–1342 (2003).
300. Rodriguez-Bayona, B., Ramos-Amaya, A., Pérez-Venegas, J.J., Rodríguez, C. & Brieva, J.A. Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in Systemic Lupus Erythematosus patients. *Arthritis Research and Therapy* **12**, 1–10 (2010).
301. Perry, D., Sang, A., Yin, Y., Zheng, Y.-Y. & Morel, L. Murine models of Systemic Lupus Erthematosus. *The Journal of Biomedicine and Biotechnology*, 1–19 (2011).
302. Roths, J.B., Murphy, E.D. & Eicher, E.M. A new mutation, *gld*, that produces lymphoproliferation and autoimmunity in C3H/HeJ mice. *The Journal of Experimental Medicine* **159**, 1–20 (1984).
303. Ma, C.S. & Deenick, E.K. Human T follicular helper (Tfh) cells and disease. *Immunology and Cell Biology* **92**, 64–71 (2014).
304. Pereira, J.P., Kelly, L.M., Xu, Y. & Cyster, J.G. EBI2 mediates B cell segregation between the outer and centre follicle. *Nature* **460**, 1122–1126 (2009).
305. Kelly, L.M., Pereira, J.P., Yi, T., Xu, Y. & Cyster, J.G. EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and non-lymphoid tissues. *The Journal of Immunology* **187**, 1–17 (2011).
306. Shulman, Z., Gitlin, A.D., Weinstein, J.S., Lainez, B., Espluges, E., Flavell, R.A., Craft, J.E. & Nussenzweig, M.C. Dynamic signalling by T follicular helper cells during germinal center B cell selection. *Science* **345**, 1058–1062 (2014).
307. Rankin, A.L., MacLeod, H., Keegan, S., Andreyeva, T., Lowe, L., Bloom, L., Collins, M., Nickerson-Nutter, C., Young, D. & Guay, H. IL-21 receptor is critical for the development of memory B cell responses. *The Journal of Immunology* **186**, 667–674 (2011).
308. Kumar, K.R., Li, L., Yan, M., Bhaskarabhatla, M., Mobley, A.B., Nguyen, C., Mooney, J.M., Schatzle, J.D., Wakeland, E.K. & Mohan, C. Regulation of B cell tolerance by the Lupus susceptibility gene *Ly108*. *Science* **312**, 1665–1669 (2006).
309. Sinai, P., Dozmorov, I.M., Song, R., Schwartzberg, P.L., Wakeland, E.K. & Wülfing, C. T/B cell interactions are more transient in response to weak stimuli in SLE-prone mice. *European Journal of Immunology*, 1–27 (2014).
310. Baumann, I., Kolowos, W., Voll, R.E., Manger, B., Gaipl, U., Neuhuber, W.L., Kirchner, T., Kladen, J.R. & Herrmann, M. Impaired uptake of apoptotic cells in tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **46**, 191–201 (2002).
311. Rivero, S.J., Díaz-Jouanen, E. & Alarcón-Segovia, D. Lymphopenia in Systemic Lupus Erythematosus. Clinical, diagnostic, and prognostic significance. *Arthritis and Rheumatism* **21**, 295–305 (1978).
312. Arce, E., Jackson, D.G., Gill, M.A., Bennett, L.B., Banachereau, J. & Pascual, V. Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with Systemic Lupus Erythematosus. *The Journal of Immunology* **167**, 2361–2369 (2001).