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
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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<sup>-/-</sup> 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<sup>-/-</sup> mice compared to the WT mice. In addition, when extrafollicular B cells were analysed in MD4 and MD4.CCR6<sup>-/-</sup> mice immunised with HEL-SRBC, the MD4.CCR6<sup>-/-</sup> 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<sup>-/-</sup> mice resulted in several findings. Immunofluorescence of splenic germinal centre follicles showed no gross structural abnormalities in the CCR6<sup>-/-</sup> mice. In addition, qRT-PCR indicated that somatic hypermutation and differentiation in CCR6<sup>-/-</sup> germinal centre B cells was not affected. However, CCR6<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice, antibody quantification by ELISA demonstrated that the production of antigen-specific IgM and IgG in CCR6<sup>-/-</sup> 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<sup>-/-</sup> mice. Taken together, the reduced extrafollicular response and increased germinal centre response likely counterbalance each other in the CCR6<sup>-/-</sup> 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<sup>gld</sup> mice, which develop a spontaneous generalised systemic autoimmune disease, have a significantly lower frequency of CCR6<sup>+</sup> B cells than WT mice. However, CCR6 expression on B cells was significantly higher in the FasL<sup>gld</sup> 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.
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<th>Description</th>
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<tr>
<td>AID</td>
<td>Activation-induced Cytidine Deaminase</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>AP-1</td>
<td>Activator Protein -1</td>
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<td>Bcl-6</td>
<td>B-cell lymphoma 6</td>
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<td>BCR</td>
<td>B cell Receptor</td>
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<td>Blimp-1</td>
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<td>B-cell linker protein</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CSR</td>
<td>Class Switch Recombination</td>
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<td>CT</td>
<td>Cycle Threshold</td>
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<td>D</td>
<td>Diversity</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DNP</td>
<td>2,4-Dinitrophenyl</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EBF1</td>
<td>Early B cell factor-1</td>
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<td>EBI2</td>
<td>Epstein-Barr virus-induced G-protein coupled receptor 2</td>
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<td>EDCI</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EF</td>
<td>Extrafollicular Foci</td>
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<tr>
<td>eGFP</td>
<td>enhanced Green Fluorescent Protein</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>FACS</td>
<td>Fluorescent Activated Cell Sorting</td>
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<td>Forkhead box P3</td>
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<td>Gut-Associated Lymphoid Tissue</td>
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<td>Gld</td>
<td>Generalised Lymphoproliferation Disease</td>
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<td>h</td>
<td>human</td>
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<td>HEL</td>
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<td>High Endothelial Venules</td>
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<td>Horseradish Peroxidase</td>
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<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
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<td>ICOS</td>
<td>Inducible T-cell Costimulator</td>
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<td>Id2</td>
<td>Inhibitor of DNA binding 2</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>Immunoglobulin Heavy Chain</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>Isolated Lymphoid Follicles</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>IRF4</td>
<td>Interferon regulatory factor 4</td>
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<td>ITAMs</td>
<td>Immunoreceptor Tyrosine-based Activation Motifs</td>
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<td>J</td>
<td>Joining</td>
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<td>JAK</td>
<td>Janus Kinase</td>
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<td>KLH</td>
<td>Keyhole Limpet Hemocyanin</td>
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<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
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<td>Lpr</td>
<td>Lymphoproliferation</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>m</td>
<td>murine</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MRL</td>
<td>Murphy Roths Large</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>MS</td>
<td>Multiple Sclerosis</td>
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<tr>
<td>MZ</td>
<td>Marginal Zone</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Nature Killer</td>
</tr>
<tr>
<td>NP</td>
<td>4-Hydroxy-3-nitrophenylacetyl</td>
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<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>Polymerase Chain Reaction</td>
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<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
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<td>RBCs</td>
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<td>Rheumatoid Arthritis</td>
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<td>Rag</td>
<td>Recombination Activating Gene</td>
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<td>ribosomal Ribonucleic Acid</td>
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<td>SAP</td>
<td>SLAM Associated Proteins</td>
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<td>s.c.</td>
<td>Subcutaneous</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>SHM</td>
<td>Somatic Hypermutation</td>
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<tr>
<td>SLAM</td>
<td>Signalling Lymphocyte Activation Molecule</td>
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<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SRBCs</td>
<td>Sheep Red Blood Cells</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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<td>TD</td>
<td>T cell-dependent</td>
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<tr>
<td>TFFH</td>
<td>T Follicular Helper cell</td>
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<tr>
<td>TFR</td>
<td>T Follicular Regulatory cell</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<td>TI</td>
<td>T cell-independent</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
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<tr>
<td>Xbp-1</td>
<td>X-box binding protein-1</td>
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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 elsewhere. 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 elsewhere⁴,⁵.

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\textsuperscript{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.

![Image of lymph node and spleen](image)

**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 Reviews\textsuperscript{8}.

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 elsewhere.

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 elsewhere, 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 development\(^1\), particularly in the bone marrow\(^1\), as well as the expansion of B cells\(^1\), their Ig rearrangement\(^1\), and transcriptional regulation\(^1\). 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)-7Rα⁺ 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 apoptosis. 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

<table>
<thead>
<tr>
<th>Stage</th>
<th>HSC</th>
<th>CLP</th>
<th>Pro-B</th>
<th>Pre-B</th>
<th>Immature B cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin Status</td>
<td>D-J IgH rearrangement</td>
<td>V-DJ IgH rearrangement</td>
<td>Pre-BCR V-J IgL rearrangement</td>
<td>IgM⁺ IgD⁻</td>
<td></td>
</tr>
<tr>
<td>Transcription Factor Profile</td>
<td>PU.1</td>
<td>Ikaros</td>
<td>E2A</td>
<td>EBF1</td>
<td>Pax5</td>
</tr>
<tr>
<td>Surface Phenotype</td>
<td>CD93⁺</td>
<td>CD43⁺</td>
<td>CD93⁺</td>
<td>CD93⁺</td>
<td>CD93⁺</td>
</tr>
<tr>
<td></td>
<td>CD43⁺</td>
<td>c-kit⁺</td>
<td>CD43⁺</td>
<td>IL-7Ra⁺</td>
<td>CD93⁺</td>
</tr>
<tr>
<td></td>
<td>CD4⁺</td>
<td>IL-7Ra⁺</td>
<td>CD4⁺</td>
<td>B220⁺</td>
<td>CD93⁺</td>
</tr>
<tr>
<td></td>
<td>c-kit⁺</td>
<td>IL-7Ra⁺</td>
<td>c-kit⁺</td>
<td>CD19⁺</td>
<td>CD93⁺</td>
</tr>
<tr>
<td></td>
<td>IL-7Ra⁺</td>
<td>B220⁺</td>
<td>IL-7Ra⁺</td>
<td>CD24⁺</td>
<td>CD93⁺</td>
</tr>
<tr>
<td></td>
<td>B220⁺</td>
<td>CD19⁺</td>
<td>B220⁺</td>
<td>CD24⁺</td>
<td>CD93⁺</td>
</tr>
<tr>
<td></td>
<td>CD24⁺</td>
<td>CD24⁺</td>
<td>CD24⁺</td>
<td>CD24⁺</td>
<td>CD24⁺</td>
</tr>
</tbody>
</table>

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 compartments\(^{19-22}\). 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

<table>
<thead>
<tr>
<th>Naïve</th>
<th>Precursor</th>
<th>Germinal Centre</th>
<th>Plasma</th>
<th>Memory</th>
<th>Marginal Zone</th>
<th>Extrafollicular Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220(^+)</td>
<td>B220(^+)</td>
<td>B220(^+)</td>
<td>B220(^+)</td>
<td>B220(^+)</td>
<td>B220(^+)</td>
<td>B220(^+)</td>
</tr>
<tr>
<td>CD38(^+)</td>
<td>CD38(^+)</td>
<td>CD38(^+)</td>
<td>CD38(^+)</td>
<td>CD38(^+)</td>
<td>CD38(^+)</td>
<td>CD38(^+)</td>
</tr>
<tr>
<td>GL7(^+)</td>
<td>GL7(^+)</td>
<td>GL7(^+)</td>
<td>GL7(^+)</td>
<td>GL7(^+)</td>
<td>GL7(^+)</td>
<td>GL7(^+)</td>
</tr>
<tr>
<td>IgM(^+)</td>
<td>IgM(^+)</td>
<td>IgM(^+)</td>
<td>IgM(^+)</td>
<td>IgM(^+)</td>
<td>IgM(^+)</td>
<td>IgG1(^+)</td>
</tr>
<tr>
<td>IgD(^+)</td>
<td>IgD(^+)</td>
<td>IgD(^+)</td>
<td>IgD(^+)</td>
<td>IgD(^+)</td>
<td>IgD(^+)</td>
<td>IgD(^+)</td>
</tr>
<tr>
<td>IgG(^-)</td>
<td>CD23(^+)</td>
<td>CD23(^+)</td>
<td>CD23(^+)</td>
<td>CD23(^+)</td>
<td>CD23(^+)</td>
<td>CD23(^+)</td>
</tr>
<tr>
<td>CD21/35(^+)</td>
<td>CD95(^+)</td>
<td>CD95(^+)</td>
<td>CD95(^+)</td>
<td>CD95(^+)</td>
<td>CD95(^+)</td>
<td>CD95(^+)</td>
</tr>
<tr>
<td>MHC-II(^{hi})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table compiled from previous studies.\(^{23-28}\)
As mentioned earlier, to improve antibody affinity for antigen, peripheral B cells undergo CSR and SHM (reviewed in 29-32). 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 apoptosis 33-35. 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.
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.

**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 proteases\(^{40}\), or by antigen-presenting macrophages\(^{39,41,42}\). Dendritic cells can also present antigen to B cells\(^{43}\). Finally, B cells that are in transit to secondary lymphoid organs can collect soluble antigen directly from circulation\(^{38}\). 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-κB), 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 elsewhere\(^{44-48}\). 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 border\(^{49}\). Here, antigen-specific B and T cells physically meet, forming conjugate pairs\(^{50}\). 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 receptor\(^{54-57}\). 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)-\(^\gamma\), 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. 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. Following T-B cell interactions, B cells must commit to either GC or EF differentiation – an important event for B cells. 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. 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 GCs. Furthermore, these decisions can also be influenced by cytokines secreted in the local environment. 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).
Only a few B cell clones are needed to form EF, which then proliferate extensively for 4-7 days following antigen challenge\textsuperscript{27,53,77}. The extrafollicular response peaks at day 8 and dissipates by day 14\textsuperscript{19,20,53}. During the extrafollicular response, B cells continue to undergo isotype switching via CSR\textsuperscript{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 cells\textsuperscript{82}. 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 clones\textsuperscript{53}. 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 differentiation\textsuperscript{83}. 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\textsuperscript{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 differentiation\textsuperscript{87–90}. In this way, plasmablasts transition into plasma cells.

\textbf{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 selection\(^94-97\). 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 challenge\(^98\).

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 follicle\(^101-103\). Furthermore, the transcription factor, interferon regulatory factor 4 (IRF4) is also required for GC formation and may contribute to Bcl-6 activation\(^104-106\). 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\(_{\text{FH}}\)) cells. In the GC, B cells must collect antigen from FDCs, and present it to T\(_{\text{FH}}\) 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\(_{\text{FH}}\) cells\(^109-111\). 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 selection\(^4\), however, they also secrete cytokines and chemokines, and engage molecules on the surface of B cells, to support the GC reaction\(^112-114\). 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 FDCs\(^117\). 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 selection\(^118-120\). In addition, the \(T_{FH}\) cells secrete cytokines, such as IL-4, IL-10, IFN-\(\gamma\) 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 apoptosis\(^115\).

**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\textsuperscript{126,127}. Clones with inappropriate mutations are eliminated by apoptosis and are cleared by tangible body macrophages\textsuperscript{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\textsubscript{FH} cells and FDCs, as well as the temporal and spatial organisation of the B cell\textsuperscript{130,131}. Indeed, \textit{in silico} modeling concurs with this theory\textsuperscript{132}, 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\textsuperscript{85,133,134}. However, a proportion of these cells can also reside in the spleen for approximately 1 year\textsuperscript{134}. Plasma cell differentiation peaks approximately 1 week after challenge, and rapidly decreases thereafter\textsuperscript{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-replenish\textsuperscript{135-137}. Memory B cells appear within 3 days of immunisation, peak at day 21 and persist until at least day 56 after immunisation\textsuperscript{135}. They can reside in both the spleen and bone marrow, until they are called upon during re-infection\textsuperscript{136}. GC-derived memory B cells can be isotype switched, or they can retain IgM\textsuperscript{137,138}. Of course, it is important to note that early TD memory B cells can also be produced independently of the GC\textsuperscript{28,81}. These cells can be isotope 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\textsuperscript{89,139}. In fact, IRF4 expression induces Blimp-1 and AID expression and consequently drives plasma cell differentiation\textsuperscript{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\textsuperscript{88,102,142}. Blimp-1 also induces XBP-1, a transcription factor required for plasma cell generation but not memory cell generation\textsuperscript{88,143,144}. In addition, Pax5, although required for early B cell development, is repressed during plasma cell differentiation by Blimp-1\textsuperscript{145-147}. Conversely, Bcl-6 not only supports GC formation, but represses Blimp-1 expression and thereby inhibits plasma cell differentiation\textsuperscript{142}. 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\(_i\)-protein coupled chemokine receptors\(^{148,149}\). Currently, 20 receptors and 50 chemokines have been identified\(^{150-152}\). Chemokine receptors are made up of an extracellular N-terminal, seven-transmembrane \(\alpha\)-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 lymphocytes\(^{153}\). 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-terminus\(^{154}\).
Reproduced from Moser and Willimann (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 ligands\(^{151}\). 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

<table>
<thead>
<tr>
<th>Classification</th>
<th>Receptor</th>
<th>Ligand/s</th>
<th>Function</th>
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</thead>
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<tr>
<td>C</td>
<td>XCR1</td>
<td>XCL1 &amp; 2</td>
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<tr>
<td>CC</td>
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<td>CCL3, 5, 7, 14, 15, 16 &amp; 23</td>
<td>Inflammatory</td>
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<tr>
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<td>CCR2</td>
<td>CCL2, 7, 8, 13 &amp; 16</td>
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</tr>
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<td>CCR3</td>
<td>CCL5, 7, 8, 11, 13, 15, 16, 24, 26 &amp; 28</td>
<td>Inflammatory</td>
</tr>
<tr>
<td></td>
<td>CCR4</td>
<td>CCL17 &amp; 22</td>
<td>Homeostatic</td>
</tr>
<tr>
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<td>Inflammatory</td>
</tr>
<tr>
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<td>CCR6</td>
<td>CCL20</td>
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<td>CCR7</td>
<td>CCL19 &amp; 21</td>
<td>Homeostatic</td>
</tr>
<tr>
<td></td>
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<td>CCL1</td>
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<td>CXCL12</td>
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<td>CXCR5</td>
<td>CXCL13</td>
<td>Homeostatic</td>
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<tr>
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<td>CXCL12</td>
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<tr>
<td>CX3C</td>
<td>CX₃CR1</td>
<td>CX₂CL1</td>
<td>Inflammatory</td>
</tr>
</tbody>
</table>

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)\textsuperscript{157,158}. Conversely, the downregulation of EBI2 enables GC formation\textsuperscript{157}. Once in the GC, B cells express high levels of CXCR5 and CXCR4, allowing activated B cells to migrate between the CXCL12\textsuperscript{+} dark zone and CXCL13\textsuperscript{+} light zones of the GC (Fig. 1.5.2.1)\textsuperscript{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\textsuperscript{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 1997\textsuperscript{162}. 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 CXCR5\textsuperscript{162}. The gene encoding CCR6 was mapped to human chromosome 6 and it was determined that multiple protein variants existed\textsuperscript{162}. Furthermore, northern blot analysis of human tissue demonstrated that CCR6 was expressed by lymphocytes in lymphoid tissue\textsuperscript{162}.

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 dye\textsuperscript{163}. 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 testis\textsuperscript{163}. CCR6 mRNA was also detected in peripheral blood leukocytes: specifically B cells, CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells, but not granulocytes, monocytes or NK cells\textsuperscript{163}. This work suggested that CCR6 may be involved in lymphocyte trafficking within secondary lymphoid organs\textsuperscript{163}. 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 chemokines\textsuperscript{164}. Finally, Greaves and colleagues also reported on the identification of the CCR6-CCL20 mediated migration of human CD34\textsuperscript{+}-derived dendritic cells\textsuperscript{165}. In addition, they confirmed that CCR6 was expressed in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells of the blood, as well as splenic B cells\textsuperscript{165}.
However, when splenic B cells were activated with anti-CD40 and IL-4, CCR6 mRNA expression was found to be reduced.\textsuperscript{165} Although CCR6 bound to CCL20 with high specificity, CCR6 was also reported to bind small antimicrobial peptides, known as β-defensins.\textsuperscript{166} Antimicrobial peptides, such as the β-defensins, are produced in the innate response and act to recruit cells to sites of infection.\textsuperscript{167-169} Briefly, β-defensins are expressed by epithelial cells and function in the recruitment of CCR6\textsuperscript{+} 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.\textsuperscript{163-165,170} The hCCL20 protein was mapped to chromosome 2 and found to share only 20–28% homology with other chemokines.\textsuperscript{170} Initially, CCL20 was largely detected in the liver, lung, and to a lesser extent in the thymus and intestine.\textsuperscript{170} Subsequently, CCL20 was identified in other lymphoid tissues including the tonsils and appendix.\textsuperscript{171} Furthermore, chemotaxis assays revealed CCL20 was chemotactic for lymphocytes isolated from human peripheral blood.\textsuperscript{170} 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.

\textbf{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 cloned.\textsuperscript{172} The mCCR6 gene encodes a 367 amino acid protein with several mRNA variants and shares 73.8% sequence homology with hCCR6.\textsuperscript{172}\textsuperscript{152,153} Northern blot analysis revealed that mCCR6 mRNA was expressed in the spleen and lymph node.\textsuperscript{172} Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated that murine B cells from the spleen, lymph node and thymus expressed CCR6.\textsuperscript{172} CCR6 expression was also detected in splenic dendritic cells, as well as CD4\textsuperscript{+} T cells from the spleen and lymph node, while mCCL20 was largely detected in the thymus, colon and small intestine.\textsuperscript{172} In addition, mCCL20 was shown to induce calcium mobilisation in human cells transfected with mCCR6, in a dose-dependent manner.\textsuperscript{172} Overall, the similarity observed between murine and human CCR6 demonstrated that \textit{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 localisation\(^{175}\). 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 cells\(^{176}\). 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 ILFs\(^{176}\). 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 GALT\(^{176-178}\). 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 tissues\(^{179}\). 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\textsuperscript{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\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and dendritic cells\textsuperscript{181}. Interestingly, altered antibody responses were observed in the absence of CCR6. Specifically, CCR6\textsuperscript{-/-} mice immunised with the protein carrier, keyhole limpet hemocyanin (KLH) generated increased IgM and decreased IgG3 levels compared to WT mice\textsuperscript{174}, while immunisation with the hapten, 2,4-dinitrophenol (DNP) conjugated to KLH, caused a reduction in IgG2b levels in CCR6\textsuperscript{-/-} mice compared to WT mice\textsuperscript{173}. Furthermore, more recent work has demonstrated that CCR6\textsuperscript{-/-} mice produce more low-affinity than high-affinity antibody forming cells than WT mice, in response to TD antigen challenge\textsuperscript{182}. In addition, preliminary evidence indicates that CCR6\textsuperscript{-/-} mice establish more TD GC follicles than WT mice\textsuperscript{182}. 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 assays\textsuperscript{183}. 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 chemokines\textsuperscript{184}. 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 levels\textsuperscript{184}. In agreement with this, Krzysiek and colleagues also documented that CCR6 expression and responsiveness to CCL20 decreased on human B cells, activated by crosslinking\textsuperscript{185}. However, it was also reported that human peripheral B cells activated by cross-linking, show enhanced responses to CCL20\textsuperscript{186}. It is important to note, that these studies used B cells isolated from peripheral blood, which were then activated \textit{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\textsuperscript{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 cells\(^{188}\). 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 PPs\(^{188}\). 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 CCL20\(^{189}\). 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 cells\(^{185-187}\). 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.
CHAPTER 1
INTRODUCTION

J. BANNAN
THE ROLE OF CCR6 IN B CELL DIFFERENTIATION DURING THE HUMORAL IMMUNE 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. 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. CCR6 autoimmune disease associations

<table>
<thead>
<tr>
<th>Disease</th>
<th>Characteristics</th>
<th>Model</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankylosing Spondylitis (AS)</td>
<td>Inflammation of axial skeleton and sacroiliac joints</td>
<td>Human</td>
<td>Increased frequency of CCR6⁺ Th cells in blood during AS¹⁹³</td>
</tr>
<tr>
<td>Autoimmune Hepatitis (AIH)</td>
<td>Liver damage</td>
<td>Mouse</td>
<td>Dysregulated CCR6 dependent migration of splenic T cells to CCL20⁺ liver induces AIH¹⁹⁴</td>
</tr>
<tr>
<td>Discoid Lupus Erythematosus (DLE)</td>
<td>Skin damage</td>
<td>Human</td>
<td>CCR6⁺ dendritic cells identified in skin lesions of DLE patients and correlated with disease activity¹⁹⁵</td>
</tr>
<tr>
<td>Granulomatosis with Polyangitis (GPA)</td>
<td>Inflammation of respiratory tract and vasculitis</td>
<td></td>
<td>Increased frequency of CCR6⁺ memory T cells¹⁹⁰</td>
</tr>
<tr>
<td>Multiple Sclerosis (MS)/Experimental Autoimmune Encephalomyelitis (EAE)</td>
<td>Central nervous system (CNS) inflammation</td>
<td>Mouse</td>
<td>CCR6⁺ cells including CD4⁺ T cells and dendritic cells, infiltrate the CNS, contributing to EAE severity¹⁹⁷⁻²⁰¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased CCL20 mRNA expression increased in CNS during EAE¹⁹²,²⁰³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased CCL20 mRNA expression in CNS and lymph nodes correlates with EAE onset and severity²⁰⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCL20 expressed by astrocytes in the CNS during EAE and acts to recruit T cells²⁰⁵</td>
</tr>
<tr>
<td>Sjögren's Syndrome (SS)</td>
<td>Inflammation of lacrimal and salivary glands</td>
<td>Human</td>
<td>Unchanged expression of CCR6 on B cells in SS²⁰⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased frequency of circulating CCR6⁺ Th17 cells in SS²⁰⁷</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>Chronic skin disease</td>
<td>Human</td>
<td>CCR6, CCL20 and β-defensin-2 expression increased in skin lesions during Psoriasis²⁰⁸,²⁰⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased frequency of CCR6⁺ T cells in skin lesions compared to blood during Psoriasis²¹⁰</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCL20 expressed by keratinocytes and PBMCs²⁰⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Memory T cells have increased CCR6 expression²⁰⁹</td>
</tr>
<tr>
<td>Rheumatoid Arthritis (RA)</td>
<td>Joint inflammation</td>
<td>Human</td>
<td>CCR6 gene polymorphisms, rs3093024, rs3093023, rs59466457, rs1571878, associated with RA susceptibility²¹¹⁻²¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCR6⁺ mononuclear cells, including B cells and Th17 cells, are recruited to CCL20⁺ joints during RA²¹⁵⁻²¹⁸</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCR6 and CCL20 expressed in bone tissue of RA patients²¹⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased CCR6 and CCL20 mRNA in RA synovial tissue²²⁰</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced frequency of CCR6⁺ B cells in RA²²¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased frequency of CCR6⁺ memory B cells in blood during RA²²⁰</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unchanged CCR6 expression on CD3⁺ T cells during RA²²²</td>
</tr>
<tr>
<td>Systemic Lupus Erythematosus (SLE)</td>
<td>Systemic disease</td>
<td>Human</td>
<td>Positive correlation between CCR6 mRNA in PBMCs and SLE severity²²³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased frequency of T[F] cells and memory T cells correlates with SLE severity²²⁴,²²⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced CCR6 expression on circulatory T cells in SLE²²⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No alteration in frequency of CCR6⁺ B cells and Th17 cells in SLE²²¹,²²⁴</td>
</tr>
<tr>
<td>Systemic Sclerosis (SSc)</td>
<td>Systemic disease</td>
<td>Human</td>
<td>CCR6 dinucleotide polymorphisms, rs3093023 &amp; rs10946216, associated with SSc susceptibility²²⁷</td>
</tr>
</tbody>
</table>
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 manifestations. Symptoms can range from cutaneous rashes, hair weakness and ulcers, to joint synovitis or tenderness, haemolytic anaemia, as well as renal and neurological problems. Immunologically, it can be characterised by leukopenia, auto-antibody production against nuclear proteins, DNA and phospholipids, and a reduction in complement proteins. The auto-antibodies generated can form immune complexes that are then deposited in organs throughout the body, causing widespread inflammation and tissue damage. SLE is also associated with a reduction in lymphocyte activity and impaired lymphocyte migration. 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 MRL1pr 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 production. 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 pathogenesis. In addition, reductions in the frequency of circulatory B cells have also been documented in SLE, whereas increased populations of GC and memory B cells have been reported in the secondary lymphoid organs of SLE participants. 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 SLE. Currently, SLE therapies used to treat disease are targeted towards inhibiting the signalling, proliferation and differentiation of B cells, and T-B cell interactions.

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. 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 controls. 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 progression$^{223}$. 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 controls$^{221}$. 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>
<thead>
<tr>
<th>Strain</th>
<th>Common Name</th>
<th>JAX® Stock Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>WT</td>
<td>000664</td>
<td>Wild-type</td>
</tr>
<tr>
<td>B6.SJL-Plpce&lt;sup&gt;a&lt;/sup&gt; Pepe&lt;sup&gt;b&lt;/sup&gt;/BoyJ</td>
<td>Ly5.1</td>
<td>002014</td>
<td>C57BL/6 congenic strain variant</td>
</tr>
<tr>
<td>B6.PL-Thyl&lt;sup&gt;+&lt;/sup&gt;/CyJ</td>
<td>Thy1.1</td>
<td>000406</td>
<td>C57BL/6 congenic strain variant</td>
</tr>
<tr>
<td>B6.129P2-Ccr6&lt;sup&gt;tm1Dgen&lt;/sup&gt;/J</td>
<td>CCR6&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>005793</td>
<td>Targeted knock-out</td>
</tr>
<tr>
<td>B6.129S6-Ccr6&lt;sup&gt;tm1(EGFP)Irw&lt;/sup&gt;/J</td>
<td>CCR6-eGFP</td>
<td>013061</td>
<td>Targeted eGFP knock-in that abolishes CCR6 function</td>
</tr>
<tr>
<td>C57BL/6-Tg(IghelMD4)4Ccg/J</td>
<td>MD4</td>
<td>002595</td>
<td>BCR (predominantly IgM &amp; IgD) specific for HEL (transgene)</td>
</tr>
<tr>
<td>B6.Cg-Tg(TeraTcrb)425Cbn/J</td>
<td>OT-II</td>
<td>004194</td>
<td>CD4 TCR α &amp; β-chain is specific for OVA (transgene)</td>
</tr>
<tr>
<td>B6(Cg)-Rag2&lt;sup&gt;tm1Lcgm&lt;/sup&gt;/J</td>
<td>Rag</td>
<td>008449</td>
<td>Targeted knock-out that arrests B &amp; T cell development in pro-stages</td>
</tr>
<tr>
<td>B6.129P2(C)-Ccr7&lt;sup&gt;tm1(Boy)&lt;/sup&gt;/J</td>
<td>CCR7&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>006621</td>
<td>Targeted knock-out</td>
</tr>
<tr>
<td>B6.129S2(Cg)-Cxcr5&lt;sup&gt;tm1Lipp&lt;/sup&gt;/J</td>
<td>CXCR5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>006659</td>
<td>Targeted knock-out</td>
</tr>
<tr>
<td>B6Smn.C3-Fasl&lt;sup&gt;gld&lt;/sup&gt;/J</td>
<td>gld</td>
<td>001021</td>
<td>Fas-ligand point mutation causing generalized lymphoproliferative disease (gld)</td>
</tr>
<tr>
<td>C57BL/6-Tg(IghelMD4)4Ccg/J x B6.129S6-Ccr6&lt;sup&gt;tm1(EGFP)Irw&lt;/sup&gt;/J</td>
<td>MD4.CCR6-eGFP</td>
<td>-</td>
<td>HEL-specific BCR &amp; targeted eGFP knock-in that abolishes CCR6 function</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Participants (n)</th>
<th>Female/Male Ratio</th>
<th>Age (yrs) Mean±SD</th>
<th>Age at Diagnosis (yrs) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>12/8</td>
<td>45.25 ± 15.17</td>
<td>-</td>
</tr>
<tr>
<td>SLE</td>
<td>14</td>
<td>13/1</td>
<td>54.71 ± 15.62</td>
<td>40 ± 15.89</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3. Standard Solutions

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

Table 2.3.1: Frequently used Solutions

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Phosphate Buffered Saline</td>
<td>80 g NaCl (Sigma-Aldrich®, S5886)</td>
</tr>
<tr>
<td>(PBS)</td>
<td>14.4 g Na2HPO4.H2O (Sigma-Aldrich®, S5136)</td>
</tr>
<tr>
<td></td>
<td>2.4 g KH2PO4 (Sigma-Aldrich®, P0662)</td>
</tr>
<tr>
<td></td>
<td>2 g KCl (Sigma-Aldrich®, P9541)</td>
</tr>
<tr>
<td></td>
<td>H2O to 1 L (pH 7.4 on dilution)</td>
</tr>
<tr>
<td>FACS Buffer</td>
<td>100 mL 10 x PBS</td>
</tr>
<tr>
<td></td>
<td>1 g Bovine Serum Albumin (BSA)</td>
</tr>
<tr>
<td></td>
<td>2 mL 10 % Sodium Azide (Sigma-Aldrich®, S2002)</td>
</tr>
<tr>
<td></td>
<td>H2O to 1 L</td>
</tr>
<tr>
<td>Red Blood Cell (RBC) Lysis Buffer</td>
<td>4.55 g NH4Cl (Sigma-Aldrich®; A0171)</td>
</tr>
<tr>
<td></td>
<td>10 mL 1 M HEPES (Sigma-Aldrich®, H3375)</td>
</tr>
<tr>
<td></td>
<td>H2O to 500 mL</td>
</tr>
<tr>
<td>MACS Buffer</td>
<td>150 mL 10 x PBS</td>
</tr>
<tr>
<td></td>
<td>7.5 g BSA</td>
</tr>
<tr>
<td></td>
<td>3 mL 10 % Sodium Azide</td>
</tr>
<tr>
<td></td>
<td>6 mL 0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8</td>
</tr>
<tr>
<td></td>
<td>H2O to 1.5 L</td>
</tr>
<tr>
<td>10 x Tris-Borate-EDTA (TBE)</td>
<td>108 g Tris (hydroxymethyl)aminomethane (Sigma-Aldrich®, T87602)</td>
</tr>
<tr>
<td></td>
<td>55 g Boric Acid (Sigma-Aldrich®, B6768)</td>
</tr>
<tr>
<td></td>
<td>40 mL 0.5 M EDTA pH 8</td>
</tr>
<tr>
<td></td>
<td>H2O to 1 L</td>
</tr>
<tr>
<td>Carbonate Buffer</td>
<td>4.2 g NaHCO3 (Sigma-Aldrich®, S6014)</td>
</tr>
<tr>
<td></td>
<td>1.78 g Na2CO3 (Sigma-Aldrich®, 223484)</td>
</tr>
<tr>
<td></td>
<td>H2O to 500 mL (pH 9.5)</td>
</tr>
<tr>
<td>Conjugation Buffer</td>
<td>0.35 M Mannitol (Sigma-Aldrich®, M4125)</td>
</tr>
<tr>
<td></td>
<td>0.1 M NaCl</td>
</tr>
<tr>
<td></td>
<td>H2O to 500 mL</td>
</tr>
</tbody>
</table>
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-SRBCs, 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 anticoagulant, 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, $1.5 \times 10^6$ 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**

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

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

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

**Table 2.5.3: Antibodies used to detect murine T cells**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Working Dilution</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-1 PE</td>
<td>1/100</td>
<td>J43</td>
<td>eBioscience</td>
<td>12-9985-81</td>
</tr>
<tr>
<td>TCRαβ PB</td>
<td>1/200</td>
<td>H57-597</td>
<td>Life Technologies™</td>
<td>HM3628</td>
</tr>
<tr>
<td>CD4 PerCP-Cy™5.5</td>
<td>1/400</td>
<td>RM4-5</td>
<td>BD</td>
<td>550954</td>
</tr>
<tr>
<td>CD8a APC-Cy™7</td>
<td>1/200</td>
<td>53-6.7</td>
<td>BD</td>
<td>557654</td>
</tr>
<tr>
<td>CD44 PeCy™7</td>
<td>1/400</td>
<td>IM7</td>
<td>BioLegend®</td>
<td>103029</td>
</tr>
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</table>
Table 2.5.4: Secondary antibodies used for flow cytometry in murine samples

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

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

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

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

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

All samples were filtered through 48 µm 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

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Application</th>
<th>Working Dilution</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA Biotin</td>
<td>IH</td>
<td>1/300</td>
<td>-</td>
<td>Vector Laboratories</td>
<td>B-1075</td>
</tr>
<tr>
<td>B220 Biotin</td>
<td>IH</td>
<td>1/300</td>
<td>RA3-6B2</td>
<td>BioLegend®</td>
<td>103204</td>
</tr>
<tr>
<td>CD4 purified</td>
<td>IH</td>
<td>1/200</td>
<td>RM4-5</td>
<td>BD</td>
<td>553043</td>
</tr>
<tr>
<td>B220 purified</td>
<td>IH</td>
<td>1/300</td>
<td>RA3-6B2</td>
<td>BD</td>
<td>553084</td>
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</table>

Table 2.6.2: Secondary antibodies used for immunofluorescence

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Application</th>
<th>Working Dilution</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Catalogue Number</th>
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</thead>
<tbody>
<tr>
<td>Streptavidin Alexa Fluor® 594</td>
<td>IH</td>
<td>1/300</td>
<td>-</td>
<td>Life Technologies™</td>
<td>S-11227</td>
</tr>
<tr>
<td>Goat α-Rat IgG Alexa Fluor® 633</td>
<td>IH</td>
<td>1/300</td>
<td>-</td>
<td>Life Technologies™</td>
<td>A-21094</td>
</tr>
<tr>
<td>Goat α-Rat IgG Alexa Fluor® 488</td>
<td>IH</td>
<td>1/300</td>
<td>-</td>
<td>Life Technologies™</td>
<td>A-11006</td>
</tr>
</tbody>
</table>
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 µL 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 µg/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 µL of 10 % (v/v) Igepal® (Sigma-Aldrich®, 1000862047), 2 µL 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 µL. 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**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product (bp)</th>
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<td>18S</td>
<td>GTAACCCGTTGAACCACCATT</td>
<td>CCAATCCAATCGGTAGTAGCG</td>
<td>151</td>
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<tr>
<td>Bcl-6</td>
<td>CTGCAATGGAGCATGTGTT</td>
<td>CGGCTGTTCAGGAACTCTTC</td>
<td>106</td>
</tr>
<tr>
<td>IL-21</td>
<td>TGAAGGCTGTGGAAGTGCAACCA</td>
<td>AGCAGATTCAACAGGACAACCCCA</td>
<td>108</td>
</tr>
<tr>
<td>CCR6</td>
<td>TGTCTCACCCTACCCTTCTTG</td>
<td>TACAGGCAAGGAGCAT</td>
<td>131</td>
</tr>
<tr>
<td>CXCR5</td>
<td>CGACATACAGAAGGACAGC</td>
<td>GTCCTGTAGGGAAATCCCGTG</td>
<td>151</td>
</tr>
<tr>
<td>AID</td>
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<td>TCCAGTCTGAGATGTAGCG</td>
<td>119</td>
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<tr>
<td>EBI2</td>
<td>CAGCTTACCACCTCAGGATA</td>
<td>AAGAAGCGGCTATGTCAAAA</td>
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<tr>
<td>Id2</td>
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<td>CTCGCAAGGACAGGATGCTGATA</td>
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</tr>
<tr>
<td>IRF4</td>
<td>CTGAGTGGCGTGATGCCCAGA</td>
<td>ATCGCAGTGGGAAGAGTCG</td>
<td>194</td>
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<tr>
<td>CCL20</td>
<td>ATAGCTTCCTTGAGCTGAGAACG</td>
<td>ATAGGATCCATCTCTTTCATCCTAGTGGCTGA</td>
<td>328</td>
</tr>
<tr>
<td>CCR6</td>
<td>CCTCACATTCTCTCAGGACTGAGAATGG</td>
<td>GGCAATCAGGCTCTCGGA</td>
<td>151</td>
</tr>
<tr>
<td>CCR7</td>
<td>CCAGCAAGACGCTCAGCAT</td>
<td>GGGATAGAAGGGATACAGAAGGA</td>
<td>92</td>
</tr>
<tr>
<td>Pax5</td>
<td>CAGACCACAGTATTACGCACAT</td>
<td>AGGGAATCTCCAGAAAATTTCGCTCC</td>
<td>259</td>
</tr>
<tr>
<td>Blimp-1</td>
<td>TCTGTTCAAGCCAGGACATCC</td>
<td>TCTGGGAACTGTGTACATTAG</td>
<td>163</td>
</tr>
</tbody>
</table>

### 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'-CGTATCGCCTCCCTCGCCATCAG-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'-TATGCGCCTTGCCAGCCGCTCAGGTGCTGGGCAGGAAGTCCCG-3’ and IgG Rv 5'-CTATGCGCCTTGCCAGCCGCTCAGRCTCAGGGAARTARCCYTTGAC-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**

<table>
<thead>
<tr>
<th>MID Name</th>
<th>Sequence (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>ACGCTCGACA</td>
</tr>
<tr>
<td>3</td>
<td>AGACGCACCTC</td>
</tr>
<tr>
<td>4</td>
<td>AGCACTGTAG</td>
</tr>
<tr>
<td>5</td>
<td>ATCAGACACAG</td>
</tr>
<tr>
<td>6</td>
<td>ATATCGCGAG</td>
</tr>
<tr>
<td>7</td>
<td>CGTGTCTCTTA</td>
</tr>
<tr>
<td>8</td>
<td>CTCGCGTGTC</td>
</tr>
<tr>
<td>9</td>
<td>TCTCTATGCG</td>
</tr>
<tr>
<td>10</td>
<td>TGATACGTCT</td>
</tr>
<tr>
<td>11</td>
<td>CATAGTAGTG</td>
</tr>
<tr>
<td>14</td>
<td>TCACGTACTA</td>
</tr>
<tr>
<td>15</td>
<td>CGTCTAGTAC</td>
</tr>
</tbody>
</table>

**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, IgG1b or IgG2ab 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.).
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**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Application</th>
<th>Working Dilution</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM Biotin</td>
<td>ELISA</td>
<td>1/1000</td>
<td>R6-60.2</td>
<td>BD</td>
<td>553406</td>
</tr>
<tr>
<td>IgG1&lt;sup&gt;b&lt;/sup&gt; Biotin</td>
<td>ELISA</td>
<td>1/2500</td>
<td>B68-2</td>
<td>BD</td>
<td>553533</td>
</tr>
<tr>
<td>IgG2a&lt;sup&gt;b&lt;/sup&gt; Biotin</td>
<td>ELISA</td>
<td>1/5000</td>
<td>5.7</td>
<td>BD</td>
<td>553504</td>
</tr>
<tr>
<td>Streptavidin HRP</td>
<td>ELISA</td>
<td>1/1000</td>
<td>-</td>
<td>BD</td>
<td>554066</td>
</tr>
</tbody>
</table>

2.14. Statistical Analysis

All data are expressed as the mean ± 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 cells\(^\text{190}\). 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 \textit{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 \textit{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\(^\text{-/-}\) 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\(^\text{-/-}\) 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. Naïve B cells were CD38+ GL7lo 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. 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, CCL20\textsuperscript{189}, which these B cells did not have access to \textit{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\textsuperscript{52,159,188}, their expression was also analysed on MD4 B cells during activation \textit{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 \textit{in vivo}, for interaction with Th cells\textsuperscript{52}. 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).
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 5μg/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 CD38hi 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. Form 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\textsuperscript{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 \textit{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⁺ 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<sup>+</sup> GL7<sup>+</sup> 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<sup>−/−</sup> 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 out\(^{182}\), 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 defined53,77,252, 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 previously53,77. 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).

\[ \text{Figure 4.1.1. The T cell-dependent Germinal kinetics in the absence of CCR6} \]

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 $\lambda$-specific GC B cells (b) and total $\lambda$-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<sup>−/−</sup> 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<sup>+</sup> border<sup>91,253</sup>. Quantification of GC follicles revealed that the CCR6<sup>−/−</sup> mice had significantly more follicles at days 3 and 5 following antigen challenge than WT mice (Fig. 4.1.2a). The CCR6<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> GC B cells correlates with the number of CCR6<sup>−/−</sup> 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<sup>−/−</sup> mice, suggests that the cause of dysregulation occurs early, during the initiation of the humoral response. As the GC response in CCR6<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>+</sup> PNA<sup>+</sup> clusters and quantified in 1 spleen cross-section/mouse (a). Representative images of the GC follicle response in WT and CCR6<sup>−/−</sup> 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<sup>−/−</sup> mice; Day 5, 14 & 42 n=4 WT mice, 4 CCR6<sup>−/−</sup> mice; Day 7 & 10 n=5 WT mice, 5 CCR6<sup>−/−</sup> 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\textsuperscript{-/-} mice, the frequency of other B cell subsets including naïve, precursor, memory and plasma cells, were also examined in these mice. Again, CCR6\textsuperscript{+/+} and WT mice were immunised with NP-KLH for 42 days and the \(\lambda\)-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\textsuperscript{+} GL7\textsuperscript{-}) generally appeared reduced in the CCR6\textsuperscript{-/-} mice compared to the WT mice (Fig. 4.2.1b). The frequency of naïve B cells was significantly reduced in CCR6\textsuperscript{-/-} 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\textsuperscript{-/-} mice and implies that more B cells are being recruited into the humoral response in CCR6\textsuperscript{-/-} 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\textsuperscript{+} GL7\textsuperscript{+}) was detected between CCR6\textsuperscript{-/-} 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\textsuperscript{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\textsuperscript{-} GL7\textsuperscript{-}) was detected 3 days after antigen challenge in both CCR6\textsuperscript{-/-} 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\textsuperscript{-/-} 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 cells. 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) CD38hi/ 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<sup>hi</sup> GL7<sup>-</sup> B cell population in the T cell-dependent humoral response

WT mice were immunised with 100µg of NP-KLH and the splenic CD38<sup>hi</sup> GL7<sup>-</sup> (memory) and CD38<sup>+</sup> GL7<sup>-</sup> (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<sup>-</sup> GL7<sup>-</sup> plasma cells, 2) CD38<sup>+</sup> GL7<sup>-</sup> naïve B cells, 3) CD38<sup>hi</sup> GL7<sup>-</sup> memory B cells and 5) CD38<sup>-</sup> GL7<sup>+</sup> GC B cells (a). CD38<sup>hi</sup> GL7<sup>-</sup> 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 (CD38hi 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.
**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<sup>−/−</sup> 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<sup>−/−</sup> mice have a higher frequency of low-affinity antibody forming cells<sup>182</sup>. 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<sup>−/−</sup> 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, 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 levels. 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. 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. 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 CD21hi CD23lo 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 (CD38hi 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 cells28,259,260. 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 cells24,28,261,262. 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 CD38hi GL7− memory B cells confirmed its identity, corresponding with the previously reported existence of IgMhi memory B cells98,137,138.

The B cell response of WT mice to NP has been well defined26-28,50,53,77,85,135. 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 B220lo 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 SW-HEL mouse model, which differs from the MD4 model used here, in that it is capable of B cell isotype switching78,263. The investigation of the EF B cell response to HEL–
SRBCs, a potent inducer of TD responses, has been published previously\(^75\). 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 cells\(^{264}\). 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 response\(^{265-267}\). 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 CXCR4$^{159}$. 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 zone$^{95-97}$.

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 zone$^{268}$. 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.
The structure of Germinal Centre follicles in the CCR6 deficient humoral response

CCR6−/− and WT mice were immunised with 100µg 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 100µm
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 \(\Delta\)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 formation\(^{102}\). 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\(^{-/-}\) mice\(^{182}\). 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, differentiation\(^{139}\). 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 \(\lambda^+\) CD38\(^-\) GL7\(^+\) B cells, were isolated using the MoFlo\(^\circledR\) Astrios\(^\text{TM}\) 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 \(\Delta\text{Ct}\) 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\(^\circledR\) Astrios\(^\text{TM}\) 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\(_{\text{int}}\) PD-1\(_{\text{int}}\) intermediate T\(_{FH}\) cells and CXCR5\(_{\text{hi}}\) PD-1\(_{\text{hi}}\) T\(_{FH}\) cells. Intermediate T\(_{FH}\) cells must upregulate CXCR5 and PD-1 to become T\(_{FH}\)
cells. As per the analysis of GC B cells, all T<sub>FH</sub> cell samples were normalised by cell number and the samples prepared for qRT-PCR.

Initially, CCR6 and CCL20 expression was quantified in WT T<sub>FH</sub> 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<sub>FH</sub> cell subsets in WT mice by qRT-PCR. This analysis showed that all three populations: non-T<sub>FH</sub> cells, intermediate T<sub>FH</sub> cells and T<sub>FH</sub> 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<sub>FH</sub> cell subsets of WT mice. A significantly higher amount of CCL20 mRNA was detected in the intermediate T<sub>FH</sub> cells, compared to the T<sub>FH</sub> cells (Fig. 5.1.3b). This is interesting as the T<sub>FH</sub> 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 expression. Thus, the finding that intermediate T<sub>FH</sub> 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<sub>FH</sub> cells were examined. Given that IL-21 production by T<sub>FH</sub> cells is fundamental for T<sub>FH</sub> cell commitment through an autocrine mechanism, and consequently GC formation, B cell differentiation and antibody production, IL-21 expression was examined in CCR6<sup>−/−</sup> and WT mice. A significantly higher amount of IL-21 mRNA was detected in CCR6<sup>−/−</sup> T<sub>FH</sub> cells than in WT T<sub>FH</sub> cells (Fig. 5.1.3c). However, this increase in IL-21 expression did not induce significant increases in Bcl-6 nor CXCR5 expression (Fig. 5.1.3d, e). Nonetheless, it has been demonstrated that IL-21 secreted by T<sub>FH</sub> cells also acts directly on GC B cells, through Bcl-6 and thereby controls GC formation and terminal B cell differentiation. Therefore, it is highly likely that the increased IL-21 production by T<sub>FH</sub> cells in the CCR6<sup>−/−</sup> 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<sub>FH</sub> cells, precursor to T<sub>FH</sub> cells, IL-21 expression was found to be similar between CCR6<sup>−/−</sup> and WT mice (Fig. 5.1.3d, e).

As mentioned earlier, no significant variation in CXCR5 expression was detected in CCR6<sup>−/−</sup> T<sub>FH</sub> cells compared to WT T<sub>FH</sub> cells, and this was also true for CCR7 (Fig. 5.1.3c). Both of these chemokine receptors are required for T<sub>FH</sub> cell localisation to the GC, 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 response$^{282}$. 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).
CHAPTER 5
THE MECHANISMS UNDERLYING CCR6 DEFICIENT B CELL DIFFERENTIATION

J. BANNAN

THE ROLE OF CCR6 IN B CELL DIFFERENTIATION DURING THE HUMORAL IMMUNE RESPONSE

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

WT and CCR6−/− mice were i.p. immunised with 100µg of NP-KLH for 5 days. 1000 cells from the T\textsubscript{FH} cell subsets: CXCR5\textsuperscript{hi} PD-1\textsuperscript{hi} T\textsubscript{FH} cells, CXCR5\textsuperscript{int} PD-1\textsuperscript{int} intermediate T\textsubscript{FH} cells and CXCR5\textsuperscript{−} PD-1\textsuperscript{−} non-T\textsubscript{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\textsubscript{FH} cell subsets of WT mice. Also, the gene expression of IL-21, CCR7, CXCR5, FoxP3 and Bcl-6 was quantified in T\textsubscript{FH} cells (c), intermediate T\textsubscript{FH} cells (d) and non-T\textsubscript{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<sub>FH</sub> cells, the T<sub>FH</sub> cell response was also examined by flow cytometry. WT and CCR6<sup>−/−</sup> mice were immunised with NP-KLH and the T<sub>FH</sub> cells were quantified 5–42 days after antigen challenge. As mentioned earlier, T<sub>FH</sub> cells are identified by their high expression of CXCR5 and PD-1. The gating strategy used to detect T<sub>FH</sub> cells is displayed in Fig. 5.1.4a. The WT T<sub>FH</sub> 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<sup>−/−</sup> mice at day 5, would be associated with an increased frequency of T<sub>FH</sub> cells. However, the CCR6<sup>−/−</sup> mice had a similar frequency of T<sub>FH</sub> cells compared to WT mice, 5 days after antigen challenge (Fig. 5.1.4b). While there was no change in the frequency of T<sub>FH</sub> cells between CCR6<sup>−/−</sup> and WT mice, the GC response is still increased in CCR6<sup>−/−</sup> mice, and thus, the T<sub>FH</sub> cell population is reduced compared to WT mice. This finding suggests that at this time point, CCR6<sup>−/−</sup> GC follicles are either under more stringent selection pressure due to the lower number of T<sub>FH</sub> cells, or the reduced number of T<sub>FH</sub> cells may directly impair B cell affinity selection. Surprisingly, a significant increase in the frequency of T<sub>FH</sub> cells was observed in CCR6<sup>−/−</sup> 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<sub>FH</sub> cell population, which is thought to be the precursor of T<sub>FH</sub> cells. Similar to the T<sub>FH</sub> cells, the intermediate T<sub>FH</sub> cell population peaked at day 7 before declining (Fig. 5.1.4c). During this time, the frequency of intermediate T<sub>FH</sub> cells was generally similar between CCR6<sup>−/−</sup> and WT mice (Fig. 5.1.4c). However, a significant reduction in the frequency of intermediate T<sub>FH</sub> cells was detected at day 21, and a significant increase was also detected at day 42 following antigen challenge, in the CCR6<sup>−/−</sup> mice compared to the WT mice (Fig. 5.1.4c). The frequency of non-T<sub>FH</sub> cells was also similar between CCR6<sup>−/−</sup> 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<sub>FH</sub> 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<sub>FH</sub> cell population in the CCR6<sup>−/−</sup> 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^hi 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 expression\(^{268}\). 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\(^{-/-}\) mice\(^{182}\).

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<sup>−/−</sup> mice. Therefore, it was hypothesised that there would be little, if any, variation in antibody production between CCR6<sup>−/−</sup> and WT mice. Antibody production was quantified in the serum of CCR6<sup>−/−</sup> 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<sub>b</sub> production between days 7–42, and IgG1<sub>b</sub> production between days 14–42 after antigen challenge (Fig. 5.1.6). An equivalent production of IgM was observed between CCR6<sup>−/−</sup> and WT mice, following antigen challenge (Fig. 5.1.6). Similarly, IgG2a<sub>b</sub> and IgG1<sub>b</sub> production was similar between CCR6<sup>−/−</sup> 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.

To conclusively reconcile the increased frequency of low-affinity antibody forming cells previously documented in the CCR6<sup>−/−</sup> mice<sup>182</sup>, which are likely to be a defect in the affinity selection due to a reduction in the T<sub>FH</sub> cell and FDC compartment, I wanted to examine the frequency of mutations introduced into CCR6<sup>−/−</sup> 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). 1.4 µg 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 iScript™ RT-qPCR sample preparation reagent, followed by 1 well of 10 000 naïve B cells in which RNA was extracted using the iScript™ 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 100 µg of NP-KLH for 5 days. 1000 GC B cells were isolated by their λ+CD38−GL7+ expression, using the MoFlo® Astrios™ cell sorter. RNA was isolated using the iScript™ 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<sup>−/−</sup> mice. In this way, antigen-specific enriched WT and CCR6<sup>−/−</sup> 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<sup>+</sup> CD38<sup>−</sup> GL7<sup>+</sup>) that were isolated from WT mice immunised for 5 days with 100µg of NP-KLH, using the MoFlo<sup>®</sup> Astrios<sup>™</sup> 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\textsubscript{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\textsubscript{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 congeneric 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 (2x10⁷) 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 2x10⁷ 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 100\(\mu\)g of NP-KLH for 7 days. The expression of CCR6, CCR7, CXCR5 and CXCR4 was analysed by flow cytometry on all \(\lambda^+\) B cells, \(\lambda^+\) CD38\(^+\) GL7\(^+\) precursor B cells and \(\lambda^+\) CD38\(^-\) GL7\(^+\) GC B cells, and representative images are shown (a). The mean fluorescent intensities of each chemokine receptor was quantified on all \(\lambda^+\) 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<sup>−/−</sup>, CCR7<sup>−/−</sup>, CCR6<sup>−/−</sup> 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<sup>52,156,281</sup>, 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<sup>−/−</sup> mice as expected, but also in the CCR7<sup>−/−</sup> 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<sup>−/−</sup> mice correlated with a significant decrease in the total number of λ<sup>+</sup> 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<sup>−/−</sup> 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<sup>−/−</sup>, CCR7<sup>−/−</sup>, CCR6<sup>−/−</sup> 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.
CHAPTER 5
THE MECHANISMS UNDERLYING CCR6 DEFICIENT B CELL DIFFERENTIATION

J. BANNAN
THE ROLE OF CCR6 IN B CELL DIFFERENTIATION DURING THE HUMORAL IMMUNE 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 100\(\mu\)g of NP-KLH. B cell differentiation was examined using antibodies against CD38 and GL7: 1) CD38\(^{hi}\) GL7\(^{-}\) plasma cells, 2) CD38\(^{hi}\) GL7\(^{-}\) naïve B cells, 3) CD38\(^{hi}\) GL7\(^{hi}\) memory B cells, 4) CD38\(^{hi}\) GL7\(^{hi}\) precursor B cells and 5) CD38\(^{hi}\) GL7\(^{hi}\) GC B cells (a). The frequency of GC B cells (b), all \(\lambda\)\(^{+}\) 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 challenge\(^{182}\), 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 cells52,158, 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\textsubscript{FH} cell subsets in disease with CCR6 expression using a mouse and human model of SLE. The FasL\textsuperscript{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\textsuperscript{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 \textit{gld} mice, and was significantly so in the lymph node (Fig. 6.1.1d).

\textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{gld} mice compared to WT mice (Fig. 6.1.2b). In addition, the frequency of memory B cells (CD38$^{\text{hi}}$ 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<sup>hi</sup> GL7<sup>-</sup>) 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<sup>-</sup> GL7<sup>-</sup>) in the lymph node and precursor B cells (CD38<sup>+</sup> GL7<sup>+</sup>) 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<sup>-</sup> GL7<sup>-</sup>) 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 p<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\textsubscript{FH} cells is displayed in Fig. 6.1.3a. A significantly larger proportion of T\textsubscript{FH} cells (CXCR5\textsuperscript{hi} PD-1\textsuperscript{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\textsubscript{FH} cells (CXCR5\textsuperscript{int} PD-1\textsuperscript{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\textsubscript{FH} cells (CXCR5\textsuperscript{-} PD-1\textsuperscript{-}) in gld mice compared to WT mice (Fig. 6.1.3b). A significant reduction in the frequency of lymph node CD4\textsuperscript{+} T cells was also observed in the gld mice compared to WT mice (Fig. 6.1.3b). Therefore, it appears that more non-T\textsubscript{FH} cells are recruited into T\textsubscript{FH} cell differentiation, resulting in the large intermediate and genuine T\textsubscript{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\textsuperscript{+} T cells and non-T\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{FH} cell differentiation in humoral responses and may contribute to the pathophysiology of systemic autoimmunity.

Consequently, excessive GC and T\textsubscript{FH} cell differentiation in the FasL\textsuperscript{gld} mouse model is associated with increased CCR6 expression on naïve, memory and precursor B cells, and reduced CCR6 expression on intermediate T\textsubscript{FH} cells. These results indicate that CCR6 could potentially be used as a biomarker to detect lymphocyte dysregulation, and therefore autoimmune disease.
The T<sub>FH</sub> cell compartment was analysed by flow cytometry in naïve WT and FasL<sup>gld</sup> mice, aged 13-15 weeks. Subsets of T<sub>FH</sub> cells were distinguished by their expression of CXCR5 and PD-1: 1) CXCR5<sup>−</sup>PD-1<sup>−</sup> non-T<sub>FH</sub> cells, 2) CXCR5<sup>int</sup>PD-1<sup>int</sup> intermediate T<sub>FH</sub> cells and 3) CXCR5<sup>hi</sup>PD-1<sup>hi</sup> T<sub>FH</sub> cells, and representative images of WT and FasL<sup>gld</sup> 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<sub>FH</sub> cell subsets of WT and gld mice are displayed (d). Figure represents data collected from 1 experiment (Spleen n=8 WT mice, 4 FasL<sup>gld</sup> 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 SLE\(^{221}\), 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\(^{\text{low/int}}\) IgD+ naïve B cells, CD38+ IgD+ Pre-GC B cells, CD38\(^{\text{low/int}}\) IgD memory B cells and CD38+ IgD- B cells represent both GC and plasma cells\(^{286}\). GC B cells were separated from plasma cells based on their expression of CD27\(^{286}\). 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<sub>FH</sub> cell population. Human T<sub>FH</sub> cells are more difficult to identify than mouse, due to the lack of consistency in defining T<sub>FH</sub> cells in the clinical field. While initial studies defined T<sub>FH</sub> cells by high CXCR5 expression on CD4+ T cells<sup>119,273</sup>, others have reported on T<sub>FH</sub> cells as CXCR5+ ICOS<sup>+</sup> CD4<sup>+</sup> T cells<sup>287,288</sup>, CXCR5<sup>+</sup> PD-1<sup>-</sup>CD4<sup>+</sup> T cells<sup>289</sup>, CXCR5<sup>+</sup> CCR7<sup>-</sup> CD4<sup>+</sup> T cells<sup>290</sup> and CXCR5<sup>+</sup> PD-1<sup>-</sup> CCR7<sup>-</sup> CD4<sup>+</sup> T cells<sup>276,291</sup>. Again, it has been questioned whether these circulatory T<sub>FH</sub> cells are in fact genuine. For instance, while the circulatory T<sub>FH</sub> cells represent the T<sub>FH</sub> cells in secondary lymphoid organs, it has been suggested that they act as memory cells, descendants of T<sub>FH</sub> cells that are capable of differentiating into fully-fledged T<sub>FH</sub><sup>224,276,292</sup>. Consequently, I chose to define the human circulatory T<sub>FH</sub> cell population as CXCR5<sup>+</sup> PD-1<sup>-</sup> CCR7<sup>-</sup> CD4<sup>+</sup> T cells and this gating strategy is presented in Fig. 6.2.2a. This classification allows the accurate detection of circulatory T<sub>FH</sub> cells and is comparable to the literature. When the T cell population was examined, a significant increase in the frequency of circulating T<sub>FH</sub> cells was detected in SLE participants compared to control participants (Fig. 6.2.2b). However, the frequency of all lymphocytes, CD4<sup>+</sup> T cells and CXCR5<sup>+</sup> CD4<sup>+</sup> 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 SLE, 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 SLE. 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 activity. 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

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

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 research. I have made use of the FasL<sup>gld</sup> 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<sup>lpr</sup> mouse model, which has a mutation in the lpr gene. The <sup>gld</sup> mouse and other such strains, are considered SLE-like models as they present with similar immunological attributes that characterise SLE. Specifically, the <sup>gld</sup> 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 <sup>gld</sup> 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 <sup>gld</sup> mice compared to WT mice. These alterations in the B cell compartment of <sup>gld</sup> mice correlated with a significant reduction in non-T<sub>FH</sub> cells and significant increases in the intermediate T<sub>FH</sub> and T<sub>FH</sub> cell populations. Furthermore, CCR6 expression was significantly increased on all CD4⁺ T cells and non-T<sub>FH</sub> cells, and it was
significantly reduced on intermediate T<sub>FH</sub> cells. Thus, the effector B and T cell populations of <i>gld</i> 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 <i>gld</i> mice, is consistent with the literature<sup>302</sup>.

In the study of SLE, I identified human B cell subsets as CD38<sub>lo/int</sub> IgD<sup>+</sup> naïve cells, CD38<sup>hi</sup> IgD<sup>+</sup> pre-GC B cells, CD38<sup>hi</sup> IgD<sup>-</sup> CD27<sup>+</sup> GC B cells, CD38<sup>hi</sup> IgD<sup>-</sup> CD27<sup>-</sup> plasma cells and CD38<sub>low/int</sub> IgD<sup>-</sup> memory B cells. This is consistent with previously published work<sup>286</sup>. 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 reports<sup>303</sup>. During SLE, the increased CCR6 expression on naïve and memory B cells, and the reduced CCR6 expression on all CD4<sup>+</sup> T cells and T<sub>FH</sub> 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<sub>FH</sub> 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 <i>gld</i> 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<sup>+</sup> 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 Fas<sub>L</sub><sup>gld</sup> 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<sub>FH</sub> 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. 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. 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 humans\(^{185-187}\). 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 formation\(^{73}\). 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 cells\(^{304}\). 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\textsuperscript{157,158}. Furthermore, EBI2 contributes to the CXCR5- and CCR7-dependent B cell organisation at the T-B cell border\textsuperscript{305}. 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 then 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\textsuperscript{-/-} B cells compared to WT, after activation in vitro. Furthermore, CCR7 expression was significantly increased on CCR6\textsuperscript{-/-} 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\textsuperscript{-/-} 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\textsuperscript{-/-} 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\textsuperscript{-/-} 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 clones\(^{306}\).

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 differentiation\(^{142}\), 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 challenge^{182}. 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^{−/−} mice^{182}. 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 efficiently\(^{304}\). 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 marrow \(^{308}\), T-B cell interactions \(^{309}\), and the precursor \(^{241}\) or GC stages \(^{310}\). 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 controls\(^{241}\). While pre-GC B cells have actually been documented to increase in children diagnosed with SLE \(^{312}\), 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 significant. 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 cells and other mononuclear cells to the joints, 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 patients.\textsuperscript{218}

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.

\textbf{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

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