The therapeutic potential of modulating inflammation in lupus

Timothy A. Gottschalk
(BBioMedSci Hons.)

Thesis is submitted in fulfilment for the degree of

Doctor of Philosophy

February 2018

Department of Immunology and Pathology
Central Clinical School
Faculty of Medicine, Nursing and Health Sciences
Monash University
Melbourne

Supervisor:
A. Prof Margaret L. Hibbs
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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes works from two review papers published in peer reviewed journals and one submitted original research publication. The core theme of the thesis is to investigate and therapeutically target inflammation in a mouse model of autoimmune disease. The ideas, development and writing up of all the published work included in the thesis were the principal responsibility of me, the student, working within the Department of Immunology and Pathology under the supervision of Associate Professor Margaret Hibbs.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. Only work that I, the student, have contributed to the hereinafter listed publications have been included in this thesis.

In the case of Chapters 1 and 5, my contribution to the work involved the following:

<table>
<thead>
<tr>
<th>Thesis Chapter</th>
<th>Publication Title</th>
<th>Status</th>
<th>Nature and % of student contribution</th>
<th>Co-author names and nature contribution</th>
<th>Co-authors, Monash student (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pathogenic Inflammation and Its Therapeutic Targeting in Systemic Lupus Erythematosus</td>
<td>Published (Front. Immunol. Oct, 2015)</td>
<td>75%. Planned, wrote and reviewed manuscript. Co-designed figures.</td>
<td>The following collectively contributed 25%: 1)ET contributed to writing and review of manuscript 2)MLH contributed to design of figures and planning and review of manuscript</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>Loss of the Leukocyte Integrin CD11b Promotes Autoimmune Disease on a Susceptible Genetic Background</td>
<td>Submitted for publication Feb, 2018</td>
<td>80%. Planned, designed and performed experiments, analysed data, constructed figures and wrote the manuscript.</td>
<td>The following collectively contributed 20%: 1)ET assisted with experiments and reviewed manuscript 2)MJM refined a protocol and assisted with experiments 3)ML assisted with experiments 4)MLH contributed to planning, design and funding of experiments and review of manuscript</td>
<td>N</td>
</tr>
</tbody>
</table>
Figure 1.4 and Table 1.1 were created by myself, and were originally published in the review article “Tsantikos E, Gottschalk TA, Maxwell MJ, Hibbs ML. Role of the Lyn tyrosine kinase in the development of autoimmune disease. Int. J. Clin. Rheumatol. (2014) 9(5), 519–535”.

I have rearranged sections of submitted or published papers with unpublished works in order to generate a consistent presentation and story within the thesis.

Some works in this thesis was performed by or in conjunction with other researchers and this is acknowledged below:

Chapter 4
Mr Scott Coutts (Lumina bacterial 16S Next Generation sequencing and data processing)

Chapter 5
Dr Evelyn Tsantikos (BALB/c Lyn⁻/⁻ survival data)

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student’s and co-authors’ contributions to this work.

Student signature: Timothy Gottschalk

Date: 02/01/2018

Main Supervisor signature: Margaret Hibbs

Date: 02/01/2018
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Publications


Communication and Conference Participation

4 Oct 2013  **Therapeutic targeting of inflammation in Systemic Lupus Erythematosus**  
Monash Department of Immunology 50TH Anniversary Symposium  
Melbourne, Victoria, Australia  
Poster Presentation

18 Nov 2015  **A high fibre diet can modulate hallmarks of inflammation and autoimmunity culminating in reduced nephritis in a model of systemic lupus erythematosus**  
Monash Central Clinical School Annual Postgraduate Research Symposium  
Melbourne, Victoria, Australia  
Oral Presentation

8 Apr 2016  **A high fibre diet can modulate hallmarks of inflammation and autoimmunity culminating in reduced nephritis in a model of systemic lupus erythematosus**  
10TH International Congress on Autoimmunity  
Leipzig, Germany  
Oral Presentation

23 Aug 2016  **A high fibre diet can modulate hallmarks of inflammation and autoimmunity culminating in reduced nephritis in a model of systemic lupus erythematosus**  
16TH International Congress of Immunology  
Melbourne, Victoria, Australia  
Poster Presentation

3 Nov 2016  **CD11b regulates inflammation, autoimmunity and associated pathology in a model of systemic lupus erythematosus**  
Monash Central Clinical School Annual Postgraduate Research Symposium  
Melbourne, Victoria, Australia  
Oral Presentation

28 Mar 2017  **CD11b regulates inflammation, autoimmunity and associated pathology in a model of systemic lupus erythematosus**  
12TH International Congress on SLE & 7TH Asian Congress on Autoimmunity  
Melbourne, Victoria, Australia  
Poster Presentation

29 Mar 2017  **A diet high in fibre diet can moderate inflammation and kidney pathology in a model of systemic lupus erythematosus**  
12TH International Congress on SLE & 7TH Asian Congress on Autoimmunity  
Melbourne, Victoria, Australia  
Oral Presentation
## Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AID</td>
<td>activation induced cytidine deaminase</td>
</tr>
<tr>
<td>ANA</td>
<td>anti-nuclear autoantibody</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>APP</td>
<td>acute phase protein</td>
</tr>
<tr>
<td>APS</td>
<td>Antiphospholipid syndrome</td>
</tr>
<tr>
<td>ARL</td>
<td>Animal research laboratory (Monash)</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor of the tumour necrosis factor family</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CD-</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor 3</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CSR</td>
<td>heavy chain class switch recombination</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic lymphocyte antigen-4</td>
</tr>
<tr>
<td>Cy7</td>
<td>cyanine 7</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DILE</td>
<td>Drug induced lupus erythematosus</td>
</tr>
<tr>
<td>DKO</td>
<td>double knockout mice (Lyn−/−CD11b−/−)</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DPX</td>
<td>distyrene, plasticiser, xylene (mounting medium)</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>e780</td>
<td>eFluor® 780</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
</tr>
<tr>
<td>Eb</td>
<td>erythroblast</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EPO</td>
<td>erythropoietin</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
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<td>FcγR-</td>
<td>Fc gamma receptor-</td>
</tr>
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<td>FDA</td>
<td>Food and drug administration (USA)</td>
</tr>
<tr>
<td>FFAR-</td>
<td>Free fatty acid receptor-</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>gMFI</td>
<td>geometric mean fluorescence intensity</td>
</tr>
<tr>
<td>gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>GPR</td>
<td>G-protein coupled receptor-</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HFD</td>
<td>high fibre diet</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IC</td>
<td>immune complex</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN-</td>
<td>interferon-</td>
</tr>
<tr>
<td>Ig-</td>
<td>immunoglobulin-</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin-</td>
</tr>
<tr>
<td>IL-6R</td>
<td>interleukin-6 receptor</td>
</tr>
<tr>
<td>IQR</td>
<td>inter-quartile range</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine based inhibition motif</td>
</tr>
<tr>
<td>JAK-</td>
<td>Janus kinase-</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LAD</td>
<td>Leukocyte adhesion deficiency</td>
</tr>
<tr>
<td>LN</td>
<td>lupus nephritis</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Mφ</td>
<td>macrophage</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MARP</td>
<td>Monash animal research platform</td>
</tr>
<tr>
<td>MAS</td>
<td>Monash animal services</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>M.ICU</td>
<td>Monash intensive care unit (animal facility)</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
</tr>
<tr>
<td>OTU</td>
<td>Operating taxonomic unit</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythin</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear cells</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
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<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>sIL-6R</td>
<td>soluble IL-6 receptor</td>
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<td>SAP</td>
<td>small adaptor protein</td>
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<tr>
<td>SCF</td>
<td>stem cell factor</td>
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<td>SCFA</td>
<td>short chain fatty acid</td>
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<td>SFB</td>
<td>segmented filamentous bacteria</td>
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<tr>
<td>SFK</td>
<td>Src family kinase</td>
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<tr>
<td>sgp130</td>
<td>soluble glycoprotein 130</td>
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<tr>
<td>sgp130Fc</td>
<td>soluble glycoprotein 130 + human IgG1 Fc region fusion protein</td>
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<tr>
<td>SHP-</td>
<td>Src homology 2 domain containing tyrosine phosphatase</td>
</tr>
<tr>
<td>SHIP-</td>
<td>Src homology 2 domain containing inositol 5’-phosphatase</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>STAT-</td>
<td>signal transducer and activator of transcription-</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>T follicular helper cell</td>
</tr>
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<td>Tg</td>
<td>transgenic</td>
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<td>T&lt;sub&gt;H1&lt;/sub&gt;</td>
<td>T helper 1 cell</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<tr>
<td>TMB</td>
<td>3,3’,5,5’ tetramethylbenzidine</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet (radiation)</td>
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## Abbreviations used in figures

<table>
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<tr>
<th>Abbreviation</th>
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<td>α-IL6</td>
<td>anti-IL-6 mAb treated mice</td>
</tr>
<tr>
<td>11b/-</td>
<td>CD11b⁻/⁻ mice</td>
</tr>
<tr>
<td>B</td>
<td>B cells</td>
</tr>
<tr>
<td>C57</td>
<td>C57BL/6 mice</td>
</tr>
<tr>
<td>DOK</td>
<td>downstream of kinase (scaffolding protein)</td>
</tr>
<tr>
<td>HF</td>
<td>high fibre diet fed mice</td>
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<tr>
<td>iso</td>
<td>isotype control treated mice</td>
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<tr>
<td>L/-</td>
<td>Lyn⁺/- mice</td>
</tr>
<tr>
<td>L/-11b/-</td>
<td>Lyn⁺/-CD11b⁺/- mice</td>
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<tr>
<td>mac</td>
<td>macrophage</td>
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<tr>
<td>Mo</td>
<td>monocyte</td>
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<td>neutrophil</td>
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<td>neut</td>
<td>neutrophil</td>
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<td>ns</td>
<td>not significant</td>
</tr>
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<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>Tr</td>
<td>Regulatory T cells</td>
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Abstract

Systemic Lupus Erythematosus (SLE) is a highly complex and multifactorial autoimmune disease characterised by deposition of immune complexes (ICs) in tissues, causing local inflammatory responses leading to tissue damage and organ failure. The kidney is one of the main sites of organ damage, with lupus nephritis (LN) developing in over 50% of patients and accounts for the majority of morbidity and mortality related to SLE. Standard therapy is heavily reliant on broad spectrum immunosuppressants and anti-inflammatory agents, and while these are generally well tolerated by many patients, they can have dose limiting toxicities and off target effects and can be ineffective at controlling disease in some patients. The B cell compartment is essential for the generation of anti-nuclear autoantibodies (ANAs) and is therefore a key mediator of pathogenesis in SLE. Therefore, unsurprisingly the focus of novel targeted therapeutics for SLE are aimed at the B cell compartment; although these approaches have met with minimal success. Amongst the failure of B cell depleting therapeutics, including Rituximab, to achieve clinical efficacy and approval, Belimumab, a monoclonal antibody targeting B cell survival factor BAFF, became the first new agent approved for the treatment of SLE in the past 50+ years; although this provides only minor improvements in a subset of non-nephritic patients. This highlights the need for novel therapeutic targets and strategies to adequately and broadly control disease manifestations in SLE and more specifically in LN patients.

Alongside hyperactivation and loss of tolerance in B cells, SLE is highly reliant on systemic inflammation to drive pathogenic processes. This has been exemplified in numerous animal model studies which demonstrate that genetic and therapeutic moderation of inflammation can rectify many systemic immune defects and improve kidney pathology. The studies within this thesis explore the impact of modulating inflammation on pathogenesis and disease outcomes in lupus, using the robust Lyn−/− mouse model of SLE which faithfully recapitulates typical pathogenic processes and kidney pathology. To further evaluate the contribution of inflammation to disease and validate the use of therapeutic approaches which moderate inflammatory processes, this thesis examined the efficacy of therapeutically targeting pro-inflammatory cytokine interleukin-6 (IL-6) using a monoclonal antibody (mAb) regimen, the impact of dietary fibre and the gut microbiome, and the contribution of leukocyte integrin CD11b to inflammatory phenotypes and disease outcomes in Lyn−/− mice. While the anti-IL-6 mAb regimen had a limited capacity to control disease, Lyn−/− mice fed a high fibre diet exhibited widespread moderation of inflammatory phenotypes including splenomegaly, myeloid cell expansion and T cell hyperactivation which led to significantly reduced kidney pathology. This effect correlated with positive shifts in the composition of the gut microbiota to a more protective profile. Furthermore, deficiency of CD11b in Lyn−/− mice enhanced typical inflammatory hallmarks and glomerulonephritis via promotion of leukocyte infiltration into the kidney, indicating that CD11b is a protective factor in SLE with therapeutic potential. Collectively, these studies offer distinct prospects for improving current treatment strategies for SLE via the inclusion of therapeutic and metabolic targeting of inflammatory processes and mediators.
Chapter 1

Introduction

This chapter contains content from the following published reviews:


1.1. Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE, lupus) is a B cell-mediated autoimmune disease characterized by the generation of autoantibodies against nuclear antigens (ANAs) and a type III hypersensitivity reaction leading to chronic systemic inflammation. SLE affects approximately 1 in every 2,500 individuals; although this can be highly variable based on geographical location, ethnicity and sex (reviewed in (1, 2)); SLE is most commonly seen in females (9:1 prevalence) with disease onset typically around child-bearing age, and in those of non-Caucasian ethnicity. The disease is polygenic and highly complex, requiring interplay between multiple immunopathogenic factors including host autoantigens and both cellular and humoral immune components which contribute to the generation of a hyper-inflammatory environment resulting in organ and tissue damage (Figure 1.1). Deposition of circulating autoantibody-autoantigen complexes can occur in various tissues and organs of the body resulting in a local inflammatory response and severe tissue destruction. Sites often affected include skin (cutaneous lupus), the nervous system (CNS lupus), joints and muscles (rheumatoid lupus, rhupus) and the kidney (renal lupus, lupus nephritis), which contributes most significantly to disease morbidity (3). Disease progression is non-linear and follows a relapse-remitting course, and due to its heterogeneous nature, it can vary widely from patient to patient, making diagnosis and treatment a challenge (3). The current diagnostic criteria requires a patient to present with 4 out of 11 symptoms/disorders including cutaneous rashes, inflammation of the pleura or pericardium, inflammation of joints and muscles, renal and/or neurologic disorders, haematologic and immunologic disorders and most significantly, autoantibodies specifically targeting nuclear antigens (such as double-stranded DNA, small nuclear riboproteins, chromatin, histone proteins etc.) or to a lesser extent cytoplasmic antigens (4). However, patients can present with fewer indications and still fulfil the diagnostic criteria as long as these symptoms are highly indicative of SLE (for example, presentation of “butterfly-like” cutaneous rash, nephritis and positive for ANAs).
Figure 1.1  Inflammation is a key factor in the pathogenesis of lupus

A hallmark of lupus is the presence of hyperactive B cells and loss of B-cell tolerance. Immune complexes containing nucleic acid autoantigens can engage and activate endosomal TLRs and promote inflammation in SLE. Plasma cell expansion and the production of autoantibodies are also features, although the autoantibodies are benign unless generated in an inflammatory milieu, wherein class-switching to pathogenic isotypes occurs. Pro-inflammatory cytokines not only drive T-cell activation and dendritic cell maturation, but they can stimulate extramedullary haematopoiesis leading to expansion of innate immune cells, and they can induce the production of acute-phase proteins (APPs). Autoantibodies become deposited in tissues such as the glomeruli of the kidney, leading to the activation of myeloid effector cells via Fcγ and complement receptors, leading to tissue destruction. Numerous factors, including genetic make-up, environment, diet, and stress, can modify disease course and severity.
1.2. Lupus Nephritis: Inflammation and Immunopathology

One or more mechanisms of B cell tolerance are lost in SLE allowing for the production of ANAs by plasma cells (reviewed in (5, 6)) (Figure 1.2A). Upward of 90% of SLE patients have elevated titres of serum ANAs, on average 2-3 years prior to clinical onset of SLE (7), with 30-70% of SLE patients developing life-limiting renal disease (8). The temporal delay between autoantibody development and disease onset coupled with incomplete penetrance of ANA-mediated disease suggests that pathogenesis of autoantibody-driven nephritis is conditional upon other factors, such as antigen availability, a pre-established inflammatory environment (Figure 1.2B) and T cell-mediated antibody isotype switching (Figure 1.2C). While a hallmark of inflammation is the elevation in levels of C-reactive protein (CRP), many lupus patients demonstrate normal or even reduced levels of CRP. CRP is involved in the clearance of apoptotic cells (reviewed in (9, 10)), and if they are inadequately cleared, this can expose nuclear antigens allowing for ANAs to extensively bind and form immune complexes (IC). Such ICs can deposit in the basement membrane of the glomerular microvessels (11), resulting in activation of the alternative complement pathway, recruitment of pro-inflammatory neutrophils, macrophages and dendritic cells to the glomeruli via chemotactic signalling which upregulate inflammatory cytokine production and activate autoreactive T cell subsets through antigen presentation and co-stimulation (Figure 1.1, 1.2) (8, 12). Endosomal toll-like receptors (TLR) -7 and -9 in activated B cells, plasmacytoid dendritic cells (pDCs) and macrophages can respond to internalized self ICs containing nucleic acids, which can contribute to the initiation and perpetuation of the inflammatory cascade (reviewed in (13)). CD4+ T helper cells play several key roles in the pathogenesis of lupus nephritis: T helper 1 (Th1) cells are responsible for high level production of pro-inflammatory cytokines such as interferon-γ (IFN-γ) which stimulates dendritic cell and myeloid cell production of interleukin(IL) 1, IL-6, IL-12, IL-18, TNF-α and BAFF creating a perpetual pro-inflammatory loop; T helper 2 cells (Th2) produce cytokines (IL-4, IL-5) which induce antibody isotype class-switching leading to the production of high affinity, pathogenic autoantibodies (reviewed in (14, 15)); Th17 cells also provide B cell support, promote plasma cell differentiation and pathogenic autoantibody production and myeloid cell hyper-activation which drives systemic inflammation (16, 17); T follicular helper cells (TFH) are now also known to contribute to autoimmune germinal centre reactions or autoantibody production in lupus-prone mice and SLE patients (18, 19)(reviewed in (20)) (Figure 1.3). Aside from autoantibody production
(Figure 1.2A), autoreactive B cells contribute to the pathogenesis of lupus nephritis via two supportive mechanisms: B cells can activate autoreactive T cells through antigen presentation and co-stimulation (Figure 1.2B) and they can produce cytokines including IL-6, a pro-inflammatory cytokine able to drive inflammation and inhibit the generation of autoimmune suppressive regulatory T cells (Treg) (Figure 1.2C, Figure 1.3) (8, 15). As well as T cell-induced isotype switching within germinal centres, evidence shows ectopic germinal centre-like congregations within the glomeruli of SLE patients suggesting B cells may undergo local somatic hypermutation of immunoglobulin (Ig) variable region genes generating both higher affinity autoantibodies and memory B cells (21). Inflammation and cytotoxicity caused by the immune response generated against glomerular ICs results in progressive renal tissue damage including immune cellular influx and progressive fibrotic, sclerotic and necrotic lesions (22). As a consequence of this, patients suffer glomerular degeneration and reduced kidney function, which may result in end-stage renal failure requiring dialysis or transplantation (23). Currently, there is an incomplete understanding of the factors driving pathogenesis in lupus nephritis which hinders the development of novel, targeted therapeutics.
B cells have multiple roles in autoimmunity through (A) their ability to produce autoantibodies and (B) via their role as antigen-presenting cells and (C) as producers of inflammatory cytokines.

Figure 1.2  The roles of B cells in lupus pathogenesis

B cells have multiple roles in autoimmunity through (A) their ability to produce autoantibodies and (B) via their role as antigen-presenting cells and (C) as producers of inflammatory cytokines.
Figure 1.3  T Cell contribution to SLE
Naïve CD4+ T helper cells undergo differentiation due to inflammatory stimuli and each subset plays a role in inflammation and pathology in SLE. Th1 cells drive inflammation and activation of effector myeloid cells by producing IFN-γ, Th2 cells can influence antibody isotype switching and activate tissue damaging granulocytes by producing IL-4 and -5, Th17 cells can promote inflammation driven immunoactivation and suppress regulatory T cells by producing IL-17, -21 and -22, and T\textsubscript{FH} promote autoreactive plasma cell differentiation in the germinal centres and production of pathogenic autoantibodies.
1.3. Genetic Associations in SLE

SLE has a strong genetic component with high familial concordance where likelihood of developing SLE over the general population is over 23 times for siblings (300 times for twins), 11 times for parents and 14 times for offspring of a patient with SLE (24, 25). The notable heterogeneity of clinical manifestations of disease and patient responses to treatment truly underlines the polygenic nature of SLE, as multiple defects, particularly in genes associated with maintaining immune system tolerance and homeostatic processes are essential for disease development. Genetic analyses and more recently genome-wide association studies (GWAS) have uncovered various human SLE susceptibility genes involved in normal immune system functions, some of which are involved in antigen processing and presentation (HLA), clearance of apoptotic debris (C1q, Dnase1), leukocyte interaction and cell surface receptors (FCGR1/II/III, ITGAM) and cell signalling and gene transcription (LYN, CSK, BLK, PTPN22, STAT4, IRF5) (26, 27). The following sections detail the contribution of some of the most significant genetic links with SLE, as well as those pertaining to the work conducted within this thesis.

1.3.1. HLA

The HLA locus is a 3.6 mega base region at 6p21.3 which encodes over 200 genes. It is subdivided into 3 regions: Class I which encodes for major histocompatibility complex (MHC) class I proteins (HLA-A, -B, -C), Class II encodes MHC class II proteins (HLA-DR, -DP, -DQ) and antigen processing channel proteins (TAP1, 2), and Class III which encodes a variety of immune related genes including Tumour necrosis factor -alpha (TNFA) and -beta (LTA; lymphotoxin-α) and the complement system (C2, C4A, C4B, CFB). Genes within the HLA locus, more specifically within the Class II region have some of the most powerful genetic links with SLE susceptibility (28). Specifically, HLA-DR, which is the most polymorphic of the HLA-D genes, exhibits the broadest association with SLE in a diverse range of populations and cohorts including Caucasian and Europeans (DR2, DR3)(29, 30), African Americans (DR2, DR3) (30, 31), Hispanic Americans (DR8) (30), Mexicans (DR3, DR7) (32), Japanese, Chinese and Koreans (DR2) (33-35) and Indians (DR4) (36). A recent meta-analysis examining 25 studies across multiple and varied ethnicities demonstrated that DR3 (OR 1.88), DR9 (OR 1.24) and DR15 (OR 1.25) are risk alleles while DR4 (OR 0.79), DR11 (OR 0.72) and DR14 (OR 0.74) are protective for SLE worldwide (37). The pathogenicity of MHC Class II is likely diverse given that it is implicated in antigen presentation, activation of T cells by DCs as well as T cell-directed B
cell activation and antibody production. DR3 and to a lesser extent DR2 have been strongly associated with production of anti-Ro and/or anti-La ANAs (38, 39); this has been hypothesized to be due to presentation of environmental T cell antigens which mimic SLE autoantigens, activating autoreactive T cells (40). The density of genes within the HLA locus accounts for the high linkage disequilibrium within this region, and risk haplotypes consisting of susceptibility alleles in various closely linked genes within the HLA locus have been identified in SLE. Within the Class II region, haplotypes containing DR2|DQ6, DR8|DQ4 or DR3|DQ2 exhibit increased susceptibility (41). Similarly, the locus spanning HLA-B8|SC01|DR17 haplotype is linked to familial SLE (42). More recently, SLE association with polymorphisms in the 3′ untranslated region of the immunoregulatory non-classical MHC Class I HLA-G gene have been identified in European (43, 44), Brazilian (45) but not in Chinese (46, 47) populations and is associated with child and early onset disease (48, 49).

1.3.2. C1q
C1q is an integral element of the initiating C1 complement complex (comprising of C1q, C1r and C1s) which contains immunoglobulin Fc binding domains allowing binding to antibody opsonized pathogens, driving the activation of the classical complement pathway (50). C1q is important for the clearance of apoptotic cell fragments via ligation to a number of identified receptors expressed on macrophages, preventing inflammatory responses driven by the release of cellular components and limiting nuclear antigen exposure (51). C1q on apoptotic cells also induces tolerogenic responses, restricting pro-inflammatory cytokine production in macrophages (52) and limiting the survival and induction of Th1 and Th17 responses (53). Genetic C1q deficiency is relatively rare, yet confers a massive risk of developing SLE, with over 90% of carriers exhibiting symptoms (54), suggesting that C1q is protective against SLE. Restoration of C1q via plasma transfusion or bone marrow transplant in deficient patients has shown curative effects, ameliorating pathology (55, 56), suggesting that genetic C1q deficiency may itself be sufficient to elicit disease, bypassing the normally heterogenic nature of SLE. More commonly, C1q deficiency can be induced by anti-C1q autoantibodies and is a strong indicator of kidney involvement in SLE (57). Predictably, mice deficient in C1q (C1qa−/−) develop lupus-like glomerulonephritis associated with ineffective clearance of apoptotic bodies (58).
1.3.3. ITGAM

ITGAM encodes for the $\alpha_M$ integrin subunit (CD11b) which is complexed with the common $\beta_2$ integrin chain (CD18) forming the $\alpha_M\beta_2$ integrin also known as Mac-1 or complement receptor 3 (CR3). Mac-1 is a cell surface receptor largely restricted to myeloid cells (macrophages and neutrophils), certain dendritic cell subsets and some B cell subsets, and its functions include cell-cell adhesion and extravasation through binding interactions with ICAM ligands and iC3b complement mediated phagocytosis. It has also been shown that Mac-1 mediated interactions between vascular endothelium and DCs can regulate their maturation and DCs can restrict T cell activation through Mac-1 mediated interactions (59, 60). More recent studies have implicated Mac-1 through intracellular mechanisms in the regulation of MyD88 and TRIF mediated signalling and cytokine production downstream of TLRs as well as moderation of autoreactive BCR signalling- both of which involve interactions with SLE-associated Src family kinase (SFK) Lyn (61-64). Therefore, it is not surprising that mutations in ITGAM, resulting in loss of these regulatory mechanisms, can contribute to the development of SLE.

Genome-wide association studies have identified single nucleotide polymorphisms (SNP) in the human ITGAM locus (16p11.2) which associate highly with incidence of SLE. Non-synonymous SNP rs1143679, which is a Guanine to Adenine substitution resulting in a missense replacement of Arginine with Histidine at position 77 (R77H) of the CD11b protein is a loss of function mutation most strongly associated with SLE and lupus nephritis across many different ethnicities including European, African and Hispanic Americans and Central and South Americans (65, 66), Brazilian (67), pan European (68, 69) and Hong Kong Chinese and Thai (70). ITGAM SNPs rs1143678 (P1146S) and rs1143683 (A858V) are also strongly linked to SLE, and together with rs1143679 show high linkage disequilibrium resulting in compound ITGAM SNPs commonly being detected in patients with SLE (66, 70). The rs1143679 SNP occurs in the extracellular ligand binding $\beta$-propeller domain influencing receptor-ligand interactions, rs1143678 occurs in the cytoplasmic tail and is associated with interactions with the cytoskeleton and signalling molecules (71). The rs1143683 SNP being a conservative amino acid substitution in the extracellular calf-1 region, does not confer any conformational or functional changes to the CD11b protein; therefore its association with SLE has been attributed to its complete linkage disequilibrium with the rs1143678 SNP (71).
While delineating the disease related effects of these mutations in humans is difficult, a number of studies have examined the SNPs in donor or transfected cell in vitro models. CD11b<sup>R77H</sup> transfected Jβ2.7 Jurkat T cells exhibit impaired ICAM binding and adhesion (72), which has also been recapitulated in neutrophils under sheer flow from humans carrying the various common SLE-linked ITGAM polymorphisms (rs1143679, rs1143678, rs1143683) (71, 73). These defects are not due to altered CD11b expression but are attributed to instability in the extracellular ligand binding region (73). CD11b<sup>R77H</sup> transfected U937 human monocyte-like cells as well as blood derived monocytes, macrophages and neutrophils from R77H donors similarly showed reduced iC3b binding and impaired phagocytosis, suggesting that leukocyte migration and clearance of apoptotic debris is hindered by the R77H mutation (72, 74). R77H transfected mouse B cell line K46-17 exhibited loss of CD11b mediated regulation of calcium influx in response to BCR stimulation and impairment in the Lyn-CD22-SHP-1 regulatory signalling axis, showing dysregulation of B cell signalling processes with the R77H mutation (62). Human blood monocytes and macrophages carrying the R77H mutation are hyper-responsive to TLR7/8 stimulation, resulting in enhanced pro-inflammatory cytokine secretion and are resistant to inhibition via Mac-1 agonists, Leukadherin-1 (LA1) and iC3b (74, 75). Similarly, the rs1143678 (P1146S) mutation promotes pro-inflammatory cytokine production through integrin clustering via recruitment of adaptor protein 14-3-3ζ (76), indicating that aberrant inflammatory responses are perpetuated in carriers of these ITGAM mutations. Indeed, ingenuity pathway analysis, which visualizes molecular interactions and signalling pathways downstream of receptor-ligand binding has determined that Mac-1 deficiencies can directly promote SLE phenotypes including immunological abnormalities, autoantibody production and nephritis through inability to regulate inflammation and clear immune complexes and nuclear debris (75).

1.3.4. LYN
LYN is a signal transduction molecule and one of nine members of the Src family non-receptor protein tyrosine kinases (SFK). It is expressed throughout the immune and haematopoietic system with the exception that it is not in T cells (77). SFKs are localized to lipid rafts within the plasma membrane (78, 79) and when activated they phosphorylate tyrosine residues within immunoreceptor tyrosine activation motifs (ITAMs) and, in the instance of LYN, immunoreceptor tyrosine inhibition motifs (ITIMs) (80, 81) within the cytoplasmic tails of cell
surface receptors resulting in the recruitment and activation of signalling molecules (Figure 1.4). LYN also directly phosphorylates and activates signal transduction molecules such as SYK (82) and regulatory phosphatases Src-homology 2 domain containing tyrosine phosphatase 1 (SHP-1) (83) and Src-homology 2 domain containing inositol 5'-phosphatase 1 (SHIP-1) (84). SFKs are highly homologous often making their activatory role functionally redundant when co-expressed within the same cell type; although, the capacity of LYN to transduce inhibitory signals and resolve cellular activation is unique, making it essential for immunoregulation and maintaining homeostasis and tolerance within the immune system (81, 85)(reviewed in (86, 87)). This is exemplified in mice lacking Lyn (Lyn−/−), which exhibit complete dysregulation and hyperactivation of immune cells resulting in loss of B cell tolerance and chronic systemic inflammation, culminating in autoantibody mediated lupus-like glomerulonephritis (85, 88)(comprehensively reviewed (87))(Further discussed in Chapter 1.7.1.). LYN, being the predominant SFK in B cells, is central in balancing appropriate activation of B cell responses (86). LYN is implicated in activation of signalling via the B cell receptor (BCR), CD19/CD21 complement co-receptor complex and phosphoinositide 3-kinase (PI3K) and is essential for regulatory signals through the inhibitory receptors FcγRIIb, CD22 and CD45 and activation of inhibitory phosphatases SHIP-1 and SHP-1 (86).

While the function of Lyn in autoimmune disease has been extensively studied in mice (reviewed in (87)), its role in human disease is far less clear. Early studies revealed aberrant expression of LYN in B cells from SLE patients (89, 90), which correlates with reports of B cell hyperactivity in SLE (91), potentially due to loss of LYN’s regulatory mechanisms. It has since been shown that ubiquitination and subsequent degradation of LYN is enhanced and translocation to lipid rafts is defective in SLE B cells, suggesting that this two-pronged dysregulation of LYN promotes B cell hyper-responsiveness in SLE (92). Conversely, transcription of LYN in whole blood is unchanged in SLE (93), but given the heterogeneous cell population, the significance of this is unclear. GWAS has linked LYN to SLE with the detection of multiple SNPs in the LYN locus (8q12), rs7829816, rs2667978 (26) and rs6983130 (94) in Americans of European ancestry which are protective against development of SLE. Association has reportedly not been established in Finnish (95), African American or Korean populations (94). The mechanisms by which these minor alleles protect against disease have, thus far, not been investigated. While a direct genetic link may not be strong, there is a clear
role for dysregulated Lyn function in contributing to pathogenesis in SLE. This is further supported by the identification of a polymorphism in SFK regulatory tyrosine kinase C-terminal Src kinase (CSK) (rs34933034) which is associated with SLE (96). This polymorphism promotes B cell activation through enhancing CSK-mediated phosphorylation of inhibitory tyrosine residues on Lyn, diminishing Lyn-mediated ITIM phosphorylation and subsequent regulation of BCR signalling (96) (Figure 1.4).

Figure 1.4  Lyn and the Src family of protein tyrosine kinases in immune cell signalling
The SFKs play an essential role in initiating activatory signalling from ITAM-containing immunoreceptors such as the BCR. While Lyn contributes to positive signalling, it also plays an essential non-redundant role in inhibitory signalling from ITIM-bearing inhibitory receptors. Solid lines signify positive signalling pathways, while dotted green lines indicate activatory signalling pathways that can also be regulated by Lyn. Dashed red lines specify inhibitory pathways that are regulated exclusively by Lyn.
1.4. Environmental Associations in SLE

Although SLE susceptibility has a clear genetic component, monozygotic twins lack complete concordance, suggesting that non-heritable factors also contribute. While epigenetics may contribute to this (97), heritability accounts for less than 44% of the phenotypic variance for SLE, whilst the remaining 56% is influenced by environmental factors (24). Causal links have been well established for ultra violet (UV) radiation, drugs and medications, and infection in SLE while, recent studies suggest that diet and the gut microbiome may influence the development of autoimmune and inflammatory diseases (98).

1.4.1. UV radiation

UV exposure is one of the major known inducers of disease flares and skin manifestations in SLE. SLE patients are photosensitive (99) and exhibit improvements in clinical outcomes using photo-protective measures (100). UV radiation from the sun can be subdivided into three grades based on wavelength; UV-C (200-290nm) is absorbed by the ozone layer and therefore has minimal impact on health while UV-A (320-400nm) and UV-B (290-320nm) both have an impact on disease (reviewed in (101)). UV-A radiation has been reported to have both exacerbatory (102) and moderating (103) effects in cutaneous lupus. The mechanism by which UV-A affects lupus is poorly understood as UV-A exhibits both pro- and anti-inflammatory functions (101), but it has been shown to improve disease scores and regulate autoantibodies in human SLE (104) and lupus prone mice (105) and is used therapeutically in both cutaneous lupus and SLE (106). Conversely, while low dose UV-B has also exhibited some immunoregulatory effects (101), UV-B potently induces cellular and DNA damage and induction of reactive oxygen species (ROS), exposing nuclear autoantigens (107, 108). European prevalence of SLE is highest in southern countries where UV-B exposure is higher (1), and UV-B concentration is linked to mortality in SLE patients in the USA (109, 110).

1.4.2. Drug induced lupus

Drug induced Lupus Erythematosus (DILE) is a distinct sub-classification of SLE that is brought on by taking medications. While over 90 known drugs can induce DILE, the most potent is the anti-arrhythmic drug procainamide, which causes DILE in 20% of patients within the first year of treatment, and the vasodilator hydralazine which promotes DILE in 5-8% of patients (111). Other drugs that are classed as low risk of DILE range from antibiotics and antifungals, antidepressants and antipsychotics, migraine medications, anti-convulsants, chelating agents,
contraceptives and hormone replacement drugs, anti-inflammatory drugs and more recently, biologicals and small molecule inhibitors specifically targeting the inflammatory mediator TNF-α and Type 1 interferon therapies (reviewed in (112)). While the clinical manifestations of DILE can resemble that of SLE, it is most prevalent in the elderly, Caucasians and does not exhibit a sex bias, contrary to that of idiopathic SLE (111). In most cases, DILE subsides after cessation of drug treatment, with more refractory disease requiring short term standard SLE treatment (111, 112).

1.4.3. Infection
The induction of autoimmune disease by infection has been largely attributed to molecular mimicry; where pathogenic antigens share epitopes with self-molecules, which leaves behind a pool of activated autoreactive lymphocytes after clearance of the initial infection (113). SLE is highly associated with previous exposure to Epstein-Barr Virus (EBV) (<99.5%) (114, 115) and antibody cross-reactivity between EBV antigen EBNA-1 and SLE autoantigens non-coding RNAs (Ro) and spliceosomal RNAs (Sm) has been well established, suggesting that EBV infection may be an initiating factor in SLE pathology (116, 117). Similar cross-reactivity has also been shown with the Coxsackie virus (118) and parvovirus (119), while cytomegalovirus (CMV) can induce production of anti-phospholipid antibodies which is a feature of both anti-phospholipid syndrome (APS) and SLE (120). Conversely, the increasing incidence of autoimmune disease inversely correlates with the decreasing incidence of infectious disease; this “hygiene hypothesis” suggests that infections can be protective against autoimmunity (121). One of the prevailing theories behind this is that pathogens can activate immunoregulatory pathways (reviewed in (121)) and produce immunosuppressive products (122, 123) which suppresses inflammation and autoreactive cells. This has been revealed in lupus prone NZBxNZW F1 mice, which after infection with malaria causing Plasmodium berghei exhibit drastic improvements in kidney function and survival (124).

1.5. Dietary fibre, the microbiome and inflammation
There has been intense recent interest in the role that diet may play in the development of autoimmune and inflammatory diseases. This has been driven by studies showing that populations that consume Western diets (highly processed, high in saturated fats, low in fibre) have a higher rate of chronic inflammatory diseases such as cardiovascular disease, colitis and asthma (125, 126). Conversely, diets low in processed foods and high in fibre derived from
complex carbohydrates are associated with a relative lack of these diseases (126-128). Much of the immunoregulatory impact of diet is due to metabolite breakdown by the gut flora or microbiome which is the microbiological makeup of the digestive tract. This complex of microorganisms performs critical functions such as the synthesis of vitamins and the fermentation of non-digestible dietary fibre or ‘resistant starches’ to produce short chain fatty acids (SCFAs) (129). The gut microbiota can vary considerably between human populations and changes in diet can lead to large changes in an individual’s microbiome (129). The most abundant phyla of gut bacteria in humans are Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria. In broad terms, Firmicutes and Bacteroidetes are responsible for the fermentation of carbohydrates to SCFAs and Actinobacteria and Proteobacteria metabolise non-digested proteins from the diet or digestive enzymes (130).

SCFAs such as acetate, propionate and butyrate are produced in the intestine by the actions of the microbiota, and either act locally (e.g. butyrate is mostly utilized by colonocytes) or are absorbed into the circulation, with acetate being the main SCFA detectable in the circulation (131). Thus SCFAs generated in the gut can have systemic effects and indeed many recent studies indicate that SCFAs can restrain inflammation and inhibit pro-inflammatory cytokine production (including IL-6) suggesting that they may be able to modulate inflammatory diseases (reviewed in (132)).

1.5.1. SCFA signalling in the immune system
SCFAs signal through a family of G-protein couple receptors (GPR) including GPR-43, -41, -109A and -109B, expressed on a variety of cells including cells of the immune system, adipocytes, pancreatic cells, neural cells and epithelial cells (reviewed in (133)). GPR-43, since renamed free fatty acid receptor 2 (FFAR2), is highly expressed on peripheral blood leukocytes and splenocytes, and is most potently activated by SCFAs acetate and propionate (134). Similarly, FFAR3 (GPR41), also potentiates SCFA signals and is largely expressed in adipose tissue (135). Expression of FFAR2 is detected on monocytes, neutrophils (136), lymphocytes (135), Tregs (137) and eosinophils (138), while DCs express FFAR3 and not FFAR2 (135, 139).

Signalling via FFAR2 restrains the development of inflammatory diseases including Dextran sulfate sodium (DSS) induced colitis, inflammatory arthritis and allergic airway disease in mice (138). FFAR2 mediated signals inhibit pro-inflammatory cytokine production, skew T cell responses away from Th17 and toward regulatory T cells (137, 138) and regulates neutrophil
recruitment and migration during inflammation (140). While the molecular mechanisms underpinning the anti-inflammatory effects of FFAR2 signalling are incompletely understood, it has been shown to activate the NLRP3 inflammasome, which in gut epithelium induces protective IL-18 production (141), but also induces pro-inflammatory IL-1β production in peripheral immune cells (142). SCFAs can also modulate gene transcription through the inhibition of histone deacetylases (HDAC). In the gut, butyrate has been shown to inhibit HDAC activity (143) which has a profound effect on driving Treg differentiation (144) and restricts macrophage release of inflammatory mediators (145), limiting local inflammation. HDAC inhibition in lupus prone MRL\textsuperscript{lpr/lpr} and Lyn\textsuperscript{-/-} mice restricted kidney pathology through the depletion of circulating plasma cells and autoantibodies (146), and in a model of renal inflammation, suppresses inflammation and fibrosis in the kidney through inhibition of NF-κB (147), highlighting this pathway as a viable target to modulate pathology in SLE.

1.5.2. Pathological dysbiosis and dietary interventions in SLE
A small cohort study has shown that SLE patients have an altered ratio of SCFA producing gut phyla Bacteroidetes and Firmicutes, indicating that some microbiota mediated metabolic pathways may be altered in these patients (148). Dysbiosis is also evident in lupus prone MRL\textsuperscript{lpr/lpr} mice with differences in intestinal flora observed between sex, age and disease progression (149). Interestingly, lupus prone Lyn\textsuperscript{-/-} mice have an expansion of segmented filamentous bacteria (SFB) (150), which can drive systemic ANA production mediated by T\textsubscript{h}17 cell responses (151), although detection of these bacteria in adult humans is questionable and therefore the significance of this in human disease is unclear (152). Evidence suggests that the restorative effects of oral Vitamin A supplementation, which has previously been shown to improve nephritis in humans and mice, may be due to alterations in the microbiota and restoration of normal microbiota function (149). MRL\textsuperscript{lpr/lpr} mice fed commercial diets high in the phytoestrogen isoflavone developed exacerbated glomerulonephritis through enhanced IC deposition and myeloid cell infiltration (153). These diets also promoted gut microbiome shifts, resulting in the expansion of disease-associated bacteria Lachnospiraceae, as well as increased lupus associated micro RNA expression (miR-148a and miR-183) and promotion of LPS induced DNA methylation (153). A model of tubule-interstitial kidney disease in rats exhibited significant amelioration of kidney pathology due to alleviation of oxidative stress, inflammation and immune cell infiltration when fed a diet high in resistant
starches (154) coinciding with shifts in the gut microbiome (155). These preliminary studies highlight the potential role that gut microbiota and dietary products, particularly carbohydrate metabolites have in regulating systemic inflammation and lupus-like disease outcomes, and further studies delineating the contributions of these micro-organisms on systemic inflammatory and pathogenic processes in SLE may uncover novel diet-based lifestyle interventions to aid in controlling disease.

1.6. Current treatments and future treatment development

Treatment of SLE has remained fairly consistent over the past 50 years utilizing non-specific anti-inflammatory agents such as non-steroidal anti-inflammatory drugs and the innate cell modulating hydroxychloroquine for mild disease and broad spectrum immunosuppressants/anti-inflammatories such as corticosteroids, azathioprine, cyclophosphamide or mycophenolate during flares or severe disease with organ involvement (156). These therapies can have severe, dose-limiting toxicities and undesirable side effects, and some patients lack an adequate response, highlighting the need for a treatment regimen tailored to individual patients (156). Development and trial of novel targeted treatments for SLE has been difficult due to the complexity and limited knowledge of disease aetiology, loose diagnostic criteria, various disease manifestations and the diverse heterogeneity of patients (reviewed in (157)).

Despite promising results in animal models (158), therapeutic targeting of B cells in SLE has had minimal success, especially in patients with lupus nephritis. B cell depletion using Rituximab, a monoclonal antibody targeting the B cell-specific receptor CD20 (anti-CD20 mAb) was unsuccessful at meeting primary and secondary endpoints in stage II/III SLE clinical trials despite showing initial promising results (159, 160). Ocrelizumab, a humanized anti-CD20 mAb also failed phase III clinical trials demonstrating insignificant renal improvement and increases in severe infection of patients (161). Inhibition of B cell survival pathways by neutralizing the survival receptor TACI, using a humanized fusion protein Atacicept was initially deemed safe in phase I trials but efficacy has not been determined due to safety concerns during a phase II/III trial which led to its premature termination (162). Modulation of B cell receptor signalling using Epratuzumab (anti-CD22 mAb) has yielded some promising results in non-renal SLE patients although there is currently no data on outcomes in patients.
with lupus nephritis (163). Recombinant small molecule inhibitor Abatacept, which blocks T cell co-stimulatory ligands (CD80 and CD86) on B cells and dendritic cells, also failed to meet primary and secondary endpoints in a phase II clinical trial of SLE patients which largely excluded lupus nephritis patients (164).

Despite this lack of success, Belimumab, a monoclonal antibody which neutralizes the soluble B cell activation and survival factor BAFF, has recently been approved for SLE patients with active disease in the USA and Europe by the Federal Drug Administration (FDA) and the European Medicines Agency after successful phase III clinical trials showing modest improvements in disease (165, 166). It is now also available for use in other countries, such as Australia. Belimumab is not only the first targeted therapeutic indicated for SLE, but it is the first new therapeutic approved for SLE in over 50 years. However, the benefits obtained with Belimumab are modest and only attained in patients with mild disease who are already receiving standard therapy (165). Given the overall lack of success of lymphocyte targeted therapeutics, coupled with the heterogeneity and diverse array of clinical manifestations of disease and the contribution of inflammatory factors to disease development in SLE, more consideration to non-B cell targets and combination therapeutic regimens are required in order to deliver a more personalized and effective approach to treating SLE.

1.7. Animal models of SLE
As SLE is highly complex, multifactorial and manifests in an array of pathologies, the disease has been difficult to study in humans. Mouse models that mimic aspects of SLE pathology and pathogenesis include the naturally occurring mutants MRL<sup>lpr/lpr</sup>, NZBxNZW F<sub>1</sub>, congenic BSXB/Yaa, NZM2410, B6.sle1.sle2.sle3, B6.sle1.Yaa, transgenic (Tg) over-expression of BAFF in BAFF-Tg, and induced models such as pristane and ALD-DNA (reviewed in (167, 168)). Much of our current understanding of SLE disease pathogenesis and many preliminary therapeutic studies for SLE have come from the identification, analysis or testing of these mouse models (reviewed in (169, 170)). One well-studied model of SLE is the Lyn-deficient mouse (Lyn<sup>−/−</sup>) (85) (Table 1.1), which exhibits clinical, pathological and biochemical features seen in human SLE (comprehensively reviewed (87)).
1.7.1. Lyn deficiency: A loss of immuno-regulation and a model of lupus nephritis

As previously discussed (Chapter 1.3.4.), Lyn is an essential signalling molecule of the immune system which has non-redundant roles in inhibitory signalling, turning off immune responses (reviewed in (86)). The effect of Lyn deletion in mice (Lyn<sup>-/-</sup>) results in widespread loss of immunoregulation which culminates in a lupus nephritis-like kidney pathology (reviewed in (87)), and the mice have been extensively studied as a model of inflammatory autoimmune disease (Table 1.1). Lyn being a major regulator of signalling in B cells, in its absence, B cells exhibit a severe deficit in both the recirculating bone marrow compartment and in the peripheral lymphoid organs, with a higher proportion of ‘short-lived’ B cells and perturbed proliferative capacity, indicating that Lyn is influential in B cell development and survival (85, 171, 172). Lyn-deficient B cells also exhibit hyper-responsiveness to signals downstream of BCR crosslinking, with Lyn being essential for phosphorylation of inhibitory receptor CD22 and the recruitment and activation of inhibitory phosphatase SHP-1 (172-174), and important in regulating signals downstream of inhibitory IgG receptor FcγRIIb (172). Lyn<sup>-/-</sup> mice also exhibit plasmacytosis which coincides with hyper-IgM production, as well as both IgM and pathogenic IgG and IgA ANAs, particularly targeting dsDNA (85, 171, 172, 175, 176). Due to ANA immune complex deposition in the microvasculature, Lyn<sup>-/-</sup> mice develop a severe kidney pathology, which is characterised by glomerular expansion and lobularity due to immune cell infiltration and hypercellularity and the development of sclerotic and necrotic lesions and vasculitis (85, 88). Altogether this leads to reduced kidney function, which impairs overall survival (85, 177). While autoreactive B cells are essential for the development of disease in Lyn<sup>-/-</sup> mice (88) (Table 1.1), disease is also dependent on systemic inflammation which becomes increasingly severe with age (reviewed in (87, 178)). Lyn<sup>-/-</sup> mice develop splenomegaly due to expansion of erythroblasts and myeloid cells, which occurs in part in response to elevated systemic inflammatory cytokines and growth factors (88, 176, 179, 180). Macrophages and neutrophils, which are potent producers of inflammatory factors, are hyper-responsive to growth factor stimulation in Lyn<sup>-/-</sup> mice (179), and over-produce B cell activating factor, BAFF (181). Lyn<sup>-/-</sup> mice also have hyperactivated DCs which exhibit elevated expression of T cell activatory molecules MHC Class II and CD80 and also overproduce inflammatory cytokines IL-6 and IL-12 (182). While T cells do not endogenously express Lyn, due to systemic inflammation and DC hyperactivation, Lyn<sup>-/-</sup> mouse CD4+ T cells exhibit a hyperactivated phenotype, which drives autoantibody isotype switching (88, 183) and on the
BALB/c background, Lyn⁻/⁻ mice have an expanded Treg cell compartment (176). Lyn⁻/⁻ mice have recently been shown to have impaired plasmacytoid DC Type 1 interferon responses (184), which is interesting given that IFN-α is a key pathogenic factor in SLE (see Chapter 1.8.1.2.). Further description of pDC contributions to disease in the Lyn⁻/⁻ mouse model is currently lacking. The afore mentioned inflammatory phenotypes are essential for disease manifestation (Table 1.1); Lyn⁻/⁻ mice lacking TLR signal transducer, MyD88 (Lyn⁻/⁻ MyD88⁻⁻), maintain B cell defects yet exhibit a resolution of DC hyperactivation culminating in ablation of autoantibody production and kidney pathology (182). Disease is maintained in mice harbouring conditional deletion of Lyn in either B cells or DCs, with both being reliant on MyD88 driven inflammatory signals to generate pathology (185, 186). Abrogation of T cell hyperactivation through the genetic modulation of the PI3K pathway (Lyn⁻⁻ p110δ⁺⁻/KD), improved kidney pathology through the regulation of B cell class switching and autoantibody production (183). Similarly, independent deletion of inflammatory cytokines IL-6 (Lyn⁻⁻ IL-6⁻⁻) or IFN-γ (Lyn⁻⁻ IFN-γ⁻⁻) resolves kidney pathology by dampening systemic inflammation and T cell hyperactivation (88, 181). These studies highlight the importance of inflammation in driving pathogenic processes which drives nephritis in Lyn⁻/⁻ mice.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic mutation</th>
<th>Effect on disease</th>
<th>Ref</th>
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<tbody>
<tr>
<td><strong>B cells and B cell signalling</strong></td>
<td></td>
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<tr>
<td>Lyn−/−μMT−/−</td>
<td>Lacking Lyn and B cells</td>
<td>Loss of T cell hyper-activation and myeloid expansion, persistence of Lyn−/− DC phenotype. Ablation of autoimmune disease</td>
<td>(88)</td>
</tr>
<tr>
<td>Lyn−/−CD19−/−</td>
<td>Lacking Lyn and CD19</td>
<td>Loss of B cell hyper-activation, autoantibody production and amelioration of glomerular disease</td>
<td>(187)</td>
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<tr>
<td>Lyn−/−Btk−/−</td>
<td>Lacking Lyn and Btk</td>
<td>Loss of B cell hyper-activation, autoantibody production and amelioration of glomerular disease</td>
<td>(188, 189)</td>
</tr>
<tr>
<td>Lyn−/−Btklo</td>
<td>Lacking Lyn and expression of Btk reduced to 25%</td>
<td>Uncoupling of autoimmune disease (absent) from B cell hyperactivity (persistent)</td>
<td>(190)</td>
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<tr>
<td><strong>Genetic background and gene interaction</strong></td>
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<tr>
<td>129Ola x C57BL/6 Lyn−/−</td>
<td>Lacking Lyn</td>
<td>Severe glomerular disease, possibly enhanced by epistatic modifiers of SLE on 129Ola background</td>
<td>(85)</td>
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<td>C57BL/6 Lyn−/−</td>
<td>Lacking Lyn</td>
<td>Moderate to severe glomerular disease</td>
<td>(88)</td>
</tr>
<tr>
<td>BALB/c Lyn−/−</td>
<td>Lacking Lyn</td>
<td>Mild glomerular disease</td>
<td>(176)</td>
</tr>
<tr>
<td>Lyn−/+</td>
<td>Haploinsufficiency of Lyn</td>
<td>Delayed, mild glomerular disease</td>
<td>(177, 191)</td>
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<td>Lyn−/−Me−/+</td>
<td>Haploinsufficiency of Lyn and SHP-1</td>
<td>Amplification of Lyn−/− phenotype, myeloid compartment defects and glomerular disease</td>
<td>(177)</td>
</tr>
<tr>
<td>Lyn−/−SHIP-1−/+</td>
<td>Haploinsufficiency of Lyn and SHIP-1</td>
<td>Amplification of Lyn−/− pathogenic autoantibody production. Mild glomerular disease</td>
<td>(177)</td>
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<td><strong>Lyn-specific mutations</strong></td>
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<td>Lynup/up</td>
<td>Constitutively active Lyn-Y508F</td>
<td>Pathogenic autoreactive antibodies and severe glomerular disease</td>
<td>(179)</td>
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<td>LynMld4</td>
<td>Kinase-dead Lyn-T410K</td>
<td>Intermediate Lyn−/− phenotype, but no development of kidney disease.</td>
<td>(192)</td>
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<td>LynWeeB</td>
<td>Kinase-dead Lyn-E260G</td>
<td>Intermediate Lyn−/− phenotype, with late onset glomerular disease</td>
<td>(193)</td>
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<td>LynΔN</td>
<td>Cytosolic Lyn</td>
<td>TNF-α dependent psoriasis-like skin inflammatory syndrome</td>
<td>(194)</td>
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<td><strong>Th2 environment</strong></td>
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<tr>
<td>Lyn−/−IL-4−/−</td>
<td>Lacking Lyn and IL-4</td>
<td>Failed to develop glomerulonephritis; kidney function rescued</td>
<td>(195)</td>
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<tr>
<td>Lyn−/−Igh-7−/−</td>
<td>Lacking Lyn and IgE</td>
<td>Failed to develop glomerulonephritis; kidney function rescued</td>
<td>(195)</td>
</tr>
<tr>
<td>Lyn−/−STAT6−/−</td>
<td>Lacking Lyn and STAT6</td>
<td>Exacerbated autoimmune traits and severe glomerular disease; uncoupling of STAT6 from expression of Th2 traits</td>
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<td><strong>T cell help</strong></td>
<td><strong>Lyn^−/−CTLA4Ig</strong></td>
<td>Lacking Lyn and overexpression of secreted CTLA4</td>
<td>Loss of IgG autoantibodies but presence of IgA autoantibodies sufficient to mediate glomerular disease</td>
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<tr>
<td><strong>Lyn^−/−p110δ+/−</strong></td>
<td>Lacking Lyn and haploinsufficiency of PI3K p110δ</td>
<td>Moderation of T cell signalling and activation, myeloid derived inflammation and glomerular disease</td>
<td>(183)</td>
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<td><strong>Lyn^−/−IL-21^−/−</strong></td>
<td>Lacking Lyn and IL-21</td>
<td>Loss of class-switched anti-DNA and histone autoantibodies, persistence of other pathogenic autoantibodies and kidney disease</td>
<td>(197)</td>
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<td><strong>Lyn^−/−TCRβ−/−TCRδ−/−</strong></td>
<td>Lacking Lyn and T cells</td>
<td>Greatly diminished levels of autoantibodies, however disease not assessed</td>
<td>(198)</td>
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<tr>
<td><strong>Lyn^−/−SAP−/−</strong></td>
<td>Lacking Lyn and SAP adaptor</td>
<td>Greatly diminished levels of autoantibodies, however disease not assessed</td>
<td>(198)</td>
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</table>

| **Interactions between SFKs** | **Lyn^−/−Fyn^−/−** | Lacking Lyn and Fyn | Severe glomerular disease thought to be due to a kidney-intrinsic mechanism | (199) |
| **Lyn^−/−Fyn^−/−Blk^−/−** | Lacking Lyn, Fyn and Blk | Immunodeficient; early block in B cell development | (200) |
| **HFL^−/−** | Lacking Hck, Fgr and Lyn | Reduced inflammation and diminished glomerular disease | (181) |

<p>| <strong>Inflammation</strong> | <strong>Lyn^−/−IL-5Rα−/−</strong> | Lacking Lyn and IL5Rα | Reduction in autoantibody production and very mild glomerular disease | (201) |
| <strong>Lyn^−/−IL-6−/−</strong> | Lacking Lyn and IL-6 | Lack of T cell and myeloid hyper-activation, abrogation of glomerular disease. Dissociation of B cell hyperactivity and disease | (88, 191) |
| <strong>Lyn^−/−IFN-γ−/−</strong> | Lacking Lyn and IFN-γ | Reduced production of BAFF, myeloid proliferation and T cell hyper-activation resulting in moderated glomerular disease | (181) |
| <strong>Lyn^−/−sgp130FcTg</strong> | Lacking Lyn and overexpression of soluble gp130Fc | Neutralization of IL-6 trans-signalling had minimal effects on B and T cell activation, autoantibody production and IC deposition but resulted in reduced myeloid inflammation, complement deposition and glomerular disease | (202) |
| <strong>Lyn^−/−IL-10−/−</strong> | Lacking Lyn and IL-10 | Exacerbation of Lyn^−/− phenotype; marked splenomegaly and lymphadenopathy, increased pro-inflammatory cytokines and severe tissue inflammation | (203) |
| <strong>Lyn^−/−MyD88−/−</strong> | Lacking Lyn and MyD88 | Attenuation of autoantibody production and protection from glomerulonephritis | (182) |
| <strong>CD11c^cre^Lyn^floxflox^</strong> | DC specific Lyn deletion | Exacerbation of Lyn^−/− phenotype, severe glomerulonephritis | (186) |</p>
<table>
<thead>
<tr>
<th>Mice Model</th>
<th>Deletion Description</th>
<th>Effect</th>
<th>Reference</th>
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<td>CD11c&lt;sup&gt;cre&lt;/sup&gt;Lyn&lt;sup&gt;flox/flox&lt;/sup&gt; MyD88&lt;sup&gt;flox/flox&lt;/sup&gt;</td>
<td>DC specific Lyn and MyD88 deletion</td>
<td>Abrogation of autoimmunity</td>
<td>(186)</td>
</tr>
<tr>
<td>CD79a&lt;sup&gt;cre&lt;/sup&gt;Lyn&lt;sup&gt;flox/flox&lt;/sup&gt;</td>
<td>B cell specific Lyn deletion</td>
<td>Similar phenotype to Lyn&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>(185)</td>
</tr>
<tr>
<td>CD79a&lt;sup&gt;cre&lt;/sup&gt;Lyn&lt;sup&gt;flox/flox&lt;/sup&gt; MyD88&lt;sup&gt;flox/flox&lt;/sup&gt;</td>
<td>B cell specific Lyn and MyD88 deletion</td>
<td>Abrogation of autoimmunity</td>
<td>(185)</td>
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1.8. Targeting inflammation in SLE

1.8.1. Myeloid derived inflammatory factors

1.8.1.1. Interleukin-1 (IL-1) and the inflammasome

The inflammasome is a complex that responds to ‘danger signals’ and induces the cleavage and release of bioactive IL-1, a cytokine upstream of many pro-inflammatory responses (204). The importance of inflammasome activation and the induction of IL-1 have been elucidated by several studies and have drawn an important link between the innate immune system and autoimmunity (205, 206). Increased IL-1 gene expression was observed in MRL\textsuperscript{lpr/lpr} mice (207, 208), and inhibition of the NLRP3 inflammasome/IL-1\textbeta axis in MRL\textsuperscript{lpr/lpr} mice attenuated proteinuria, autoantibody production, systemic pro-inflammatory cytokines and kidney pathology (209, 210). When SLE was induced in mice deficient in IL-1, they showed reduced levels of autoantibody and milder disease manifestations (211). It was also found that IL-1 induced IgG production by cells from SLE patients and healthy controls (212), and increased IL-1 levels were produced by B cells from SLE patients (213) and were found in the cerebrospinal fluid (CSF) of patients with neural SLE (214). A single nucleotide polymorphism in the IL1B gene (rs1143629) is associated with juvenile onset SLE, implicating IL-1\textbeta in the early stages of SLE pathogenesis (215). Treatment of MRL\textsuperscript{lpr/lpr} and NZBxNZW F\textsubscript{1} mice with anti-IL-1R antibody reduced autoantibody titres (216, 217). A preliminary study treating patients with the IL-1R antagonist Anakinra showed promising results (218); however no further studies have since been published.

In contrast with the concept of a critical role for inflammasome activation in autoimmunity, inflammasome deficiency due to a point mutation in the NLRP3 gene has recently been identified in lupus-prone NZBxNZW F\textsubscript{1} mice correlating with reduced IL-1\textbeta release (219). In addition, the NLRP3/ASC inflammasome protected against kidney damage independent of IL-1 signalling due to regulation of the anti-inflammatory activity of transforming growth factor-\textbeta (TGF-\textbeta) in C57BL6/J\textsuperscript{lpr/lpr} mice, which represent a mild spontaneous model of lupus (220). Collectively, these studies suggest that therapeutic targeting of the inflammasome in SLE may exacerbate disease in some patients or only be suitable for patients with a certain inflammatory profile. Recently, emphasis has been placed on understanding how the
inflammasome contributes to pro-inflammatory cell death (pyroptosis) and the subsequent modification of autoantigens and generation of autoimmune responses in lupus (221).

1.8.1.2. Interferon alpha (IFN-α)

IFN-α is one of the most strongly implicated cytokines in the pathogenesis of SLE (222). Early studies showed that IFN-α was increased in the serum and found to be associated with increased disease activity (223-226). Correlations were also found between the levels of IFN-α and IC in the serum (224), as well as the deposition of IC in kidney sections from SLE patients (227). This may be due to IFN-α having an antagonistic effect on CRP production, leading to the elevation of available nuclear antigens (9). In addition, IFN-α was expressed at high levels in the CSF of patients with neural lupus (228, 229) and CSF from these patients had strong IFN-α-inducing ability when added to cell cultures (230). Treatment of a patient harbouring a malignant tumour with IFN-α induced SLE-like symptoms (231) and it is also epidemiologically interesting that SLE patients with high IFN-α levels showed low rates of Hepatitis B infections (232); highlighting the role of IFN-α in anti-tumour and anti-viral responses, but also in the pathogenesis of SLE. Interestingly, viral DNA and RNA as well as self nucleic-acid-containing ICs stimulate IFN-α production by plasmacytoid dendritic cells via TLR-7 and -9; specific inhibitors towards these TLRs potently inhibited IFN-α production (233), while treatment of lupus-prone NZBxNZW F1 mice with these inhibitors significantly reduced serum ANAs, glomerulonephritis and organ damage while improving survival (234). In addition, increased TLR7 and 9 expression in PBMC from SLE patients was correlated strongly with high levels of IFN-α mRNA (235), thus bridging innate immunity with autoimmunity. Clinical assessment of targeting IFN-α has shown safety (236). A Phase II study did not reach its primary endpoint, however exploratory analysis of the results did reveal positive reductions in disease (237). A phase I/II trial in SLE patients examining the safety of IFNα kinoid (IFN-K) which induces a host polyclonal antibody response to IFN-α is currently underway (NCT01058343). A phase I trial in lupus patients testing sifalimumab (MEDI-545), a human anti-IFN-α mAb, showed safety, tolerability and clinical activity (238), which has led to a phase IIb trial, which has now been completed although no data has been published (NCT00299819). Studies targeting TLRs in nephritic SLE are currently lacking; however small molecule TLR antagonists are starting to be assessed for efficacy in animal models (239). Very interestingly, it is now apparent that hydroxychloroquine, a mainstay in lupus treatment, is a TLR-7 and -9 antagonist. Thus,
manipulation of TLR signalling with new agents promises to be a future growth area in the clinical management of inflammatory and autoimmune diseases such as SLE (reviewed in (240)).

1.8.1.3. Tumour Necrosis Factor alpha (TNF-α)

TNF-α is an interesting and controversial cytokine in the field of SLE, due to its apparent dual role (241). Similar to other pro-inflammatory cytokines, increased levels of TNF-α have been observed in the serum of lupus-prone animals and SLE patients (242). In particular, elevated serum levels and gene expression levels are positively associated with disease activity as well as renal involvement in SLE patients (243, 244). While TNF-α blockade has been successful as a mainstay treatment for rheumatoid arthritis (RA) (245), the assessment of this therapy in SLE patients has not been straightforward. In SLE patient studies, anti-TNF-α therapy increases the serum levels of anti-dsDNA and anti-phospholipid autoantibodies (246). It would follow that a further increase in anti-phospholipid antibodies in patients may lead to vascular events, which although rare, have the potential to be life-threatening. In addition, the risk of bacterial infection is increased as a result of anti-TNF-α therapy (247). Despite the risks, treatment has led to syndromes that are transient, mild in nature, have not induced flares, and have resulted in reductions in proteinuria and provided benefit to patients with lupus arthritis (248). A more recent study has demonstrated safety and efficacy of anti-TNF-α therapy in SLE (249). It is suggestive that any consideration of anti-TNF-α for the treatment of SLE patients must be for a short duration only, and not recommended for patients with antiphospholipid syndrome (up to 15% of SLE patients (250, 251)). It is still debatable whether the risks associated with therapeutic targeting of TNF-α in SLE are worth the benefits obtained.

1.8.1.4. B Cell activating factor (BAFF)

BAFF is an important B cell survival factor with well-known pathogenic roles in SLE. It is expressed by numerous cells in the immune system but is highly expressed by innate immune cells. Mice engineered to over-express BAFF developed autoimmune manifestations (252), while over-expression of BAFF in lupus-prone congenic strains accelerated renal pathology (253). BAFF levels are increased in the serum of patients with SLE (254, 255), are associated with increased anti-dsDNA antibody levels (256) and disease activity (257, 258). Neutralization of BAFF in lupus-prone NZBxNZW F1 mice depleted B cells, prevented progressive T cell activation and dendritic cell accumulation and prolonged survival (259).
Lyn−/− mice show excessive BAFF production by myeloid cells and treatment with anti-BAFF mAb attenuated their lupus-like disease (181). BAFF neutralization also reduced glomerulonephritis and improved survival in lupus-prone BXYB/Yaa mice (260) and NZM2410 mice (261). T cells from SLE patients produced large amounts of BAFF in culture (262) and had significantly up-regulated BAFF mRNA (263) compared to control T cells. Increased BAFF expression was also found in SLE B cells and was positively associated with anti-dsDNA autoantibody and disease activity scores (264), indicating that as well as being produced by other cell types, B cells from SLE patients can produce BAFF in an autocrine manner. Especially pertinent is the FDA approval of Belimumab (trade name Benlysta®), a neutralizing antibody against BAFF, for the treatment of SLE (166), spurred by the success of a randomized double-blind placebo-controlled trial which demonstrated efficacy and safety in SLE patients treated with Belimumab over placebo (165). This pathway continues to dominate the focus of SLE clinical trials.

1.8.2. Myeloid Growth Factors
Extramedullary haematopoiesis is often a hallmark of infectious and inflammatory diseases, driven by excess production of myeloid growth factors and is evident in models of lupus such as Lyn−/−, NZBxNZW F1 and MRLlpr/lpr mice (Figure 1.1) (88, 265). This phenomenon may contribute to the splenomegaly seen in some lupus patients and lupus-prone mice (88, 265, 266). Myeloid growth factors stimulate progenitor cell release from bone marrow, myeloid cell production and cellular activation, and this may promote enhanced inflammatory responses and tissue damage depending on tissue context. Several studies have implicated myeloid growth factors including IL-3, M-CSF and GM-CSF in the inflammatory pathways and pathology in SLE.

1.8.2.1. Interleukin-3 (IL-3)
IL-3 is a pleiotropic, synergistic growth factor that is involved in the differentiation, activation and support processes of many immune cells including dendritic cells (267). Although it has been long known that IL-3 can be elevated in SLE patients (268), very few studies on the role of IL-3 in SLE have since been conducted. In the Lyn-deficient mouse model of lupus, IL-3-responsive progenitor cells are elevated in spleen (88), and IL-3 induces enhanced signalling and survival of Lyn−/− plasma cells suggesting it may play a role in the support of autoreactive plasma cells (269). Recently, a study has shown that IL-3 can drive glomerulonephritis in
MRL<sup>lpr/lpr</sup> mice; hypothesized to be due to enhanced antigen presentation by dendritic cells, elevated Ig secretion and/or basophil mediated support functions (270). Treatment of MRL<sup>lpr/lpr</sup> mice with an anti-IL-3 mAb ameliorated nephritis, improved kidney function and restrained production of certain autoantibodies (270). This suggests that the IL-3 axis might be an undervalued contributor to inflammation and pathology in SLE, and future studies to further our understanding of this system may benefit the development of superior treatment regimens.

1.8.2.2. Macrophage colony-stimulating factor (M-CSF)

M-CSF is a myeloid growth factor that induces the differentiation of myeloid precursor cells into monocytes/macrophages or dendritic cells as well as regulating macrophage functions, survival, trafficking and proliferation and is associated with inflammatory pathology (271). M-CSF is elevated in the serum of SLE patients and correlates with active disease, renal pathology and myeloid activation syndrome (272, 273). Local M-CSF production by renal mesangial cells is elevated in lupus nephritis and contributes to proteinuria, local macrophage infiltration and proliferation and glomerular proliferation (274, 275). M-CSF is also detectable in the urine of lupus nephritis patients with levels correlating with flares in renal disease (276). Elevated M-CSF is also observed in MRL<sup>lpr/lpr</sup> mice systemically as well as in the kidney (277) and is heavily implicated in driving autoantibody production, glomerular infiltration of myeloid cells and nephritis (278). M-CSF is involved in driving a pro-inflammatory, immunopathogenic phenotype in MRL<sup>lpr/lpr</sup> macrophages (279), and in Lyn<sup>−/−</sup> mice, hematopoietic progenitors responsive to M-CSF are enhanced in spleen (88). Inhibition of M-CSF signalling with a selective M-CSF receptor kinase inhibitor (GW2580) prevents macrophage and T cell accumulation in the kidney, restricts the local renal inflammatory profile and improves kidney pathology in an induced model of lupus nephritis (280). This highlights that the M-CSF pathway may be a novel target for therapeutic trials in SLE.

1.8.2.3. Granulocyte Macrophage colony-stimulating factor (GM-CSF)

GM-CSF is a growth factor that drives differentiation of myeloid lineage cells (granulocytes and monocyte/macrophages) and it can act on mature immune cells to upregulate an inflammatory phenotype, enhance antigen presentation and migration (281). Although there is conflicting evidence as to whether GM-CSF levels are altered in SLE (282, 283), the frequency of systemic GM-CSF-secreting immune cells are elevated and correlate with anti-
dsDNA titres in SLE (284). It has also been suggested that high concentrations of GM-CSF can drive Ig secretion and leukocyte activation marker CD69 expression in lupus patients (285). GM-CSF can also be produced locally by glomerular mesangial cells and levels correlate with lupus nephritis (274). Interestingly, some studies suggest that neutrophils and dendritic cells may demonstrate resistance to GM-CSF responses in SLE (286, 287). Colony formation by splenic progenitor cells induced by GM-CSF is increased in Lyn−/− mice (88). Therapeutic targeting of the GM-CSF axis is not currently being explored in SLE, but has shown promise in RA (281). MOR103, a humanized anti-GM-CSF mAb has recently successfully completed a phase I/II trial in RA reporting safety and preliminary efficacy (288), whilst a fully human anti-GM-CSF receptor mAb, Mavrilimumab is currently in phase II trials (NCT01712399). Further studies delineating the role of GM-CSF in inflammation and pathology in SLE may highlight this system as a potential target for treatment in a subset of SLE patients.

1.8.3. T helper cells and their inflammatory mediators

1.8.3.1. T_{H1} cells and Interferon gamma (IFN-γ)
IFN-γ is a prototypic T_{H1} cytokine that activates a pro-inflammatory program in macrophages (Figure 1.3). Like IFN-α, IFN-γ is also elevated in the serum of SLE patients (244, 289, 290). Increased IFN-γ levels were also found in lymphoid organs of pre-diseased MRL^{lpr/lpr} mice (207), while increased IFN-γ-producing T cells were correlated with autoantibody titres and proteinuria in aged diseased mice (291). Treatment of NZBxNZW F_{1} mice with IFN-γ accelerated disease, while neutralization of IFN-γ resulted in reduced disease symptoms and improved survival (292, 293); furthermore, genetic deletion of IFN-γ receptor in these mice impaired autoantibody production and glomerulonephritis (294). Studies in the Lyn^{−/−} mouse model showed that genetic deletion of IFN-γ led to reduced production of BAFF and decreased myeloid proliferation and T cell hyperactivation, thereby resulting in moderation of glomerular disease (181). Analysis of PBMC from SLE patients showed that they had significantly higher IFN-γ transcripts compared to control PBMC (295) and that T cells from SLE patients produced more IFN-γ which induced BAFF production by monocytes (296), while SLE NK cells produced higher IFN-γ (297). There are currently no completed trials on the effect of neutralizing IFN-γ in SLE patients (298); however a recent single dose study treating SLE patients with AMG811, an anti-INF-γ IgG1 mAb, was well tolerated and showed reductions in IFN-γ-mediated gene expression (299).
1.8.3.2. T\(h_2\) cells, interleukin-4 (IL-4) and IgE

Typically, autoimmune diseases are driven by a T\(h_1\) skewed response (Figure 1.4). Individuals with SLE display elevation in classical T\(h_1\) cytokines, such as IL-6, IFN-\(\alpha\) and IFN-\(\gamma\). Furthermore, SLE disease in mouse models almost exclusively relies on a T\(h_1\) signature. For instance, the autoimmune disease phenotype of Lyn\(^{-/-}\) mice is promoted by IL-6 and IFN-\(\gamma\), and is characterized by high IgG2a/IgG2c serum immunoglobulin titres. Interestingly, more recent studies have revealed that Lyn\(^{-/-}\) mice represent a unique situation, where prominent T\(h_2\) traits such as atopy, mast cell hyper-responsiveness and eosinophilia are coexistent with autoimmunity (300, 301). It has been proposed that the T\(h_2\) environment is an important contributor to the development of lupus in Lyn\(^{-/-}\) mice, supported by the reduction of ANAs and glomerular disease in mice lacking IL-4 or IgE (195). This finding has now been extended to other lupus models. In the Lyn-deficient model, T\(h_2\) cell induced autoreactive IgE induces basophil activation and homing to lymphoid organs where it is hypothesized that they can influence B and T cell activity (195). The studies in Lyn\(^{-/-}\) mice prompted an examination of human disease, finding IgE autoantibodies and activated basophils correlating with disease flairs (195). This has now been extended to two independent cohorts of patients showing that approx. two-thirds have IgE autoantibodies to a spectrum of autoantigens and their presence was associated with increased disease activity (302). At present it is unclear what role autoreactive IgE is playing in disease pathogenesis given that the levels of this isotype are extremely low in serum and tissues, and the fact that this isotype does not fix complement, which is an important mechanism underlying tissue damage in lupus. The role of the T\(h_2\) environment in the development of lupus in Lyn\(^{-/-}\) mice was also examined more directly by genetic deletion of STAT6, a key mediator of T\(h_2\) immunity downstream of IL-4 signalling. This resulted in significant amplification of autoimmune disease pathology, leading to increased serum ANA titres, exaggerated immune cell activation and worsened glomerulonephritis but very interestingly, IgE autoantibodies were still present albeit at low levels (196).

1.8.3.3. T\(h_17\) cells, Interleukin-17 (IL-17), Interleukin-21 (IL-21), Interleukin-22 (IL-22), Interleukin-23 (IL-23)

T\(h_17\) cells and their related pro-inflammatory cytokine profile are associated with many chronic inflammatory and immunopathological conditions including RA, multiple sclerosis and SLE (16). Circulating T\(h_17\) cells are elevated in SLE patients (303, 304) and positively correlate with INF-\(\alpha\) and IL-6 activity (305) and negatively correlate with Treg numbers and function
The Th17 cytokine IL-17 and the Th17 activation/differentiation factor IL-23 are elevated systemically (308-310) as well as locally in the kidney (303), and IL-17 gene products can be found in the urine (311) of SLE patients. IL-21 has been shown to be both elevated (312, 313) and reduced (314) in different subsets of SLE patients whilst IL-22 levels increase with disease duration and activity (315). Frequencies of Th17 cells including IL-21+ and IL-22+ CD4+ cells as well as levels of IL-17, IL-21 and IL-23 positively correlate with disease severity in lupus nephritis patients (303, 304, 310, 316-318). The Th17 system is also implicated in the pathogenesis of lupus nephritis in animals. IL-17 is essential for the development of ANAs and nephritis in pristane-induced murine lupus (319); although deficiency of IL-17A in MRL<sup>lpr/lpr</sup> mice did not improve nephritis (293). Similarly, IL-17 produced by γδ T cells is crucial for pathogenesis in a model of autoimmune glomerulonephritis (320). IL-21R deficiency in BXSB/Yaa mice completely abrogated autoimmunity and nephritis (321), and production of pathogenic IgG anti-dsDNA autoantibodies but not kidney pathology is reliant on IL-21 in Lyn<sup>-/-</sup> mice (197). Proof of concept studies therapeutically targeting the Th17 axis in murine models of lupus have shown some promising results. While therapeutic neutralization using an anti-IL-17A mAb in NZBxNZW F<sub>1</sub> mice did not impact disease progression (293), anti-IL-21R mAb therapy improved survival and proteinuria although kidney disease was not assessed (322). IL-21 neutralization using an IL-21R-Fc fusion protein improved kidney function and showed potential mild improvements in nephritis in BXSB/Yaa mice (323). Inhibition of Th17 differentiation using the small molecule inhibitor Stattic, which constrains STAT3 activation, delayed the onset of immunopathology and nephritis in MRL<sup>lpr/lpr</sup> mice (324). At present, there is no clinical data on targeting Th17 cells and their cytokines in human SLE, but safety and clinical improvements have been demonstrated in a range of inflammatory autoimmune diseases (17).

1.8.4. Interleukin-6 (IL-6) as a major pathogenic inflammatory mediator

1.8.4.1. IL-6 signalling

IL-6 is a pleiotropic cytokine that acts on a range of cell types; it can influence growth and differentiation, antibody production, and mediates the acute-phase inflammatory response. It is also highly implicated in the pathogenesis of many inflammatory and autoimmune diseases (reviewed in (325)). IL-6 signals via the heterotetrameric IL-6 receptor complex which consists of two unique IL-6R alpha subunits that have a limited cellular distribution, and two
subunits of gp130, which are ubiquitously expressed (Figure 1.5A, 1.5B). Upon ligation of IL-6 by the IL-6R/gp130 complex, common cytokine signal transduction mediators are activated; JAK family of tyrosine kinases (JAK1, JAK2, JAK3 and TYK) (326) and subsequently Signal Transducer and Activator of Transcription 3 (STAT3) which translocates to the nucleus to mediate transcription of target genes (327). IL-6R cell surface expression is limited to hepatocytes and some immune cells (Figure 1.5A), but can be cleaved during inflammation or differentially spliced during transcription to produce a soluble form (sIL-6R) capable of signalling via cell membrane expressed gp130 on all cells, in a phenomenon known as trans-signalling (Figure 1.5C) (328). Soluble IL-6R mediated trans-signalling is regulated by circulating soluble gp130 (sgp130) which can bind to and inhibit IL-6/sIL-6R complexing with membrane bound gp130 (Figure 1.5D) (329). It has been hypothesized that classic IL-6 signalling via membrane bound IL-6R does not contribute to chronic inflammation and mediates regulated pathogen clearing processes through anti-inflammatory pathways, whilst trans-signalling through sIL-6R/gp130 complexes is associated with chronic inflammatory conditions by driving hyper-inflammatory and immunopathogenic processes (328).
Figure 1.5  Duplicitous signalling roles of IL-6

(A) Classical IL-6 signalling occurs via direct interaction of IL-6 with the membrane-bound IL-6 receptor, which has a limited cellular distribution, and the ubiquitously expressed gp130. gp130 lacks intrinsic kinase activity; IL-6 signals are transduced intracellularly via the recruitment and activation of the JAK/STAT pathway. (B) Cells lacking the IL-6 receptor are not receptive to IL-6 except (C) in the presence of the IL-6/soluble IL-6 receptor complex which interacts with gp130 expressing cells and this is defined as IL-6 trans-signalling. (D) Inhibition of IL-6 trans-signalling can be achieved via the presence of excess soluble gp130Fc fusion protein.
1.8.4.2. IL-6 drives disease in lupus nephritis

IL-6 is a pleiotropic cytokine that acts on a range of cell types; it can influence growth and differentiation, antibody production, and mediates the acute-phase inflammatory response. It is also highly implicated in the pathogenesis of many inflammatory and autoimmune diseases (reviewed in (325)). IL-6 has consistently been shown to be elevated in the serum of SLE patients and it has been suggested that levels correlate with disease activity, making it a suitable biomarker for tracking disease activity (330-333). Similarly, IL-6 can be elevated in the urine of lupus nephritis patients with higher levels correlating with active renal inflammation and pathology (334, 335). IL-6 has been implicated in driving autoantibody production and loss of tolerance in SLE through the upregulation of recombination-activating gene (RAG) activity (336). Studies have identified polymorphisms in the IL-6 gene which are associated with SLE susceptibility (337). In mice, elevated levels of IL-6 are observed in numerous models including NZBxNZW F1 (338), MRL\textsuperscript{lpr/lpr} (339), Lyn\textsuperscript{−/−} (88), ALD-DNA (340), B6.sle1.sle2.sle3 (341), B6.sle1.Yaa (342), and it is heavily implicated in pathology. Studies in IL-6-deficient mice have shown that they are resistant to ALD-DNA-induced lupus which ordinarily promotes anti-dsDNA autoantibody titres, proteinuria, CD4\textsuperscript{+} T cell activation and glomerulonephritis (340). The mechanism was thought to be due to an expansion of regulatory T cells in the absence of IL-6, which ordinarily suppresses their generation (340). Impaired Treg maturation and activity was observed in B6.sle1.sle2.sle3 mice due to overproduction of IL-6 by dendritic cells (341). IL-6 has also been shown to promote disease in Lyn\textsuperscript{−/−} mice as Lyn\textsuperscript{−/−}IL-6\textsuperscript{−/−} show moderated B cell hyperactivity and plasmacytosis, and abrogation of T cell hyperactivity and splenic myeloid cell expansion (88). In addition, class-switched pathogenic ANAs, glomerular IgG and complement deposition are absent in Lyn\textsuperscript{−/−}IL-6\textsuperscript{−/−} mice, and glomerular structural integrity is significantly improved (88). Similarly, IL-6-deficient MRL\textsuperscript{lpr/lpr} mice showed greatly improved survival with significant amelioration of renal immunopathology (343), and in B6.Sle1.Yaa mice, IL-6 deficiency eliminated autoantibody production and nephritis (344).

Specific downregulation of IL-6 trans-signalling in Lyn\textsuperscript{−/−} mice through transgenic overexpression of soluble gp130-Fc fusion protein (Lyn\textsuperscript{−/−}sgp130FcTg) resulted in a loss of splenomegaly, a decrease in splenic myeloid cells and reduced systemic BAFF levels (202). Although pathogenic ANA production was sustained, as was IgG IC deposition in kidney
glomeruli, renal complement deposition was significantly reduced, which suppressed renal leukocyte infiltration, thereby markedly attenuating glomerulonephritis and improving kidney function (202). These findings suggest that more emphasis should be put on examining the role of IL-6-trans-signaling in lupus nephritis with the possibility of targeting this inflammatory pathway in disease.

**1.8.4.3. Therapeutic targeting of IL-6 in inflammatory and autoimmune disease**

There is much evidence to suggest that IL-6 plays a significant role in a number of inflammatory, autoimmune and proliferative diseases, making it a strong candidate for targeted novel biological therapeutics (reviewed in (345)). This notion is further enhanced by the improvement seen in IL-6-targeted murine SLE models. A preliminary study treating NZBxNZW F₁ mice monthly with anti-IL-6 in combination with anti-IL-1α concluded that this regimen only had a partial effect on disease, alleviating proteinuria (346). Young NZBxNZW F₁ mice treated weekly with a rat anti-IL-6 monoclonal antibody from 3 months to 9 months of age showed reduced ANA production, improved proteinuria and increased survival, although these mice required initial tolerising treatments of anti-CD4 to prevent a rapid onset anti-rat Ig response (347). A more thorough study in young NZBxNZW F₁ mice without anti-CD4 tolerance showed that anti-IL-6 mAb treatment suppressed systemic serum amyloid A (SAA), and anti-dsDNA antibody levels, suppressed hyperactivation of B and T cells and greatly diminished the development of kidney pathology (348).

The use of murine IL-6 targeting monoclonal antibodies in human disease has been unsuccessful as IL-6/mAb immune complexes form which can further drive pathology in inflammatory settings (349). A fully humanized anti-IL6 mAb, Sirukumab, has shown tolerance in humans (350, 351), and efficacy in treating RA (352). A phase I trial in cutaneous and systemic lupus patients (NCT01702740) concluded that Sirukumab is generally well tolerated in SLE although mild leukopenia, neutropenia and decreases in platelet count were observed (353). A phase II trial in lupus nephritis patients (NCT01273389) has recently been completed with official outcomes not yet reported, although unofficial accounts indicate a high frequency of serious adverse events, the majority of which were infections.

A study examined the effect of targeting IL-6R in MRL⁺/-pr mice by treating 15 week old animals every 1-3 days for 5 weeks with a neutralizing rat anti-IL-6R mAb. These mice saw an initial reduction in anti-dsDNA autoantibodies which rebounded and elevated in response to
increasing titres of anti-rat Ig antibodies (354). The treatment was reported as successful as both kidney function and structure was improved at the endpoint compared to controls (354). Tocilizumab, a humanized murine anti-IL6-R mAb has demonstrated efficacy in treating multiple myeloma (355) and RA (356). A phase I safety trial demonstrated that Tocilizumab is well tolerated in SLE patients although similarly to Sirukumab, temporary dose-dependent neutropenia was observed (357). A recent study in SLE patients showed that Tocilizumab treatment can reduce B and T cell activation, memory B cells and autoantibody producing plasma cells without impacting naïve B cells populations (358).

Given that anti-IL-6 and anti-IL6R inhibition may lead to unwanted neutropenia and infection, targeting IL-6 trans-signalling without disrupting classical signalling pathways may prove to be a superior approach in SLE as many anti-inflammatory and regulatory mechanisms mediated by IL-6 classic signalling will not be impacted (Figure 1.5D) (202, 328, 329). A fusion protein joining sgp130 to the IgG Fc region, sgp130Fc, has demonstrated effective inhibition of soluble IL-6R mediated trans-signalling (329). Proof of concept for sgp130Fc treatment has been demonstrated in a range of inflammatory disease models including RA (359, 360), atherosclerosis (361), and Crohn’s disease (329). Given its efficacy, it is surprising that no clinical trials utilizing this agent in lupus appear to be in the pipeline.

Components of signalling pathways that are aberrantly activated in disease are attractive drug targets. The JAK/STAT signalling pathway is activated downstream of IL-6 and gp130 (Figure 1.5) and can be targeted by small molecule inhibitors, which have already shown promise in cancer therapy (362). However, there are few studies on JAK/STAT inhibitor treatments in inflammatory diseases; one study demonstrated clinical efficacy of the JAK1/2 inhibitor AZD1480 in five models of experimental autoimmune encephalitis (EAE, modelling human multiple sclerosis) by inhibiting myeloid cell hyper-activation, Th1 and Th17 differentiation, pro-inflammatory cytokine production as well as showing improvements in disease and clinical pathology (363). A signalling study in Lyn−/− mice showed that treatment with AZD1480 resulted in reduced splenic B cells and plasma cells as well as reduced numbers of splenocytes and thymocytes; however effects on lupus pathology were not reported (269). While this may be an attractive approach, inhibition of the JAK/STAT pathway in autoimmune disease should be undertaken cautiously and the use of single-specificity inhibitors may be more beneficial. Indeed, treatment using the JAK2 selective inhibitor CEP-33779 in both NZBxNZW F1 and
MRL<sup>lpr/lpr</sup> mice saw dose-dependent improvements in lymphadenopathy and splenomegaly, reductions in systemic C3 and pro-inflammatory cytokines including IL-1, IL-12, IFN-α IL-17A and TNF-α, reductions in autoantibody-producing plasma cells, increased survival and significant improvements in glomerulonephritis through blockade of STAT3-mediated signalling (364, 365). These animal data lend support for trailing a JAK2 inhibitor in human disease. Interestingly, GSK recently halted development of the JAK1 inhibitor GSK2586184 following disappointing results in a Phase II study in SLE (366).

1.9. Research Rationale

With the advancement of our understanding of the pathology and contributing factors to disease onset and propagation in SLE, it is disappointing that treatment has remained relatively consistent. With the sole exception of Belimumab, which confers only minor improvements on a limited subset of responsive patients, no other treatment has achieved clinical approval. There is great necessity to expand upon the therapeutic targets which so far have been largely focussed at the effector (B cell) level and to thoroughly investigate the validity of targeting supportive mechanisms (inflammation), which has been a successful approach in other autoimmune and inflammatory disorders. Systemic inflammation in SLE is largely driven by hyper-responsive myeloid cells where regulatory mechanisms (such as those mediated by CD11b) are lost; investigating these axes may provide insight into novel therapeutic strategies. There is also evidence that lifestyle interventions (diet) can drastically influence the severity of inflammatory disease and this has, so far, been overlooked in SLE. Therefore this project will investigate the effect of therapeutic targeting of inflammatory factors, dietary modulation as well as further understanding the role CD11b plays in regulating inflammation and disease in lupus, using the Lyn-deficient mouse as an experimental model.

1.9.1. Aims

1. To examine the effect of targeting the inflammatory mediator IL-6 in aged Lyn-deficient mice.
2. To investigate the effect of dietary fibre and the microbiome on disease development in Lyn-deficient mice.
   a. High Fibre diet from weaning (4.5% Fat, 20% Cellulose + 20% Guar Gum)
   b. High Fibre diet from disease onset (4.5% Fat, 20% Cellulose + 20% Guar Gum)
3. Characterise the contribution of the ‘myeloid’ receptor CD11b to lupus disease pathogenesis
Chapter 2

General Methods
2.1. Mice
Mice were housed under specific pathogen free conditions at the Monash ICU Animal Facility (M. ICU) (AMREP Campus). Mice were housed in micro-isolator cages with fibrecycle (paper based) bedding and, with the exception of mice in chapter 4, were fed standard mouse chow and autoclaved tap water. All experiments and procedures were conducted in accordance with the Australian code for the care and use of animals for scientific purposes (National Health and Medical Research Council of Australia, EA28) and were approved by the AMREP Animal Ethics Committee (applications E/1025/2010/M, E/1377/2013/M, E/1688/2016/M).

2.1.1. C57BL/6
C57BL/6/MARP mice were used as wild type healthy controls for all mutant strains and were purchased from Monash Animal Services (MAS) (Monash University, Clayton, Australia).

2.1.2. Lyn⁻/−
The Lyn⁻/− mice that are the subject of this thesis were initially generated on a mixed 129Ola x C57BL/6 genetic background (85), then crossed for 20 generations to the C57BL/6 background (B6.Lyn⁻/−) (88). Only C57BL/6 background mice were used for all studies. The B6.Lyn⁻/− mouse strain was maintained at the Animal Research Laboratory (ARL) - Monash Animal Research Platform (MARP) (Monash University, Clayton, Australia).

2.1.3. CD11b⁻/−
B6.129S4-Iltgamtm1Myd/J mice (Jackson Laboratory; Bar Harbor, ME, USA) were obtained from Prof Karlheinz Peter (Baker IDI Heart and Diabetes Institute, Melbourne, Australia) (367). This strain (designated B6.CD11b⁻/−) was maintained at ARL- MARP (Monash University, Clayton, Australia).

2.1.4. Lyn⁻/−CD11b⁻/−
C57BL/6 background Lyn⁻/−CD11b⁻/− mice were derived from crossing B6.Lyn⁻/− and B6.CD11b⁻/− mice and then inter-crossing the subsequent heterozygous F₁ generation. F₂ generation mice were genotyped via PCR to detect homozygous double mutant mice (Lyn⁻/−CD11b⁻/−) which were then inter-crossed. The progeny were confirmed by PCR to be homozygous double mutants, and the double knockout colony was maintained by brother
sister mating. The B6.Lyn^-/-;CD11b^-/- double knockout (DKO) mouse strain was maintained at ARL-MARP (Monash University, Clayton, Australia).

2.2. Flow Cytometry
Multi-colour flow cytometry was used to assess immune cell populations and their activation phenotype. Single cell suspensions of spleen were prepared by extruding cells from under the capsule in FACS buffer (PBS + 2% FCS + 2 mM EDTA) and then passing them through a Falcon 40 μm cell strainer (Corning Life Sciences, Tewksbury, MA). 20x10^6 cells/ml (counted using Z2 Coulter Counter, Beckman Coulter, Mount Waverley, VIC, Australia) were pre-incubated with anti-FcγR mAb (clone 2.4G2) to block non-specific binding followed by a combination of the following mAbs: Ter119-PE, CD71-FITC, B220-APC-e780, CD138-biotin, IgM-APC, IgD-PE, FcyRIIb-PE, CD40-APC, CD4-APC, CD8-APC-e780, CD25-PE, CD44-FITC, CD62L-PECy7, CD69-V450, CD11b-APC-e780 or CD11b-APC, CD11c-APC, CD25-PE, IgM-APC, IgD-PE, FcγRIIb-PE, CD40-APC, CD4-APC, CD8-APC-e780, CD25-PE, CD44-FITC, CD62L-PECy7, CD69-V450, CD11b-APC-e780 or CD11b-APC, CD11c-APC, CD86-FITC, cfms-PE, Gr-1-APC, Ly6G-APC. Cells were incubated with streptavidin-Pacific Blue before being resuspended in Propidium Iodide + FACS buffer and assayed using BD LSR-Fortessa (BD Biosciences, San Jose, CA, USA). Output files were analysed using FlowJo software (Windows V10, FlowJo LLC, Ashland, OR, USA). Populations were determined as follows: cytotoxic T cells (CD8^+CD4^-), T helper cells (CD8^-CD4^+), activated/regulatory T helper cells (Treg) (CD4^+CD25^+), effector T helper cells (CD4^-CD25^-), B cells (B220^+CD138^{lo-mid}), Plasma cells and Plasmablasts (B220^+CD138^{hi}), erythroblasts (Ter119^+ and/or CD71^+), dendritic cells (CD11b^-CD11c^+), macrophages (CD11b^-cfms^- or CD11b^-Gr-1^-cfms^- or Ly6G^-cfms^-), neutrophils (CD11b^-Ly6G^- or CD11b^-Gr-1^-cfms^- or Ly6G^-cfms^-), monocytes (CD11b^-Gr-1^-cfms^- or Ly6G^-cfms^-). Total numbers of individual cell populations were quantified by multiplying total cell counts by frequencies obtained from flow cytometry. T cell activation was defined as CD4^+ or CD8^+ cells expressing CD44 and/or CD69, and/or exhibiting downregulation of CD62L. Expression of cell surface activation markers was determined by geometric mean fluorescence intensity (gMFI) and normalised to the mean gMFI of the control group (C57BL/6 group under the most standard conditions) to control for instrument differences between experiments.

2.3. Kidney Histopathology
Kidneys were fixed in 10% neutral buffered formalin for 24-48 hrs, processed and embedded in paraffin. 3 µm sections were cut and mounted onto SuperFrost slides and stained with
Mayer’s Haematoxylin and Eosin. Slides were imaged using an Olympus BX-51 light microscope with 20x objective lens (Olympus Australia, Notting Hill, VIC), capturing ≈8 images of the kidney cortex per slide. Images were analysed using ImageJ software, measuring the area of each glomerulus using the polygon tool. The median glomerular area for each animal was plotted to derive the median area of each genotype/treatment group.

2.4. Immunohistochemistry

Kidneys were fixed in 10% neutral buffered formalin for 24-48 hrs, processed and embedded in paraffin. 3 µm sections were cut and mounted onto SuperFrost slides. Slides were dewaxed in histolene and rehydrated with PBS. For CD45 and F4/80 staining, antigen retrieval was performed using DAKO antigen retrieval buffer (Agilent, Santa Clara, CA, USA), microwaved for ~12 min. For Gr-1 staining, antigen retrieval was performed using 20µg/ml Proteinase-K in Tris-HCl pH8.0 at 37°C for 20 mins. Sections were blocked with 1% BSA + PBS. Antibody was diluted in 0.2% BSA + PBS; primary antibody (anti-mouse CD45-biotin, anti-mouse F4/80-biotin) was incubated for 2 hrs, slides washed in PBS, then incubated in secondary antibody for 1 hr at room temperature. DAKO diaminobenzidine (DAB) chromogen solution (Agilent) was added to sections for <10 min, and the colour change reaction was stopped by washing slides in PBS. Slides were counter-stained in haematoxylin then coverslip adhered with DPX (Sigma-Aldrich, Saint Louis, MO, USA). Slides were imaged using an Olympus BX-51 light microscope with 20x objective lens (Olympus Australia), capturing ≈6 images of the kidney cortex per slide. Images were analysed using ImageJ software. For CD45 immunostaining, the area fraction of CD45 stained glomeruli was determined by splitting the images into RGB colour channels, measuring the blue channel intensity (at threshold 100) of each glomeruli, selected using the polygon tool. For F4/80 immunostaining, the proportion of tissue F4/80 staining was calculated by dividing the intensity of the total tissue counterstain of each image, as determined by measuring the whole image intensity (at threshold 185), by the total tissue F4/80 staining, as determined by splitting the images into RGB colour channels and measuring the blue channel intensity (at threshold 100) for the entire image.

2.5. Serum collection

Regular serum collections were conducted throughout treatment periods by tail vein bleed. Mice were warmed under an infrared lamp, restrained and tail vein pricked with a 26 G needle.
Blood pooled at the site of the needle prick was collected by pipette and stored in a 1.7 ml collection tube and left at room temperature for ≈1 hr to clot. At experimental endpoints, blood was collected via cardiac puncture, where a syringe and 26 G needle was injected under the sternum and blood directly drawn from the heart. Clotted blood was centrifuged at 15000 RPM for 15 minutes to separate the haematocrit from serum. Serum was gently pipetted out and stored either neat or diluted 1:10 in 50% BD assay diluent (BD, 333213) + PBS at -80°C.

2.6. Autoantibody Enzyme Linked Immunosorbent Assay (ELISA)
To determine autoantibody titres, Nunc maxisorb 96 well immunoplates (Nunc, 430341) were coated overnight with 50 µg/ml grade 1 calf thymus DNA (Sigma-Aldrich, D1501) in PBS, 50 µl/well. Plates were washed and then blocked for 60 min with BD assay diluent (BD, 333213). Where ‘relative titre’ is stated (Chapters 4, 5), High ANA titre reference serum (in-house) was serially diluted (starting from 1:100) to create a standard curve from which arbitrary titre values could be derived; serum samples were diluted on plates at 1:50 (C57BL/6, CD11b−/−), 1:500 (young Lyn−/−) or 1:1000 and 1:2000 (aged Lyn−/−, Lyn−/−CD11b−/−). In instances of ‘O.D.’ being reported (Chapter 3), serum samples were added to the plate at a 1:100 dilution. Plates were washed and secondary detection antibody, goat anti-mouse IgG(H+L)-HRP (Southern Biotech, 1030-05) (1:4000 in 50% BD assay diluent + PBS, 50µl/well) was added to wells. TMB colorimetric substrate reagents A (hydrogen peroxide) and B (3,3’,5,5’ tetramethylbenzidine) (BD, 555214) was added to washed wells and the colour change reaction was stopped by adding 1.5 M sulphuric acid. Plates were read on MultiSkan GO microplate spectrophotometer (Thermo Fisher Scientific) at 450 nm with correction at 595 nm.

2.7. Immunoglobulin isotype ELISA
To determine Ig isotypes in serum, Nunc maxisorb 96 well immunoplates (Nunc, 430341) were coated overnight with 0.5 µg/ml goat anti-mouse Ig(H+L) polyclonal Ab (Southern Biotech, 1010-01) in PBS, 50 µl/well. Plates were blocked with BD assay diluent (BD, 333213). Standards (serially diluted 1:2 starting from: mouse IgA (0106-01) 12.5 ng/ml, mouse IgG1 (0102-01) 50 ng/ml, mouse IgG2b (0104-01) 50 ng/ml, mouse IgG2c (0122-01) 12.5 ng/ml, mouse IgG3 (0105-01) 50 ng/ml, mouse IgM (0101-01) 50 ng/ml, Southern Biotech) and diluted sample serum were added to wells. Plates were washed and secondary detection antibody (goat anti-mouse IgA+biotin (1040-08) 1:2000, goat anti-mouse IgG1+HRP (1070-05)
1:2000, goat anti-mouse IgG2b+HRP (1090-05) 1:1000, goat anti-mouse IgG2c+HRP (1079-05) 1:2000, goat anti-mouse IgG3+HRP (1100-05) 1:2000, goat anti-mouse IgM+HRP (1020-05) 1:2000, Southern Biotech; streptavidin+HRP (BD Pharmingen, 554066) 1:1000) was added to appropriate wells. TMB substrate reagents A and B (BD, 555214) colorimetric substrate were added to washed wells and colour change reaction was stopped by adding 1.5 M sulphuric acid. Plates were read on MultiSkan GO microplate spectrophotometer (Thermo Fisher Scientific) at 450 nm with correction at 595 nm.

2.8. Statistical Analysis
Distribution of data was assessed by D’Agostino & Pearson omnibus normality test and in most cases was not normally distributed; therefore graphs are presented as individual points with horizontal bar representing median or bar graphs of the median ± IQR. Unless otherwise stated, in-text descriptions of values are the median and interquartile range. For most studies unless indicated, Mann-Whitney non-parametric U tests were performed to determine significance, where no significance is left blank (Chapters 3 and 4) or labelled ‘ns’ (Chapter 5), trending significance (0.05<p<0.1) p value is stated, and significance is indicated by p < 0.05 *, p < 0.01 **, p < 0.001 ***, p < .0001 ****. All statistical analyses were performed using GraphPad Prism software (version 6.01 and 7.01, La Jolla, CA, USA), unless otherwise indicated.
Chapter 3
Targeting the inflammatory mediator, IL-6 in Lyn-deficient mice as a therapy for lupus
3.1. Abstract
Systemic Lupus Erythematosus (SLE), an autoimmune disease requiring both autoreactive B cell mediated anti-nuclear antibody (ANA) production and chronic systemic inflammation to mediate tissue pathology has largely utilized the same treatment options for the last 50 years. The use of broad spectrum anti-inflammatory and immunosuppressive drugs, while effective for many, can have off target effects and dose limiting toxicities, and in some patients, are insufficient to control disease. Studies on novel, targeted therapeutics for SLE have largely focussed on the B cell compartment, which has been met with limited success. Comparatively, therapeutics targeting inflammatory mediators have been poorly investigated for SLE, highlighting a potential avenue for new therapeutic targets. Mice lacking immunoregulatory Src-family tyrosine kinase, Lyn (Lyn−/−), develop autoimmune disease mediated by autoantibodies and systemic inflammation culminating in lupus-like glomerulonephritis. Inflammation in these mice is dependent on the pro-inflammatory factor IL-6, which drives myeloid cell expansion and T cell hyperactivation that underpins the generation of class switched pathogenic ANAs. To determine the therapeutic potential of targeting the inflammatory mediator IL-6 in lupus-like disease, Lyn−/− mice underwent biweekly anti-IL-6 mAb treatments from 20 weeks, a time-point at which early disease is present in the mice and resembles initial presentation of a patient to the clinic. Mice were analysed after 12 weeks of treatment for resolution of autoimmune and inflammatory phenotypes. Anti-IL-6 mAb therapy was efficacious in moderating splenomegaly by inhibiting erythrocytosis and myeloid cell hyperplasia, had a modest impact on restricting lymphocyte hyperactivation, restrained pathogenic autoantibody production but had no significant impact on glomerulonephritis. These findings indicate that treatment regimens which include IL-6 targeting agents may be of therapeutic benefit in SLE.
3.2. Introduction

Systemic Lupus Erythematosus (SLE) is a complex, heterogenic autoimmune disease mediated by the production of autoreactive antibodies to nuclear components which can form immune complexes and deposit in tissues and microvessels resulting in severe inflammatory tissue destruction. While SLE is dependent on B cells to produce the autoantibodies, disease propagation and perpetuation is also heavily reliant on an inflammatory environment largely driven by the production of pro-inflammatory cytokines (178). Current therapeutic regimens in SLE utilise broad spectrum immunosuppressive agents, which dampen inflammatory innate responses. These agents include hydroxychloroquine, which suppresses type I interferons, and glucocorticoids, which antagonise the pro-inflammatory NF-κB pathway (156). While generally well tolerated, these drugs can have undesired side-effects or lead to dose dependent toxicities, and in some patients, they are ineffective at controlling disease (156). Clinical studies for SLE have focused mainly on B cell targeted therapeutics with limited efficacy; notably B cell depleting mAbs Rituximab (anti-CD20) and Epratuzumab (anti-CD22) did not meet primary endpoints and have not been pursued further in SLE (159, 160, 163, 368). The most recent drug approved for SLE, Belimumab, a mAb which targets BAFF, a B cell survival factor produced by activated myeloid cells, only has a modest impact on patients with mild disease and without major kidney involvement (165). This highlights a need for novel targeted therapeutics which can be tailored to the individual patient.

Similar to human SLE, mice deficient in Lyn (Lyn−/−), a Src-family tyrosine kinase essential for the regulation of inhibitory signalling in the immune system, develop a B cell-mediated systemic autoimmune disease culminating in deposition of immune complexes in the kidneys resulting in a lupus nephritis-like pathology (85, 88). Lack of inhibitory signals in Lyn−/− mice drive B cell hyper-responsiveness and hyperactivation leading to plasmacytosis and hyper IgM and pathogenic IgG ANAs (85). Studies in these mice have confirmed that while B cells are necessary for disease (88), it is also dependent on an inflammatory environment which is essential for pathology. Lyn−/− mice lacking TLR-mediated inflammatory responses through the deletion of MyD88 (Lyn−/−MyD88−/−), have restrained autoantibody production and glomerulonephritis, through the inhibition of dendritic cell mediated IL-6 and IL-12 responses (182). Indeed, this observation is recapitulated in dendritic cell specific Lyn deficiency; where exacerbated glomerulonephritis is abrogated by the dendritic cell specific deletion of MyD88
Furthermore, Lyn−/− mice lacking the inflammatory cytokine IL-6 (Lyn−/−IL-6−/−) maintain B cell hyper-responsiveness and plasmacytosis, but due to limiting myeloid cell expansion and T cell hyperactivation, these mice fail to generate class-switched autoantibodies and therefore do not develop glomerulonephritis (88, 191). This not only highlights the importance for inflammation in driving autoimmune disease, but dissociates B cell intrinsic defects from inflammation driven pathology in Lyn−/− mice and indicates that the IL-6 pathway may be targeted for therapeutic benefit.

The aim of this chapter was to investigate the effect of targeting the inflammatory factor IL-6 in Lyn−/− mice, with therapy initiated at age of disease onset, and assess the impact of therapy on inflammatory phenotypes and autoimmune pathology. We found that anti-IL-6 mAb therapy moderated some systemic inflammatory phenotypes including myeloid cell hyperplasia, splenomegaly and lymphocyte hyper-activation, and while this diminished pathogenic autoantibody production, it had no significant impact on kidney pathology. This study highlights a potential therapeutic avenue for targeting IL-6 in SLE.
3.3. Specific Methods

3.3.1. Anti-IL-6 mAb treatment
Mice were housed in the M.ICU and aged until 20 weeks before receiving treatment. Lyn<sup>-/-</sup> mice received twice weekly intraperitoneal injections with 10 mg/kg anti-IL-6 IgG1 (BioXCell, MP5-20F3) (n=12) or 10 mg/kg IgG1 isotype control (anti-HRP IgG1; BioXCell, HRPN) (n=7) for 12 weeks. Mice were bled via tail vein bleed fortnightly throughout the treatment period. One mouse receiving the isotype control treatment died prematurely at 30 weeks of age. Mice were killed at 32 weeks of age, alongside aged-matched C57BL/6 control mice (n=6), and organs were collected for assessment of autoimmune disease development to determine efficacy of treatment.
3.4. Results

3.4.1. Splenomegaly and splenic haematopoiesis is ablated by anti-IL-6 mAb treatment in Lyn\(^{-/-}\) mice

To assess the efficacy of IL-6 targeted therapy in lupus prone Lyn\(^{-/-}\) mice, animals were treated from 20 weeks of age with either anti-IL-6 mAb or isotype control for 12 weeks. At 32 weeks, mice were then assessed for hallmarks of inflammation and autoimmune disease. Previous studies have revealed that due to systemic inflammation, Lyn\(^{-/-}\) mice develop splenomegaly in part as a result of splenic haematopoiesis (88); therefore to assess whether anti-IL-6 mAb therapy had an effect on this phenotype, the weights of the spleens were recorded at the end of the treatment period. As expected, isotype control mAb treated Lyn\(^{-/-}\) mice had splenomegaly, exhibiting significantly larger spleens than C57BL/6 control mice (Figure 3.1A). Interestingly, the spleen weights of anti-IL-6 mAb treated Lyn\(^{-/-}\) were significantly lower than those of isotype control mAb treated Lyn\(^{-/-}\) mice and in fact resembled those of the C57BL/6 mice indicating that splenomegaly is resolved by anti-IL-6 mAb therapy in Lyn\(^{-/-}\) mice (Figure 3.1A).

To further investigate these changes to the spleen, splenic erythropoiesis by quantifying erythroblasts in the spleen was assessed using flow cytometry. Proportionally, erythroblasts (CD71\(^{+}\) and/or Ter119\(^{+}\)) made up 5.44% (4.01-10.55%) of splenocytes in C57BL/6 control mice, whereas in the isotype control mAb treated Lyn\(^{-/-}\) mice, this was significantly expanded to 40.95% (33.40-44.78%) (Figure 3.1B). This typical erythroblast expansion in the spleen was moderated in the anti-IL-6 mAb treated Lyn\(^{-/-}\) mice to 14.90% (12.75-30.00%) (Figure 3.1B). Overall this suggests that splenic haematopoiesis which drives erythroblast expansion is a major contributor to splenomegaly in Lyn\(^{-/-}\) mice and that this is ablated by treatment with anti-IL-6 mAb.
Figure 3.1  Splenomegaly and erythrocytosis is largely ablated in Lyn⁻/⁻ mice treated with anti-IL-6 mAb
Spleens from anti-IL-6 mAb and isotype control mAb treated Lyn⁻/⁻ mice and C57BL/6 control mice were (A) weighed to determine splenomegaly and (B) proportions of splenic erythroblasts (CD71+ and/or Ter119+) were determined by flow cytometry. The dot plots shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, ** p<0.01, *** p<0.001.
3.4.2. Myeloid cell expansion is moderated in Lyn⁻/⁻ mice treated with anti-IL-6 mAb

As myeloid cells accumulate in the spleen due to splenic myelopoiesis and are heavily implicated in driving pathology in Lyn⁻/⁻ mice (88, 181), neutrophil, macrophage and monocyte cell numbers in the spleen we quantified by flow cytometry to determine whether this was impacted by anti-IL-6 mAb treatment. Myeloid cell subsets were determined by firstly gating on CD11b+ cells (not shown), then assessing differential expression of cfms (CSF-1/M-CSF receptor) and Ly6C/Ly6G (Gr-1). Neutrophil (CD11b+Gr-1<sup>hi</sup>cfms-) numbers were significantly expanded in isotype control mAb treated Lyn⁻/⁻ mice, and although still significantly higher than C57BL/6 controls, this expansion was significantly moderated in anti-IL-6 mAb treated Lyn⁻/⁻ mice (Figure 3.2A). A similar observation was seen for monocytes (CD11b+Gr-1<sup>mid</sup>cfms+) but not macrophages (CD11b+Gr-1-cfms+) (Figure 3.2A). Collectively, this indicates that myeloid cell expansion in the spleen is partially moderated by anti-IL-6 mAb treatment.

Next, we assessed the myeloid activation phenotype of these mice using flow cytometry. Expression of activation marker CD69 was significantly elevated on macrophages in Lyn⁻/⁻ mice treated with isotype control mAb compared to C57BL/6 control mice; this was moderated by anti-IL-6 mAb treatment (Figure 3.2B). During the course of these studies, we noticed that CD11b expression was elevated on neutrophils from isotype control mAb treated Lyn⁻/⁻ mice compared to C57BL/6 control mice (Figure 3.2C). This increased expression of CD11b was significantly moderated, albeit still observed in Lyn⁻/⁻ mice treated with anti-IL-6 mAb (Figure 3.2C). Conversely, macrophages from Lyn⁻/⁻ mice expressed lower levels of CD11b, which was not influenced by anti-IL-6 mAb treatment (Figure 3.2C). These phenomena have not previously been reported in Lyn⁻/⁻ mice and prompted further investigation into the role of CD11b in autoimmune disease (Chapter 5). These results suggest that anti-IL-6 mAb therapy can restrain myeloid cell activation and potentially moderate a newly identified inflammatory marker in Lyn⁻/⁻ mice.
Figure 3.2  Myeloid expansion and activation is restrained by anti-IL-6 mAb treatment in Lyn−/− mice

(A) Populations of splenic neutrophils (CD11b+Gr-1hi cfms−), macrophages (CD11b+Gr-1-cfms+) and monocytes (CD11b+Gr-1mid cfms+) were determined by flow cytometry and cell counts. Plots are pre-gated on CD11b+, proportions are representative of total live splenic cells. Geometric mean fluorescence intensity (gMFI) of myeloid cell activation markers (B) CD69 and (C) CD11b were quantified by flow cytometry. The dot plots and histograms shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; trending significance is labelled, * p<0.05, ** p<0.01.
3.4.3. B cell lymphopenia and plasmacytosis is not restored yet hyper-activation is moderately controlled in Lyn−/− mice treated with anti-IL-6 mAb

Lyn−/− mice typically present with severely reduced B cell numbers and expanded plasma cells (85), and therefore flow cytometry was used to assess the B cell compartment for changes as a result of anti-IL-6 mAb therapy. Anti-IL-6 mAb treated Lyn−/− mice maintained their reduced B cell numbers (B220+CD138lo) compared to C57BL/6 control mice and no improvement was observed compared to isotype control mAb treated Lyn−/− mice (Figure 3.3A). Similarly, plasma cell numbers (B220lo-midCD138hi) were elevated in anti-IL-6 mAb treated Lyn−/− mice to similar levels observed in the isotype control mAb treated Lyn−/− mice compared to the C57BL/6 control mice (Figure 3.3A). This suggests that IL-6 does not play a role in B cell development in Lyn−/− mice.

The B cell and plasma cell hyperactivation phenotype was also examined by flow cytometry. T cell costimulatory molecule CD86 expression is elevated in isotype control mAb treated Lyn−/− mice compared to C57BL/6 control mice and this elevation is slightly moderated on anti-IL-6 mAb treated Lyn−/− mouse B cells (Figure 3.3B). Similarly, expression of B cell receptor IgM on B cells is downregulated on isotype control mAb treated Lyn−/− mice when compared to C57BL/6 control mice and there is a trending restoration of IgM expression on anti-IL-6 mAb treated Lyn−/− mouse B cells although not to the levels on C57BL/6 B cells (Figure 3.3B). Inhibitory IgG receptor FcγRIIb expression is elevated on isotype control mAb treated Lyn−/− mouse plasma cells compared to the plasma cells of C57BL/6 control mice and this is significantly moderated on the plasma cells of anti-IL-6 treated Lyn−/− mice (Figure 3.3C). These findings suggest that despite not controlling B cell lymphopenia or plasmacytosis, anti-IL-6 mAb therapy can modulate the B cell and plasma cell hyper-activatory phenotype typically evident in Lyn−/− mice.
Figure 3.3  B cell and plasma cell hyperactivation is moderated in anti-IL-6 mAb treated Lyn−/− mice
(A) Populations of splenic B cells (B220+CD138lo) and plasma cells (B220lo-midCD138hi) were
determined by flow cytometry and cell counts. (B),(C) Geometric mean fluorescence intensity
(gMFI) of B cell and plasma cell activation markers were quantified by flow cytometry. The
dot plots shown are representative of the median of each group. Graphs represent the
median of each group. Significance was determined by Mann-Whitney non parametric T test;
no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, ***
p<0.001.
3.4.4. Dendritic cells are further reduced and hyperactivation is unchanged in Lyn^-/- mice treated with anti-IL-6 mAb

Dendritic cells are hyperactivated (88, 182) and are central to the development of autoimmune kidney pathology in Lyn^-/- mice (186). Therefore the splenic dendritic cell compartment of Lyn^-/- mice was assessed by flow cytometry for changes in activation state in response to anti-IL-6 mAb therapy. While the proportion of splenic dendritic cells in Lyn^-/- mice was consistent with C57BL/6 control mice (Figure 3.4A), due to reduced splenic cellularity, Lyn^-/- mice had fewer numbers of dendritic cells, which were even further reduced by anti-IL-6 mAb therapy (Figure 3.4B). The hyperactivated DC phenotype in Lyn^-/- mice, as determined by increased expression of surface CD86 and CD62L, is unresolved by anti-IL-6 mAb treatment (Figure 3.4C). This indicates that although there are fewer splenic DCs in Lyn^-/- mice treated with anti-IL-6 mAb, this therapy is unable to resolve DC hyperactivation.
Figure 3.4  Dendritic cell hyperactivation is unresolved by anti-IL-6 mAb treatment in Lyn^-/^- mice
(A) Splenic DCs (CD11b+CD11c+) were analysed by flow cytometry and (B) absolute numbers were quantified by flow cytometry and cell counts. (C) Geometric mean fluorescence intensity (gMFI) of DC activation markers CD86 and CD62L were quantified by flow cytometry. The dot plots shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, * p<0.05, ** p<0.01, *** p<0.001.
3.4.5. T cell lymphopenia is unresolved and hyper-activation is only slightly improved in Lyn<sup>−/−</sup> mice treated with anti-IL-6 mAb

A feature of aged disease-bearing Lyn<sup>−/−</sup> mice is a diminished T cell compartment that exhibits a hyperactive phenotype, driven by systemic inflammation and DC hyperactivation (88). Therefore splenic T cell numbers and activation was examined using flow cytometry. Despite displaying higher proportions of both CD4+ and CD8+ T cells, there was no restoration of T cell numbers in Lyn<sup>−/−</sup> mice treated with anti-IL-6 mAb (Figure 3.5A). Similarly, CD4+ T cell subsets, helper T cells (CD4+CD25−) and T regulatory cells (CD4+CD25+) were also unchanged in anti-IL-6 mAb treated Lyn<sup>−/−</sup> mice compared to isotype control treated Lyn<sup>−/−</sup> mice (Figure 3.5B).

T cell activation was assessed through the upregulated expression of surface markers CD44 and CD69 and downregulation of the cell adhesion molecule CD62L. Proportions of CD69+ and CD62L− but not CD44+ activated CD4+ T cells were elevated in both the isotype and anti-IL-6 mAb treated Lyn<sup>−/−</sup> groups (Figure 3.5C). There were no differences in the activated CD4+ T cell proportions observed between isotype and anti-IL-6 mAb treated Lyn<sup>−/−</sup> mice (Figure 3.5C). The proportion of CD62L− activated CD8+ T cells was significantly reduced and there was a trending reduction observed for CD69+ T cells in anti-IL-6 mAb treated Lyn<sup>−/−</sup> mice (Figure 3.5C). This suggests that anti-IL-6 mAb therapy may only confer a mild improvement to CD8+ T cells in Lyn<sup>−/−</sup> mice.
Figure 3.5  T cell numbers are not restored by anti-IL-6 mAb therapy in Lyn⁻/⁻ mice

(A) Populations of CD8+ T cells (CD4-CD8+), CD4+ T cells (CD4+CD8-) and (B) T helper cells (CD4+CD25-) and T regulatory cells (CD4+CD25+) were determined by flow cytometry and cell counts. (C) Proportions of activated CD4+ and CD8+ cells were determined by expression of activation markers CD44 and CD69 and downregulation of CD62L using flow cytometry. The dot plots and histograms shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, *** p<0.001.
3.4.6. Pathogenic autoantibody production is restrained in Lyn⁻/⁻ mice treated with anti-IL-6 mAb

The impact of anti-IL-6 mAb therapy on pathogenic autoantibody production was assessed over the course of treatment by ELISA. Levels of anti-dsDNA IgG were elevated in both isotype and anti-IL-6 mAb treated Lyn⁻/⁻ groups compared to control mice over the entire course of treatment. In addition, there was no significant change in anti-dsDNA IgG titres over time between the isotype and anti-IL-6 mAb treated groups (Figure 3.6A). We next assessed whether IgG2c autoantibodies, which are pathogenic due to their complement binding ability, were moderated with anti-IL-6 mAb therapy. While titres of anti-dsDNA IgG2c were significantly elevated in both isotype and anti-IL-6 treated groups, the anti-IL-6 mAb treated group had comparatively lower titres at week 8 and were trending lower at week 10 of treatment (Figure 3.6B). There was no significant difference observed at the endpoint between isotype and anti-IL-6 mAb treated groups.

To determine whether reduced autoantibody titres were a result of impaired class switching, serum antibody titres were measured at the endpoint by ELISA. Although titres of IgA, IgM, IgG1, IgG2b, IgG2c and IgG3 were all elevated in both isotype and anti-IL-6 mAb treated groups, a moderation in IgA, IgG2b and IgG2c titre was observed after anti-IL-6 mAb therapy (Figure 3.6C). This indicates that anti-IL-6 mAb treatment can restrain pathogenic complement binding autoantibody production likely through non-class switching related mechanisms.
Figure 3.6  Pathogenic autoantibody production is restrained in anti-IL-6 mAb treated Lyn⁻/⁻ mice

(A) Total serum IgG anti-dsDNA and (B) serum IgG2c anti-dsDNA autoantibody titres were determined in anti-IL-6 mAb and isotype control mAb treated Lyn⁻/⁻ mice by ELISA. (C) Serum immunoglobulin titres were determined by ELISA. Graphs in A and B represent the median; graphs in C represent the median ± IQR of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01.
3.4.7. Kidney pathology is not improved in Lyn−/− mice treated with anti-IL-6 mAb

To assess development of glomerulonephritis, kidneys were analysed for glomerular expansion. Quantification of median glomerular area revealed that both isotype and anti-IL-6 mAb treated Lyn−/− groups had enlarged glomeruli compared to C57BL/6 control mice and there was no quantifiable difference between the isotype and anti-IL-6 mAb groups (Figure 3.7). This indicates that despite inducing some changes to immune cell compartments, anti-IL-6 mAb therapy is unable to moderate kidney pathology in Lyn−/− mice.

Figure 3.7  Anti-IL-6 mAb treatment doesn’t prevent development of glomerulonephritis in Lyn−/− mice

Kidneys from 32 week old mice were stained with H&E and glomerular area quantified. Pictographs are representative of the median of each group. Graphs represent the median. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, ** p<0.01.
3.5. Discussion

IL-6 being a pleiotropic yet predominantly pro-inflammatory cytokine has long been implicated as a driver of inflammation and autoimmune disease in human and murine lupus (178). A previous study from our lab examined the role of IL-6 in driving inflammation and autoimmune pathology in autoimmune disease prone Lyn−/− mice. IL-6 deficiency ameliorated a number of inflammatory phenotypes in Lyn−/− mice including splenic myelopoiesis and myeloid cell accumulation, T cell hyperactivation and overexpression of T cell interaction molecules on B cells which coincided with ablation of pathogenic autoantibody titres and kidney pathology (88). These findings highlighted IL-6 as a novel and highly promising therapeutic target to treat autoimmune disease in Lyn−/− mice and by extension suggestive of therapeutic benefit in human SLE. Therefore we conducted a pre-clinical, proof of concept study to determine the curative potential of an anti-IL-6 mAb therapy regimen on inflammatory and autoimmune pathology in Lyn−/− mice. Mice were treated twice weekly with 10mg/kg antibody for 12 weeks; this regimen was designed to be maximally efficacious while avoiding the induction of an anti-rat antibody immune response. We found that anti-IL-6 mAb therapy suppressed many inflammatory phenotypes including splenomegaly and myeloid cell expansion yet only had a modest impact on the lymphocyte hyperactivation phenotype and did not ultimately improve kidney pathology.

Splenomegaly occurs in Lyn−/− mice due to extramedullary haematopoiesis; where haematopoietic stem cells migrate from the bone marrow to the spleen in response to inflammatory signals where excess growth factor production and Lyn-deficiency mediated increased sensitivity to growth factor signalling promotes their uncontrolled differentiation and expansion (179, 180). This results in the expansion of erythroblasts and myeloid cells observed in the spleens of Lyn−/− mice. After anti-IL-6 mAb treatment, Lyn−/− mice exhibited a complete resolution of splenomegaly coincident with normalised erythroblast, neutrophil, and monocyte populations in the spleen. IL-6 has been implicated in driving erythropoiesis via trans-signalling mechanisms (369) or by stimulating autocrine erythropoietin (EPO) production (370). Similarly, IL-6, historically referred to as a macrophage granulocyte inducer (371), works synergistically with IL-3, M-CSF, or stem cell factor (SCF) to promote the proliferation and differentiation of myeloid precursors (372-374). Indeed Lyn−/− mice lacking IL-6 exhibited similarly ablated splenomegaly, myeloid cell expansion and restricted splenic
myeloid colony formation, indicating that IL-6 is a key factor promoting this phenotype, either acting directly on this compartment, or indirectly by driving systemic inflammation as previously suggested by our group (88). Secondary to the changes in myelopoiesis, anti-IL-6 mAb treated Lyn−/− mice exhibited moderation in neutrophil CD11b expression, suggestive of dampening of IL-6 signalling having a resolving effect on neutrophil hyperactivation.

IL-6 was initially attributed as a B cell differentiation factor that directed the maturation of B cells into antibody secreting plasma cells (375). This is exemplified in mice overexpressing IL-6 (IL-6 transgenic mice), which exhibit profuse plasmacytosis and hypergammaglobulinemia (376). Interestingly, the effects of IL-6 on the B cell compartment of Lyn−/− mice are modest; Lyn−/− mice lacking IL-6 exhibited no changes in splenic B cell populations but showed a mild albeit significant reduction in plasma cells (88, 191). Similarly, anti-IL-6 mAb treatment was unable to resolve these B cell compartment anomalies in Lyn−/− mice. While autoimmune pathology and systemic inflammatory phenotypes are manipulable in Lyn−/− mice, induced either genetically (summary of compound genetic crosses with Lyn−/− mice, Table 1.1; extensively reviewed in (87)) or by therapeutic intervention, including the anti-IL-6 mAb regimen described in this chapter, the typical B cell deficiency and, to a lesser extent, plasmacytosis exhibited by Lyn−/− mice is largely refractory to change. This is indicative of the hardwired maturational, developmental and survival defects Lyn-deficiency has on the peripheral B cell compartment (85, 171), yet also highlights that these defects do not necessarily influence autoimmunity and disease manifestations in these animals. Lyn−/− mice also exhibit a range of humoral defects including hypergammaglobulinemia, especially hyperproduction of IgM antibodies and the production of pathogenic, isotype switched ANAs (85, 171, 172). Whilst IL-6-deficient Lyn−/− B cells maintain hyperactivity and autoreactivity through the production of low affinity IgM ANAs, these mice fail to generate high affinity, isotype switched autoantibodies (88, 191). This was only partially recapitulated by anti-IL-6 mAb therapy in Lyn−/− mice; total IgG autoantibodies persisted yet highly pathogenic, complement recruiting IgG2c ANAs were reduced. This was not due to defective isotype switching processes as these mice maintained high titres of IgA and IgG subclass antibodies. Despite not expressing Lyn, T cells are heavily implicated in the autoimmune pathogenesis of Lyn−/− mice, due to their influence on driving antibody isotype switching and the subsequent production of pathogenic autoantibodies (88, 183). With age, T cells from Lyn−/− mice exhibit a
hyperactivated phenotype, correlating with autoantibody production and disease progression (88, 183). IL-6, it seems, is central to this phenotype, as Lyn⁻/⁻IL-6⁻/⁻ mice exhibited normalisation of T cell hyperactivation in aged mice, correlating with minimal IgG ANAs (88). The inability of anti-IL-6 mAb therapy to control T cell activation is likely to be promoting the persistence of isotype switched, pathogenic autoantibodies in Lyn⁻/⁻ mice.

Two previous studies have examined the efficacy of anti-IL-6 mAb (clone MP5-20F3) therapeutically in lupus prone NZB/NZW F₁ mice. The first study commenced treatment from approximately 12 weeks of age, prior to the onset of disease, and mice received weekly injections of 500µg of antibody which continued for 6 months (approx. 38-40 weeks of age) (347). This same study identified a strong and rapid anti-rat antibody immune response toward the therapeutic anti-IL-6 mAb which they were able to suppress with a tolerising treatment of anti-CD4 mAb alongside the initial anti-IL-6 mAb dose (347). This study reported that their tolerised anti-IL-6 mAb treatment regime was able to suppress ANA levels while improving kidney function and survival of the NZBxNZW F₁ mice, yet did not examine kidney pathology directly (347). The second study similarly commenced treatment at 10-12 weeks and continued to 34 weeks, with mice receiving weekly doses of 1mg antibody without the use of a tolerising dose of anti-CD4 mAb (348). This study reported suppressed activation of T and B cells, reduced ANAs and improved kidney pathology by anti-IL-6 mAb treatment (348). Disappointingly, and contrary to the improvements observed in NZBxNZW F₁ mice and the abrogation of autoimmune kidney pathology observed in the Lyn⁻/⁻IL-6⁻/⁻ mice (88), kidney pathology was not improved by anti-IL-6 mAb treatment in Lyn⁻/⁻ mice. While we did manage to observe a therapeutic effect on the splenic myeloid cell compartment, the lack of improvement of kidney pathology may be due to ineffective dosage. While the Lyn⁻/⁻ mice in this current study received a similar weekly dose (approx. 300µg per 30g mouse, twice weekly) compared to the first NZBxNZW F₁ study (500µg per mouse per week), this was approximately half that of the second study (1mg per mouse per week), so therefore it is possible that it was insufficient to moderate lymphocyte activation and subsequent kidney pathology. The first NZBxNZW F₁ study demonstrated that their tolerising dosage of anti-CD4 mAb had a depletive effect on circulating CD4+ T cells; and while CD4+ T cell levels returned to normal (347), they had effectively depleted the repertoire of pathogenic T cells which was restored with “IL-6 naïve” cells, which are likely to exhibit less activation and therefore restrict ANA production.
and subsequently kidney pathology. Therefore it is likely that the tolerising dose of anti-CD4 mAb had a greater contribution to the improvements observed in the anti-IL-6 treated NZBxNZW F₁ mice than reported. An effective anti-rat antibody immune response assay in the anti-IL-6 treated Lyn⁻/⁻ mice in this study was not possible due to the high cross reactivity between mouse and rat antibodies, but given a physiological response was observed, it is unlikely that this is the cause for the lack of kidney pathology improvement. The two regimens used in the NZBxNZW F₁ studies commenced treatment at approximately 12 weeks of age (347, 348) which precedes the onset of disease and as such is acting prophylactically, which is not representative of the mild disease observed in patients when they present to the clinic, as we had tried to recapitulate in our study. However, with a slight modification to the treatment regimen to adopt the dosage and frequency of the second NZBxNZW F₁ study, yet still treating the mice from disease onset (approx. 18 weeks), the clinical efficacy of the anti-IL-6 mAb treatment regimen in Lyn⁻/⁻ mice may be improved.

IL-6 has been targeted in human SLE; Sirukumab, a humanized anti-IL-6 mAb, which was generally well tolerated in phase I trials (NCT01702740) (353) has stalled at phase II trials (NCT01273389) due to lack of efficacy and excessive adverse events (377). Early studies determined that antibodies cross-linking soluble factors, such as IL-6, can themselves form ICs and promote organ damage in deposited tissues (349). However, this has been overcome by the development of an antibody targeting the IL-6 receptor (tocilizumab), which effectively blocks IL-6 signalling, and this agent has since been indicated for a number of diseases including Castleman disease (lymphoproliferative) (378, 379), RA (380-383) and I is in trials for Neuromyelitis optica (neurodegenerative) (384). IL-6R blockade has shown efficacy in lupus prone NZWxNZB F₁ mice (although this utilised a tolerising dosage of anti-CD4 mAb) (385), and has since entered into trials for SLE, showing safety and tolerability in a phase I trial (357) as well as showing resolution of B and T cell activation, suppressing autoantibody producing plasma cells (358). In conjunction with the anti-IL-6 mAb therapy studies in NZBxNZW F₁ mice and the Lyn⁻/⁻ mice study described here, these collectively validate the IL-6 signalling axis as a therapeutic target for SLE.

In summary, we have evaluated the efficacy of an anti-IL-6 mAb treatment of Lyn⁻/⁻ mice and observed that this therapy moderated splenomegaly through regulation of erythrocytosis and myeloid cell expansion, and somewhat moderated activation of myeloid cells and
lymphocytes, although not to the extent seen in IL-6 deficient Lyn−/− mice. This only had a modest impact on ANA production yet did not improve kidney pathology. Although our regimen was only partially efficacious, it supports the previous findings of our lab that IL-6 is an important driver of inflammation and disease processes in Lyn−/− mice and adds to the collective data supporting inclusion of IL-6 targeting therapeutics in SLE treatment approaches.
Chapter 4

The anti-inflammatory effects of dietary fibre and the modulation of the gut microbiome in Lyn-deficient mice
4.1. Abstract
Systemic Lupus Erythematosus (SLE) is a complex, multifactorial autoimmune disease mediated by the deposition of immune complexes in tissues such as the kidney, skin and brain, with the ensuing inflammatory cascade driving progressive tissue damage and dysfunction. Mice lacking Lyn tyrosine kinase (Lyn−/− mice) develop an autoimmune disease similar to SLE, driven by dysregulation of the immune system, immune complex deposition in tissue and systemic inflammation culminating in progressive glomerulonephritis. The gut microbiome has been shown to have an immunoregulatory effect on the development of autoimmune and inflammatory diseases, in large part due to the production of short chain fatty acids from the fermentation of dietary fibre. To determine whether dietary fibre could moderate systemic autoimmune and inflammatory pathology, Lyn−/− mice and control C57BL/6 mice were fed a high fibre diet (HFD) or a standard control diet from weaning until 42 weeks old. On the control diet, Lyn−/− mice developed dysbiosis, lymphopenia, splenomegaly from enhanced splenic erythropoiesis and myelopoiesis, hyperactivation of immune cells, and pathogenic IgG anti-dsDNA autoantibodies that deposited in the kidney glomeruli leading to glomerulonephritis. These hallmarks of inflammation and autoimmune disease were significantly reduced in Lyn−/− mice fed a HFD, indicating that dietary intervention is effective at dampening chronic systemic inflammation and glomerular pathology. The effects of initiating high fibre supplementation from overt disease onset, which mimics the timepoint when patients present to the clinic for diagnosis was also examined; interestingly, this resulted in modulated inflammatory phenotypes and restricted the development of glomerulonephritis. These findings highlights the contribution of diet and the gut microbiome in regulating systemic immune responses and controlling autoimmunity, inflammation, and preventing the progression of immunopathology and indicates that fibre supplementation may improve outcomes for those living with SLE or other chronic systemic inflammatory diseases.
4.2. Introduction

Autoimmune diseases, which have a strong genetic predisposition, are also heavily influenced by environmental factors. While genetic influence has remained relatively constant, the comparatively higher incidence of inflammatory autoimmune diseases in the developed “Western World” which has been steadily increasing since the 1950s, compared to the lower prevalence observed in developing countries, is indicative that the “Western lifestyle” supports the development of these pathologies (386, 387). The Western lifestyle favours better socio-economic stability, lower susceptibility of infection, lower exposure to and reduced richness of gut commensal bacteria, and a diet high in fats, salt and sugar offset by less dietary fibre (98, 131). The interplay between dietary components and the gut microbiome has been shown to direct local immune responses and modulate inflammation. Soluble fibre and resistant starches from the diet can be fermented by gut resident bacteria into short chain fatty acids (SCFAs), namely butyrate (carboxylic chain containing 4 carbons, C4), propionate (C3) and acetate (C2) (131). Bacteria of the phyla Bacteroidetes (gram negative) and Firmicutes (gram positive), which comprise the majority of the gut microbiome, are responsible for the fermentation of SCFAs from dietary carbohydrates; with Bacteroidetes prioritising production of acetate and propionate and Firmicutes producing butyrate (388). These SCFAs can act locally within the intestines to regulate gut immunity, but propionate and acetate can also move into the circulation, where they can illicit systemic immunoregulatory effects (131). SCFAs signal via cell membrane receptors Free Fatty Acid Receptor 2 (FFAR2, previously known as GPR43) which is expressed on monocytes/macrophages, neutrophils (136), lymphocytes (135), regulatory T cells (137) and eosinophils (138), and FFAR3 (GPR41) which is expressed on DCs (135, 139). SCFAs can also repress gene transcription via inhibition of Histone Deacetylases (HDACs) (143), which can moderate inflammatory responses (144, 145). SCFAs can regulate inflammatory cytokine responses in monocytes/macrophages (389) and neutrophils, and repress the activity of inflammatory transcription factor NF-κB (390-392). SCFAs can also restrict neutrophil migration and infiltration to sites of aberrant inflammation (390, 392), and influence their survival (393). Studies on mice lacking GPR43 (GPR43−/−), which have lost most of the immunoregulatory effects of SCFAs, exhibit exacerbated pathologies in induced models of colitis, arthritis, airway inflammation (138) and food allergy (394), indicating that these SCFA derived immunoregulatory mechanisms can restrict the development of inflammatory
pathology. Indeed, supplementation of SCFAs, primarily acetate, in GPR43-sufficient animals significantly moderated these pathologies (138, 394), as well as in models of lung injury (390) and asthma (395). Protection against disease has even been conferred in models of asthma, Type 1 Diabetes and food allergy by feeding animals a diet high in fibre or resistant starches (394-396). While these studies exemplify the protective role that dietary fibre derived SCFAs have on inflammatory immunopathologies in models of localised disease, the impact of these immunoregulatory effects are yet to be determined in systemic autoimmunity.

Mice deficient in immunoregulatory tyrosine kinase Lyn (Lyn−/−), develop autoimmunity and pathology resembling human Systemic Lupus Erythematosus (SLE) (85, 171). These mice, through loss of peripheral tolerance, develop autoantibodies targeting primarily nuclear components (ANAs), which form immune complexes (IC) that deposit in the microvasculature of the kidney, initiating a lupus nephritis-like glomerulonephritic pathology (85, 171). Lyn−/− mice exhibit leukocyte hyperactivation that perpetuates a systemic hyper-inflammatory environment, which is essential for disease manifestation (88, 181, 182), and similar to human SLE patients, exhibit aberrant dysregulation of gut bacterial communities (148, 150, 397). Therefore, we hypothesise that a diet high in fibre will promote normalisation of the gut microbiome and in turn, illicit an anti-inflammatory protective effect on the development of autoimmune pathology in Lyn−/− mice. In this study, aged Lyn−/− mice fed a high fibre diet from weaning exhibited moderation of systemic inflammatory phenotypes including splenomegaly, myeloid cell expansion, and T cell hyperactivation; and while there was only a modest impact on the B cell compartment and autoantibody production, kidney pathology was largely attenuated. A similar effect was observed in Lyn−/− mice that were switched onto a high fibre diet at 18 weeks of age, which is a time-point when early disease is manifest. These findings endorse the implementation of diets high in fibre as a lifestyle intervention to support the treatment of SLE and other inflammatory autoimmune diseases.
4.3. Specific Methods

4.3.1. High fibre diet regimen
To assess the role of high fibre diet on autoimmune development, pups born at ARL-MARP were shipped to M.ICU at weaning age (3-4 weeks) where they were placed on high fibre diet (SF11-029; Specialty Feeds, Western Australia) (Lyn\(^+/\) n=12, C57BL/6 n=10) or standard diet (AIN93G; Specialty Feeds, Western Australia) (Lyn\(^+/\) n=11, C57BL/6 n=10) (Table 4.1).

To determine the effect of high fibre diet as a lifestyle intervention from disease diagnosis, mice were housed under normal conditions until 18 weeks of age when they were then placed on either high fibre (Lyn\(^+/\) n=14, C57BL/6 n=8) or standard (Lyn\(^+/\) n=13, C57BL/6 n=8) diet.

Mice were bled via tail vein bleed and faecal samples were collected every \(\approx 4\) weeks throughout the treatment period, starting from week 12 for the weaning group and week 18 for the intervention group. Mice were killed at 42 weeks of age and organs were collected for assessment of autoimmune disease development.

<table>
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<th>Table 4.1 Composition of experimental diets</th>
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<tr>
<td><strong>Fibre</strong></td>
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<td>Standard AIN93G</td>
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<td>High Fibre SF11-029</td>
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20% Guar Gum | 40.2% Starch | 12.6% Casein |

4.3.2. Gut bacterial communities analyses
Mouse faecal samples were collected monthly and stored at -80°C. Bacterial DNA was purified from the faeces (QIAamp® Fast DNA Stool Mini Kit, Qiagen), with DNA yield determined by UV spectrophotometry (NanoDrop 2000c, Thermo Scientific). To prepare samples for downstream sequencing, V3V4 amplicon template sequences were amplified from samples by PCR (forward 5’- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’, reverse 5’- GTCTCGTGGGCTCGAGATGTGTATAAGACAGCAGACGGGTGATCTAATCC-3’, Sigma-Aldrich) using KAPA HiFi HotStart Ready Mix (KAPA Biosystems) ran using the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) with the following protocol: 95°C 3min, 25x 95°C 30sec > 55°C 30sec > 72°C 30sec, 72°C 5min. Amplified samples underwent Lumina bacteria ribosomal 16S next generation sequencing at MicroMon.
Outputs were analysed using Qiime workflow and Greengenes database using closed referencing Operational taxonomic unit (OTU) picking (398). Beta diversity was analysed using the browser-based visualisation tool, EMPeror (399). Relative abundance of gut bacteria was determined at the phyla and genera level. To determine the relative abundance of classifiable gut bacteria genera, the abundance of each genus for Lyn⁻/⁻ mice reared on a standard diet were ranked and graphed, with the same genera for the Lyn⁻/⁻ high fibre diet group overlaid. Abundance below 0.005% was considered insignificant. Taxa which lacked distinct family and genera classification were excluded. Significantly different genera, as determined by unpaired T test, are listed on the graph, and for taxa which were unable to be classified at the genera level, the family is listed and underlined (indicative of a genera belonging to that family, not the family as a whole). Mean abundance is stated in the text for comparison of bacterial community shifts due to high fibre diet in Lyn⁻/⁻ mice.
4.4. Results

4.4.1. Splenomegaly and splenic haematopoiesis are abrogated in Lyn\(^{-/-}\) mice on a high fibre diet

Lyn\(^{-/-}\) mice were fed a high fibre diet from weaning until 42 weeks of age, and the effect of this dietary supplementation on inflammatory and autoimmune features in the mice was then examined. To assess whether a high fibre diet can impact the development of splenomegaly, spleen weights were recorded. As expected, significantly elevated spleen weights were observed in Lyn\(^{-/-}\) mice on standard diet when compared to control mice (Figure 4.1A), whereas Lyn\(^{-/-}\) mice fed a high fibre diet had lower spleen weights compared to their standard diet counterparts and were of a similar size to the control mice (Figure 4.1A).

Splenomegaly in Lyn\(^{-/-}\) mice is driven by extramedullary haematopoiesis and the expansion of both erythroid and myeloid cells (180). To determine whether the abrogation of splenomegaly in high fibre fed Lyn\(^{-/-}\) mice was due to moderation of splenic erythropoiesis, populations of erythroblasts in the spleen were determined by flow cytometry. High fibre had a profound impact on splenic erythrocytosis, reducing the splenic erythroblast population in Lyn\(^{-/-}\) mice from 50.6% (44.4-56.4%) in the control diet fed mice to 20.9% (14.6-25.2%) in the high fibre fed mice (Figure 4.1B). Collectively this data indicates that inflammatory splenomegaly and extramedullary erythropoiesis in Lyn\(^{-/-}\) mice can be regulated by a high fibre diet.
Figure 4.1 Splenomegaly and erythrocytosis is moderated in Lyn\textsuperscript{-/-} mice reared on a high fibre diet

Spleens from Lyn\textsuperscript{-/-} and C57BL/6 control mice fed either a standard or a high fibre diet from weaning were (A) weighed to determine splenomegaly and (B) analysed by flow cytometry to determine proportions of splenic erythroblasts (CD71\textsuperscript{+} and/or Ter119\textsuperscript{+}). The dot plots shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, ** p<0.01, *** p<0.001, **** p<0.0001.
4.4.2. Myeloid cell expansion is moderated in Lyn−/− mice on a high fibre diet

To determine whether inflammatory myelopoiesis and myeloid cell accumulation was impacted by a high fibre diet, splenic macrophages and neutrophils were quantified by flow cytometry. Typical expansion of splenic neutrophil populations which was observed in Lyn−/− mice fed a standard diet was ameliorated in Lyn−/− mice on a high fibre diet (Figure 4.2A). Similarly, splenic macrophage expansion which was also observed in standard diet fed Lyn−/− mice was significantly moderated in high fibre fed Lyn−/− mice (Figure 4.2B). These data indicate that a high fibre diet can control expansion of myeloid cells in the spleen of Lyn−/− mice.

Next, the activation phenotype of splenic myeloid cells were assessed by flow cytometry. While Lyn−/− macrophages showed no improvement in their expression of activation markers (CD86, MHC Class II, CD62L) on a high fibre diet (data not shown), neutrophils demonstrated a modest recovery of CD62L expression indicating a potential inhibition of activation (Figure 4.2C). As previously demonstrated in Chapter 3, CD11b expression was elevated on neutrophils and reduced on macrophages from Lyn−/− mice on a standard diet, this phenotype was not rescued by high fibre from weaning (Figure 4.2D).
Figure 4.2  Myeloid expansion and activation is restrained in high fibre fed Lyn⁻/⁻ mice
(A) Populations of splenic neutrophils (CD11b+Ly6G+) and (B) macrophages (CD11b+cfms+)
were quantified by flow cytometry. Geometric mean fluorescence intensity (gMFI) of (C)
neutrophil activation marker CD62L and (D) myeloid cell CD11b expression were quantified
by flow cytometry. The dot plots and histograms shown are representative of the median of
each group. Graphs represent the median of each group. Significance was determined by
Mann-Whitney non parametric T test; trending significance is labelled, * p<0.05, ** p<0.01,
*** p<0.001, **** p<0.001.
4.4.3. B cell lymphopenia and plasmacytosis are not restored in Lyn\textsuperscript{-/-} mice on a high fibre diet

To determine the impact that high fibre diet has on the B cell compartment, populations of splenic B cells and plasma cells were examined by flow cytometry. B cell lymphopenia and plasmacytosis persisted in Lyn\textsuperscript{-/-} mice on a high fibre diet (Figure 4.3A), indicating that high fibre cannot moderate the B cell compartmental defects in Lyn\textsuperscript{-/-} mice.

The B cell and plasma cell hyperactivation phenotype was also assessed by flow cytometry. Lyn\textsuperscript{-/-} mice typically express reduced surface BCR (IgM and/or IgD), CD40 and FcγRIIb on B cells; this phenotype was slightly moderated in Lyn\textsuperscript{-/-} mice on a high fibre diet (Figure 4.3B). Overexpression of T cell costimulatory ligands CD80 and CD86 on Lyn\textsuperscript{-/-} B cells was not moderated by high fibre diet (data not shown). Plasma cells also did not show improvement in their expression of any activation/maturation markers (data not shown). This suggests that a high fibre diet can modestly moderate the B cell hyperactivation phenotype in Lyn\textsuperscript{-/-} mice.

Interestingly, expression of the myeloid receptor CD11b on B cells has been associated with regulation of BCR signalling through the promotion of Lyn-mediated regulatory mechanisms (62). Therefore, to determine whether CD11b expression on B cells is altered in Lyn-deficient mice and whether this is further impacted by a high fibre diet, expression was assessed using flow cytometry. Expression of B cell CD11b was elevated in Lyn\textsuperscript{-/-} mice on standard diet compared to control C57BL/6 mice, this phenotype was significantly moderated in Lyn\textsuperscript{-/-} mice fed a high fibre diet (Figure 4.3C), suggesting that Lyn-deficiency leads to an upregulation of CD11b on B cells and this phenotype can be modulated by dietary fibre.
Figure 4.3 B cell deficit remains unchanged but the hyperaction phenotype is moderated in Lyn−/− mice fed a high fibre diet

(A) Populations and absolute numbers of splenic B cells (B220+CD138lo) and plasma cells (B220lo-hiCD138hi) were determined by flow cytometry and cell counts. (B) Geometric mean fluorescence intensity (gMFI) of B cell activation markers were quantified by flow cytometry. The dot plots shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, **** p<0.0001.
4.4.4. T cell activation is moderated in Lyn-/- mice on a high fibre diet
While Lyn is not expressed in T cells, Lyn-/- mice develop T cell lymphopenia and exhibit a hyperactivation phenotype with age, due to chronic systemic inflammation, which contributes to pathogenesis by promoting autoantibody class switching (88, 183). Therefore, to assess the impact of high fibre diet on the T cell compartment, populations of CD4+ and CD8+ T cells were assessed by flow cytometry. While proportions of CD4+ and CD8+ T cells were restored in high fibre fed Lyn-/- mice, absolute numbers of T cells were unaffected by high fibre in Lyn-/- mice (Figure 4.4A) due to reduced splenic cellularity, suggesting that high fibre is insufficient to restore T cell populations in Lyn-/- mice.

The activation phenotype of T cells was also determined by assessing expression of activation markers CD44, CD69 and CD62L using flow cytometry. Proportions of activated CD44+, CD69+ and CD62L-CD4+ and CD8+ T cells, which were consistently elevated in aged Lyn-/- mice reared on standard diet, were significantly moderated in Lyn-/- mice on a high fibre diet, with proportions of CD44+ T cells similar to C57BL/6 mice (Figure 4.4B). This indicates that a high fibre diet can significantly moderate T cell hyperactivation in aged Lyn-/- mice.
Figure 4.4  T cell hyperactivation is significantly moderated in high fibre fed Lyn⁻/⁻ mice

(A) Proportions and absolute numbers of CD8+ T cells (CD4⁻CD8+) and CD4+ T cells (CD4⁺CD8⁻) were determined by flow cytometry and cell counts. (B) Proportions of activated CD4+ and CD8+ cells in the spleen were determined by expression of activation markers CD44 and CD69 and downregulation of CD62L using flow cytometry. The dot plots and histograms shown are representative of the median of each group. Graphs represent the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, **** p<0.0001.
4.4.5. Autoantibody production is restrained and kidney pathology is largely ameliorated in Lyn−/− mice reared on a high fibre diet

The impact of high fibre diet on the development of autoantibodies was assessed by ELISA. Production of anti-dsDNA IgG autoantibodies was restrained significantly at 12 weeks and trending at 18 weeks of age in mice on a high fibre diet compared to those on a standard diet (Figure 4.5A). At the experimental endpoint, autoantibody production was comparable in high fibre fed Lyn−/− mice compared to standard diet fed Lyn−/− mice (Figure 4.5A). This suggests that a high fibre diet can acutely restrain the production of autoantibodies in Lyn−/− mice.

To determine whether a high fibre diet can modulate kidney pathology in Lyn−/− mice, histopathology was performed and glomerular size was measured. Typical glomerular expansion indicative of glomerulonephritis was observed in Lyn−/− mice fed a standard diet from weaning (5012 µm², 4069-7253 µm²), however this was significantly moderated in Lyn−/− mice reared on a high fibre diet (3439 µm², 3154-3918 µm²) (Figure 4.5B). Although glomerular area was slightly enlarged in high fibre fed Lyn−/− mice compared to C57BL/6 control mice on a high fibre diet (2996 µm², 2327-3232 µm²), there was no significant difference when compared to C57BL/6 control mice on a standard diet (3109 µm², 2733-3808 µm²) (Figure 4.5B). Lyn−/− mice reared on a high fibre diet did show minor indications of glomerular hypercellularity and lobularity, although this was considerably moderated compared to Lyn−/− mice on a standard diet. This indicates that a high fibre diet can significantly restrict the development of autoimmune kidney pathology in Lyn−/− mice.
A

**anti-dsDNA IgG**

<table>
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<tr>
<th>relative titre</th>
<th>std</th>
<th>HF</th>
<th>std</th>
<th>HF</th>
<th>std</th>
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<tr>
<td></td>
<td>12wks</td>
<td>18wks</td>
<td>26wks</td>
<td>34wks</td>
<td>42wks</td>
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B

**C57**

**Lyn**

**standard**

**high fibre**

**glomerular area (μm²)**

- C57 std
- C57 HF
- Lyn standard
- Lyn HF

**Statistical Significance**

- ***** p-value
- ** ** p-value

**Scale:** 100μm
Figure 4.5  Pathogenic autoantibody production is restrained and glomerulonephritis is ablated in Lyn/- mice fed a high fibre diet

(A) Total IgG anti-dsDNA autoantibody titres were determined in high fibre and standard diet fed Lyn/- mice by ELISA. (B) Kidneys from 42 week old mice were stained with H&E and glomerular area quantified. Graphs in A and B represent the median. Images in B are representative of the median of each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
4.4.6. Characterisation of the gut microbiome of high fibre diet reared Lyn\(^{-/}\) mice

To determine the effect that a high fibre diet has on the gut microbiome, gut bacterial communities were analysed using ribosomal 16S Next-Generation Lumina sequencing. Microbial communities at the phyla level largely correlate with diet (Figure 4.6A). With the exception of an expansion in Verrucomicrobia, few changes occurred in the microbial communities in C57BL/6 mice fed a standard diet versus a high fibre diet. However, Lyn\(^{-/}\) mice reared on a high fibre diet exhibited an expansion of Bacteroidetes, Proteobacteria, Verrucomicrobia and Tenericutes, and a restriction of Firmicutes and Deferrribacteres communities (Figure 4.6A). Similar shifts were also observed in C57BL/6 control mice on a high fibre diet, although these weren’t statistically significant (Figure 4.6A). Interestingly, Cyanobacteria which is significantly expanded in standard diet fed Lyn\(^{-/}\) mice, was not restricted by a high fibre diet, suggesting that the increase in these bacteria is intrinsically linked to Lyn-deficiency (Figure 4.6A). While Bacteroidetes and Firmicutes are both fibre fermenting phyla, higher ratios of Bacteroidetes to Firmicutes is associated with moderation of inflammation especially in the context of obesity (400), and Bacteroidetes species produce high quantities of systemically active propionate and acetate (388), while lower ratios are indicative of dysbiosis in C57BL/6 background mice (401). Lyn\(^{-/}\) mice fed a high fibre diet had a higher ratio of Bacteroidetes to Firmicutes compared to their standard diet counterparts (Figure 4.6B), indicating a positive shift in the microbiome away from an inflammatory profile.

The comparison of the gut microbial community diversity between each of the different genotype and diet groups (beta diversity) revealed unique, yet overlapping clustering for each group (Figure 4.6C). On the standard diet, overlapping of the C57BL/6 and Lyn\(^{-/}\) clusters was minimal, indicating that Lyn\(^{-/}\) mice exhibit dysbiosis compared to C57BL/6 mice (Figure 4.6C). In C57BL/6 mice, a high fibre diet drove a unique gut microbiome profile compared to standard diet, as indicated by the complete lack of overlap of these clusters (Figure 4.6C). Lyn\(^{-/}\) mice fed a high fibre diet also exhibited a complete lack of cluster overlap with their standard diet fed counterparts and in fact showed high similarity with the C57BL/6 high fibre diet and lesser similarity to the C57BL/6 standard diet cluster, indicating that a high fibre diet can override dysbiosis in Lyn\(^{-/}\) mice (Figure 4.6C).
At the genera level, a high fibre diet in Lyn⁻/⁻ mice drove the expansion of an unclassifiable bacteria of the family S24-7 (Bacteroidetes) (standard diet 47.1% vs. high fibre diet 72.5%), Akkermansia (Verrucomicrobia) (0.62% vs. 4.75%), Sutterella (Proteobacteria) (0.41% vs. 1.28%), Prevotella (Bacteroidetes) (0.16% vs. 2.99%), Blautia (Firmicutes) (0.007% vs. 0.34%), Proteus (Proteobacteria) (0.006% vs. 0.35%), Roseburia (Firmicutes) (<0.005% vs. 0.01%), Anaeroplasma (Tenericutes) (<0.005% vs. 0.10%), Turicibacter (Firmicutes) (<0.005% vs. 0.05%), an unclassifiable bacteria of the family Enterobacteriaceae (Proteobacteria) (not detected vs. 0.01%) and Enterbacter (not detected vs. 0.006%) (Figure 4.6D). This was offset by the recession of Parabacteroides (Bacteroidetes) (15.1% vs. 3.67%), Bacteroides (Bacteroidetes) (11.9% vs. 5.81%), an unclassifiable bacteria of the family Rikenellaceae (Bacteroidetes) (3.27% vs. 0.40%), Oscillobia (Firmicutes) (1.39% vs. 0.31%), Allobaculum (Firmicutes) (1.14% vs. <0.005%), Ruminococcus (Firmicutes) (1.05% vs. 0.21%), an unclassifiable bacteria of the family Ruminococcaceae (Firmicutes) (0.93% vs. 0.11%), Mucispirillum (Deferribacteres) (0.83% vs. 0.06%), Bilophila (Proteobacteria) (0.32% vs. 0.05%), Dehalobacterium (Firmicutes) (0.25% vs. 0.04%), Lactobacillus (Firmicutes) (0.17% vs. 0.03%), Coprococcus (Firmicutes) (0.14% vs. 0.04%), an unclassifiable bacteria of the family Coriobacteriaceae (Actinobacteria) (0.09% vs. 0.02%), Dorea (Firmicutes) (0.07% vs. <0.005%), Streptococcus (Firmicutes) (0.02% vs. 0.006%) and Adlercreutzia (Actinobacteria) (0.02% vs. 0.006%) (Figure 4.6D).
Figure 4.6  Shifts in the gut microbiota of high fibre diet fed Lyn⁻/⁻ mice toward a less inflammatory profile

Bacterial DNA was extracted from faeces and analysed by Lumina ribosomal 16S sequencing. Outputs were analysed using Qiime workflow to determine gut bacterial composition. Gut microbiome proportions in 42 week old C57BL/6 and Lyn⁻/⁻ mice on standard or high fibre diet at the (A) Phyla level with (B) the ratio of the bacterial phyla Bacteroidetes to Firmicutes in the gut calculated, (C) the diversity of gut microbial communities between genotype and diet groups and (D) at the Genera level for Lyn⁻/⁻ mice at 42 weeks on the two different diets. Graphs in A, B represent the median ± IQR of each group, significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, **** p<0.0001. Graph in C represents the mean + SEM, significance was determined by unpaired T test; all labelled points are significantly different between diets.
4.4.7. Leukocyte populations and inflammatory phenotypes are similarly moderated in Lyn−/− mice fed a high fibre diet from disease onset

Given the profound impact that a high fibre diet administered from weaning had on moderating inflammation driven immune cell phenotypes and ablating autoimmune kidney disease in Lyn−/− mice, we sought to determine whether this diet would be effective as an intervention from disease onset. Lyn−/− and C57BL/6 mice were aged under standard conditions until 18 weeks of age, at which point Lyn−/− mice exhibited elevated titres of autoantibodies and mild kidney pathology, resembling some patients at time of diagnosis; mice were then placed on either a high fibre or a standard diet and analysed at 42 weeks for hallmarks of inflammation and autoimmune disease as above.

Similar to feeding from weaning, high fibre intervention reduced splenomegaly, in part due to a trending moderation of erythrocytosis in Lyn−/− mice (Figure 4.7A). While high fibre intervention was unable to moderate splenic macrophage accumulation, neutrophils were significantly reduced, to levels similar to control mice (Figure 4.7B). Neutrophil activation was similarly moderated, with increased expression of CD62L and downregulated CD11b expression (Figure 4.7B). As found previously in mice fed modified diets from weaning, high fibre intervention was unable to restore B cell numbers or regulate plasma cell expansion, but was able to moderate IgM expression in B cells and also potentially moderate B cell CD11b overexpression (Figure 4.7C). Interestingly, Lyn−/− mice fed a high fibre diet from disease onset demonstrated a small albeit significant restoration in CD4+ T cell numbers which was not seen in Lyn−/− mice fed high fibre from weaning; there was no impact of high fibre intervention on CD8+ T cell numbers (Figure 4.7D). Unlike from weaning, high fibre from disease onset had no significant impact on the T cell hyperactivation phenotype (data not shown). This indicates that high fibre can modulate several inflammatory phenotypes when administered as a lifestyle intervention in Lyn−/− mice, highlighting the therapeutic potential of this diet in human disease.
Figure 4.7  High fibre diet implemented from disease onset is effective at controlling many of the inflammatory phenotypes in Lyn−/− mice

(A) Spleens collected from mice at the endpoint were weighed and proportions of erythroblasts (CD71+ and/or Ter119+) were determined using flow cytometry. (B) Absolute numbers of macrophages (CD11b+cflks+) and neutrophils (CD11b+Ly6G+) and expression of activation markers CD62L and CD11b on neutrophils were determined using flow cytometry (C) Absolute numbers of B Cells (B220hiCD138lo) and plasma cells (B220lo- hiCD138hi) and expression of activation markers IgM and CD11b were determined by flow cytometry (D) Populations of CD4+ T cells (CD4+CD8−) and CD8+ T cells (CD4-CD8+) were determined by flow cytometry. Graphs in A-D represent the median. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled, trending significance is labelled, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
4.4.8. High fibre diet from disease onset regulates autoimmunity and protects against kidney pathology in Lyn−/− mice

As a high fibre diet fed to Lyn−/− mice from disease onset was able to moderate several of their inflammatory phenotypes, we next examined whether autoimmunity and immunopathology was similarly moderated. ELISA analyses of serum from mice fed a high fibre diet from disease onset showed that autoantibody production was acutely moderated in Lyn−/− mice at 26 weeks and 34 weeks; at the endpoint, autoantibody levels were comparable between high fibre and standard diet fed Lyn−/− mice (Figure 4.8A). Histopathological analysis of kidneys from mice fed a high fibre diet from disease onset revealed that kidney pathology was markedly diminished; while cellular infiltrates and glomerular lobularity is still evident, glomerular expansion was minimal, resembling standard diet fed C57BL/6 control mice. (Figure 4.8B). This indicates that high fibre diet as a lifestyle intervention, when implemented during early stages of disease, is effective at controlling disease progression in Lyn−/− mice.
Figure 4.8  High fibre diet implemented from disease onset can regulate autoantibody production and protect against kidney pathology in Lyn⁻/⁻ mice
Mice were reared on a standard diet and then switched to a high fibre diet at 18 weeks of age and aged until 42 weeks. They were assessed every 8 weeks for (A) total serum IgG anti-dsDNA autoantibody titres by ELISA. (B) Kidneys from mice at the experimental endpoint (42 wks) were stained with H&E and glomerular area quantified. Graphs in A, B represent the median. Images are representative of the median for each group. Significance was determined by Mann-Whitney non parametric T test; no significance is unlabelled or labelled ns, * p<0.05, ** p<0.01.
4.5. Discussion
The fermentation of soluble dietary fibre by the gut microbiome to produce SCFAs has demonstrated anti-inflammatory effects, improving disease outcomes in a number of models of inflammatory autoimmune diseases (394-396, 401). In this study we have shown that a diet high in fibre, promotes an anti-inflammatory shift in the gut microbiome and resolves many inflammatory phenotypes including splenomegaly via the moderation of myeloid cell expansion and erythrocytosis and T cell hyperactivation culminating in amelioration of kidney pathology in Lyn<sup>−/−</sup> mice, a model of systemic inflammation and autoimmune kidney pathology.

The body of work examining the diet-microbiome-disease axis in SLE is currently in its infancy. While correlations have been made between known microbiome-altering dietary metabolites, such as vitamin D, vitamin A and polyunsaturated fatty acids and disease outcomes in SLE patients (402), only a handful of studies have directly examined the gut microbiota of SLE patients (148, 397, 403), and prior to our study, the impact of high fibre or SCFAs has not been examined in any model of SLE. The gut microbiome is attributed with shaping both local and systemic immune responses, and is implicated in modulating the course of pathology for many diseases (404, 405). While the advent of 16S ribosomal bacterial sequencing has simplified the characterisation of the gut microbiome, the inability to isolate and culture the majority of these organisms prohibits the conducting of functional studies to determine the impact these bacteria have on heath and disease outcomes. This largely limits analysis to the correlation of microbiome profiles or “enterotypes”, with disease states and outcomes and inferences based on genetic analyses or limited studies on cultivable strains. Analysis of the gut microbiome of Lyn<sup>−/−</sup> mice fed a high fibre diet revealed an expansion of bacteria of the phyla Bacteroidetes, which are responsible for producing acetate and propionate, the major SCFAs detectable in the circulation. This positively shifted the ratio of Bacteroidetes to Firmicutes, indicating a shift toward a protective microbial composition. The expansion of Bacteroidetes in high fibre fed Lyn<sup>−/−</sup> mice was largely due to the expansion of the family S24-7. S24-7 has been identified as one of the major components of C57BL/6 mouse gut microbiome in a number of studies, as well as being abundant in the human gut (401, 406-408). Genome sequencing and analysis has found that this family comprises at least 27 distinct species, the majority of which contain the metabolic machinery capable of carbohydrate fermentation and production of acetate and propionate (408). These preliminary studies
suggest that S24-7, like other families within the Bacteroidetes phyla can illicit systemic anti-inflammatory effects through production of SCFAs, which may be confirmed in future studies using the newly isolated cultivable S24-7 strain (409) and its expansion correlates positively with improvements in disease outcomes in Lyn−/− mice. We also observed an expansion of Prevotella (Bacteroidetes) and diminution of Bacteroides and Parabacteroides (Bacteroidetes) genera in high fibre fed Lyn−/− mice, which in humans, is typical of a diet high in carbohydrates and lower in typical ‘Western diet’ components such as animal fats and protein, and correlates with increases in SCFAs (410-413); although over-expansion of Prevotella copri has been linked to inflammatory complications in susceptible individuals (414-416).

Akkermansia muciniphila (Verrucomicrobia), the only species of the genus Akkermansia that has been identified in the mammalian gut (including humans (417)), is associated with moderation of inflammation and improvements in disease outcomes in models of obesity, type 2 diabetes and metabolic syndrome (418-420), colitis (421) and atherosclerosis (422). Similarly, we observed expansion of Akkermansia in high fibre fed Lyn−/− mice which correlated with resolution of many inflammatory phenotypes and amelioration of autoimmune kidney pathology. While the phyla Verrucomicrobia is not known to be SCFA producing, A. muciniphila elicits regulation of gut immunology through crosstalk with the innate immune system via TLR-2 interactions (419), and promotion of anti-inflammatory and anti-microbial peptide production (418). A previous study characterised the microbiome of MRL mice, and found that the dominant constituent of the gut microbiota in these mice was the Lactobacillaceae family of the phylum Firmicutes (149). MRL mice carrying a lupus-susceptibility mutation (MRLlpr/lpr) exhibited a reduction in the abundance of Lactobacillaceae, which when restored, correlated with improvements in renal function (149). Conversely, in high fibre fed Lyn−/− mice, improvements in kidney pathology correlated with restoration of the Bacteroidetes family S24-7, and reduced abundance of Lactobacillus (of the family Lactobacillaceae). This suggests that in mice, improvements in pathology and disease is driven by the restoration of a normal gut microbiome more so than the universal expansion of any one specific organism. Whether this is solely due to the positive functions of these highly abundant bacteria or also due to out-colonising pathogenic bacteria is still undetermined.

There are two distinct arms which drive pathogenesis in Lyn−/− mice: intrinsic B cell hyperactivation and loss of tolerance, and the systemic, self-perpetuating inflammatory
milieu (85, 88). While B cells are essential for manifestation of disease and inflammation in Lyn−/− mice (88), inflammation, largely generated by myeloid cells, DCs and T cells, is essential for induction of pathogenic autoantibody production and activation and migration of myeloid effector cells which cause kidney pathology (88, 182, 186). Given that the B cell compartment, especially the lymphopenia/plasmacytosis and hyper-responsive signalling phenotypes are largely refractory to change by moderating inflammation, either therapeutically (Chapter 3), or genetically (88, 175), it is unsurprising that our high fibre diet did not resolve any of the defects of the splenic B cell compartment, aside from slight improvements in the expression of B cell hyperactivation markers. Previous studies on Lyn−/− mice have shown that improvements in disease outcomes correlate with improvements in inflammatory phenotypes, mainly the inhibition of peripheral myeloid cell expansion and normalised expression of T cell hyperactivation markers (88, 182, 183, 198, 202). In Lyn−/− mice, high fibre diet moderated the expression profile of activation markers on T cells, shifting these toward normal levels. Interestingly, moderation of T cell hyperactivation restrained autoantibody production only during the early stages of high fibre diet feeding in Lyn−/− mice from weaning. In contrast, Lyn−/− mice fed a high fibre diet from disease onset displayed moderated autoantibody titres during early-mid stages despite lack of suppression of T cell hyperactivation. This suggests that in addition to restraining T cell activation, inhibitory effects on autoantibody production may be, at least in part, driven by SCFAs moderating myeloid effector functions.

SCFAs have been shown to exert anti-inflammatory effects on the myeloid cell compartment by restricting their production of Th1 cell activating pro-inflammatory cytokine IL-12 and promoting the production of immunoregulatory IL-10 (389). This in turn can dampen pro-inflammatory Th1 cell responses including perpetuation of inflammation (181), myeloid cell activation and IFN-γ mediated pathogenic IgG2c autoantibody isotype switching (Figure 1.3). SCFAs can also influence growth and survival of myeloid cells both indirectly, via promotion of IL-10 production (389), which represses production of growth factors IL-3 and GM-CSF (423) and inhibits signalling downstream of the GM-CSF receptor (424), or directly via induction of apoptosis in neutrophils via inhibition of HDACs (393). Consumption of a high fibre diet prevented expansion of myeloid cells from both weaning and from disease onset in Lyn−/− mice, indicative of moderated systemic inflammation. Kidney pathology was also moderated in
Lyn−/− mice fed a high fibre diet, from both weaning and disease onset. Not only was glomerular expansion ameliorated, but enlargement of the sub capsular space, lobularity and hyper-cellularity were also moderated in high fibre fed Lyn−/− mice. Glomerular expansion and damage occurs due to infiltration of myeloid cells which drives local inflammation and tissue destruction (Figure 1.1) (85, 171). SCFAs not only influence myeloid cell survival but they can also control migration and infiltration of neutrophils to sites of aberrant inflammation and can also moderate their activation and pathogenic effector functions (390, 392), which are enhanced in Lyn−/− mice (425). Therefore it seems that high fibre diet derived SCFAs are protecting against kidney pathology by dampening systemic inflammation and moderating the hyper-expansion, activation and infiltration of myeloid effector cells, despite having minimal impact on autoantibody production. Further histological studies examining humoral and cellular infiltrates into the kidneys of high fibre fed Lyn−/− mice will further support this hypothesis. The improvements in kidney pathology were conferred regardless of whether mice commenced the high fibre diet feeding from weaning or as an intervention from disease onset, suggesting that dietary modifications to include foods rich in fibre may be an excellent adjunct to standard therapy in human SLE.

Altogether, our study has shown that high fibre feeding can normalise the gut microbiome profile by promoting the expansion of systemically active SCFAs acetate and propionate producing gut bacteria. This correlated with resolution of inflammatory splenomegaly, erythrocytosis and myeloid cell expansion, moderation of T cell hyperactivation and restraint of autoantibody production, which culminated in significant improvements in kidney pathology in lupus prone Lyn−/− mice. While superior suppression of systemic inflammatory phenotypes was achieved in Lyn−/− mice fed a high fibre diet from weaning, improvements in autoimmune pathology were still achieved in mice which commenced high fibre feeding from disease onset, highlighting the potential for high fibre to be implemented as a cost-effective lifestyle intervention in SLE patients; although further studies addressing the impact and efficacy of high fibre in human disease would be beneficial.
Chapter 5

The protective role of CD11b on disease progression in Lyn-deficient mice

This chapter contains content from the submitted original research article:

5.1. Abstract
Systemic Lupus Erythematosus (SLE) is a highly complex, heterogeneous autoimmune disease characterized by circulating self-reactive antibodies that deposit in tissues including skin, kidneys and brain, alongside a chronic inflammatory response that leads to progressive tissue damage and impaired function. Genome-wide association studies (GWAS) have identified a number of receptors, cytokines and signal transduction molecules specific for the immune system that predispose to the development of SLE. A single nucleotide polymorphism (SNP) in the ITGAM gene encoding CD11b (rs1143679) that leads to a loss-of-function, associates with an increased incidence of SLE, implicating CD11b as a protective factor against disease development. CD11b has both pro-inflammatory and regulatory functions in myeloid and dendritic cells and more recently, has been implicated in promoting antibody class-switching and moderating autoreactive B cell responses, suggesting that CD11b functions are complex and duplicitous in nature. To determine if CD11b plays a role in altering susceptibility to autoimmune disease, we crossed CD11b-deficient mice (CD11b−/−) with Lyn-deficient (Lyn−/−) mice, a well-studied, robust model that mimics human SLE, and mice were analysed over time for characteristics of autoimmune disease and inflammation. While CD11b−/− mice presented with mild splenomegaly and lymphadenopathy, lymphocyte compartments were unchanged and pathogenic IgG anti-dsDNA autoantibody titres and glomerulonephritis were undetected in 30-week-old mice suggesting that CD11b deficiency alone does not lead to autoimmune disease at this time-point. Conversely, deficiency of CD11b on the Lyn-deficient autoimmune-prone background exacerbated disease, driving splenomegaly and lymphadenopathy, extramedullary haematopoiesis, autoantibody production and glomerulonephritis, which impacted survival. These findings confirm that CD11b is an autoimmune susceptibility gene with loss-of-function mutations in CD11b exacerbating the severity of disease on a susceptible genetic background. This work highlights an important role for CD11b in regulating and controlling the progression of inflammation and autoimmune disease.
5.2. Introduction

CD11b is a member of the leukocyte integrin family comprising CD11a (LFA-1), CD11b (Mac-1/CR3), CD11c (p150,95/CR4) and CD11d (αDβ2). Members of this family share a common beta subunit (CD18) but have a unique alpha subunit (CD11a, b, c, d) and they are expressed only in leukocytes. Their importance for immune cell function has been highlighted by the identification of Leukocyte Adhesion Deficiency (LAD) patients that harbor genetic mutations in CD18 resulting in reduced, absent or non-functional expression of all family members and they are unable to clear infections due to the inability of immune cells to migrate to sites of inflammation (426). CD11b is ordinarily expressed on neutrophils, monocytes, macrophages, (DCs), eosinophils, natural killer cells and subpopulations of B cells (427). CD11b has numerous ligands including counter-receptors (ICAM-1, ICAM-2), the complement component C3bi, extracellular matrix proteins (fibrinogen, collagen, vitronectin, laminin), blood coagulation proteins (fibrinogen, factor X), and enzymes such as myeloperoxidase and elastase (428). Although LAD patients exhibit defective immune cell trafficking, mice lacking CD11b are not impaired in neutrophil recruitment during inflammation indicating other family members can compensate (367, 429-431), however CD11b is important for phagocytosis, oxygen radical generation and neutrophil apoptosis (367, 429-431), and CD11b-deficient mice are defective in bacterial clearance (432).

While these early reports indicated that CD11b serves a pro-inflammatory function to promote innate immune responses, more recent studies suggest that CD11b is duplicitous in nature also having protective immunomodulatory roles (433). CD11b is implicated in regulating DC maturation in inflammatory settings and restricts antigen-presenting cell (APC) directed T cell activation (59, 60), but it is also required for pro-inflammatory cytokine production in response to LPS mediated toll like receptor (TLR)-4 activation in DCs (64). Conversely, in macrophages, CD11b regulates MyD88 and TRIF degradation downstream of TLR activation, moderating hyper-inflammatory cytokine responses (61, 63). In B cells, CD11b has a similarly duplicitous role. Its expression is upregulated during B cell activation and it has been reported to drive antibody somatic hyper-mutation (SHM) and heavy chain class-switch recombination (CSR) via modulating expression of activation-induced cytidine deaminase (AID) (434), as well as maintaining tolerance by regulating autoreactive B cell antigen receptor (BCR) signals (62). Interestingly, CD11b’s regulatory functions in B cells and potentially macrophages...
are reliant on the Src family tyrosine kinase Lyn, a regulatory kinase that is critical for inhibitory signalling and heavily implicated in autoimmunity (61, 62).

More recent evidence suggests that loss of CD11b can promote autoimmunity. Polymorphisms in ITGAM, the locus encoding human CD11b, confer a strong risk of developing the autoimmune disease systemic lupus erythematosus (SLE) (26, 65, 435). A number of SNPs have been identified that lead to mutations in CD11b in particular the SNP rs1143679, which encodes an Arg to His change at amino acid 77 (R77H) in the extracellular beta propeller domain of CD11b. While this mutation does not affect expression of CD11b or appearance of the CD11b activation state, it promotes pro-inflammatory cytokine production, impairs myeloid cell phagocytosis and adhesion to a range of ligands (74), and leads to dysregulated BCR signalling (62), processes which collectively contribute to autoimmune pathogenesis. Another SNP associated with SLE, rs1143678 which induces mutations in the cytoplasmic domain of CD11b, alters intracellular protein binding and promotes inflammation (76). Thus, these findings suggest that loss of CD11b activity heightens the susceptibility of patients to SLE. Indeed, CD11b deficiency promotes cell-mediated kidney pathology in an animal model of induced glomerulonephritis (436).

Animal models of SLE have enriched our understanding of the genetic, cellular and molecular mechanisms that contribute to the disease and one model that has been used extensively for the past two decades is the Lyn-deficient mouse. Lyn−/− mice spontaneously develop a disease akin to SLE manifesting immune cell defects, antinuclear antibodies (ANAs), systemic inflammation and glomerulonephritis due to immune complex deposition in kidney, which leads to renal dysfunction (85, 88, 171). These mice have been used widely to delineate the contributions of specific genes to autoimmunity and to examine genetic interactions in autoimmune disease development (87). Thus, to determine if loss of CD11b would alter susceptibility to SLE-like autoimmune disease, we combined Lyn deficiency with CD11b deficiency. We show that CD11b deficiency on its own does not result in autoimmunity, although aged CD11b−/− mice exhibit evidence of inflammatory traits. However, when CD11b deficiency is combined with Lyn insufficiency, this leads to dramatic exacerbation of autoimmunity and augmented autoimmune disease development. These studies indicate that loss of CD11b function can drive autoimmune disease progression in individuals with a genetic predisposition.
5.3. Specific Methods

5.3.1. CD11b\textsuperscript{−/−} mouse autoimmune phenotyping
CD11b\textsuperscript{−/−} mice (n=10) were housed and aged in the M.ICU and killed at 30 weeks of age, alongside aged matched C57BL/6 control mice (n=6) and organs collected for assessment of autoimmune disease development.

5.3.2. Survival Study
Lyn\textsuperscript{−/−} (n=23) and Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} (n=19) mice on C57BL/6 background were housed in the M.ICU under standard Optimice housing conditions. Mice received daily health and condition checks by animal technicians and further assessments by technicians, vets and researchers when showing signs of illness. While some aged mice were found dead on a daily health check, most animals were culled when their condition had deteriorated to predefined stages, ethically set by researchers in consultation with veterinary staff. Deaths were recorded at these time-points. Lifespan (days) was documented and plotted to determine median age and to derive a Kaplan-Meier survival curve. The published survival data of BALB/c Lyn\textsuperscript{−/−} mice generated by our lab (176) was reanalysed to examine survival differences between males and females. Significance was determined by Gehan-Breslow-Wilcoxon test (earlier deaths are more heavily weighted) and Mantel-Cox test (all deaths are weighted evenly) where p<0.05 is significant.

5.3.3. Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} autoimmune phenotyping
Mice were housed in the M.ICU under standard Optimice housing conditions. Mice were bled via tail vein bleed every four weeks throughout the observation period. To determine the contribution of CD11b to autoimmune disease development in Lyn\textsuperscript{−/−} mice over time, mice were killed at 12 weeks (C57BL/6 n=12, Lyn\textsuperscript{−/−} n=14, CD11b\textsuperscript{−/−} n=13, Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} n=18), 24 weeks (C57BL/6 n=12, Lyn\textsuperscript{−/−} n=7, CD11b\textsuperscript{−/−} n=11, Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} n=10) and 36 weeks (C57BL/6 n=12, Lyn\textsuperscript{−/−} n=8, CD11b\textsuperscript{−/−} n=11, Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} n=9) and tissues and organs were collected for assessment of immune cell changes and autoimmune disease development.
5.4. Results

5.4.1. Lyn-deficiency drives altered expression of CD11b on myeloid cells and B cells

As previously reported in Chapter 3 and Chapter 4, Lyn\(^{-/-}\) mice display altered expression of CD11b on myeloid cells and B cells which can be moderated by therapeutics (Figure 3.2) or high fibre diet (Figure 4.3, 4.7), suggestive of a novel marker of inflammation/activation. To examine whether the phenotype changes with age, we measured CD11b expression on B cells, neutrophils and macrophages from Lyn\(^{-/-}\) mice at young (12wk), mid (24wk) and aged (36wk) time points. While expression is low, B cells from Lyn\(^{-/-}\) mice express mildly elevated levels of CD11b at 12 weeks (1.2 fold, 1.0-1.4 fold), which increased moderately at 24 weeks (2.4 fold, 1.6-3.4 fold) and was further enhanced at 36 weeks (4.6 fold, 3.8-8.5 fold) compared to C57BL/6 control mice (Figure 5.1A). Similarly, Lyn\(^{-/-}\) neutrophils expressed moderately elevated levels of cell surface CD11b at 12 weeks (1.9 fold, 1.7-2.2 fold) and 24 weeks (1.6 fold, 1.2-2.2 fold) which was further increased by 36 weeks of age (3.5 fold, 1.8-4.4 fold) relative to control mice, suggesting that it was tracking with disease development (Figure 5.1B). Conversely, Lyn\(^{-/-}\) macrophages consistently expressed lower levels of CD11b at 12 (0.79 fold, 0.67-0.90 fold), 24 (0.86 fold, 0.79-0.91 fold) and 36 weeks (0.80 fold, 0.71-0.97 fold) (Figure 5.1C).
Figure 5.1 CD11b expression is modulated on cells from Lyn⁻/⁻ mice
Flow cytometry was used to assess the expression of CD11b on (A) B cells, (B) neutrophils and (C) macrophages from 12, 24 and 36 week old C57BL/6 (C57) and Lyn⁻/⁻ (L⁻/-) mice. Expression is indicated as geometric mean fluorescence intensity (gMFI) and normalised to the mean gMFI of aged-matched C57BL/6 mice. Representative histograms of CD11b staining of cells from 24 week old C57 and Lyn⁻/⁻ mice are shown on the RHS of the figure. Data are from 2-5 experiments per timepoint, n=1-6 mice per genotype per experiment. For all data, ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
5.4.2. CD11b-deficiency alone is not sufficient to generate autoimmune disease in aged mice
To determine whether deficiency of CD11b confers autoimmunity, CD11b<sup>−/−</sup> mice were analysed at 30 weeks of age, a time point where autoimmune disease development is significant in Lyn<sup>−/−</sup> mice (88, 202), for traits of autoimmunity and inflammatory immunopathology. CD11b<sup>−/−</sup> mice showed splenomegaly at 30 weeks of age (Figure 5.2A), correlating with a trending increase in numbers of splenic macrophages (Figure 5.2B), and significantly elevated splenic neutrophil populations (Figure 5.2C). Lymphocyte subsets including CD4+ and CD8+ T cells, B cells and plasma cells were not significantly impacted by CD11b-deficiency (Figure 5.2D-5.2G). Autoantibody titres in 30 week old CD11b<sup>−/−</sup> mice were similar to levels in matched C57BL/6 mice and were significantly lower than the titres in 30 week old Lyn<sup>−/−</sup> mice (Figure 5.2H), and examination of kidney sections from 30 week old CD11b<sup>−/−</sup> mice revealed no signs of glomerulonephritis (Figure 5.2I). This indicates that while CD11b-deficiency has a moderate impact on the splenic myeloid compartment, there is no evidence of autoimmunity or immunopathology in CD11b<sup>−/−</sup> mice at 30 weeks of age.
Figure 5.2 CD11b-deficient mice have minor changes to their immune system at 30 weeks of age but do not exhibit autoimmunity

CD11b-deficient mice have minor changes to their immune system at 30 weeks of age but do not exhibit autoimmunity. C57BL/6 (C57) and CD11b\(^{-/-}\) mice of 30 weeks of age were evaluated for (A) spleen weight. Flow cytometry and cell counting was used to determine absolute numbers of splenic (B) macrophages, (C) neutrophils, (D) CD4\(^{+}\) T cells, (E) CD8\(^{+}\) T cells, (F) B cells, and (G) plasma cells. Mice were also analysed for (H) titres of IgG anti-dsDNA using serum from C57BL/6 mice as a negative control and Lyn\(^{-/-}\) mice as a positive control, and (I) glomerulonephritis. Data are from 1-2 experiments, n=2-5 mice per genotype per experiment. For all data, ns = non-significant, \(*p < 0.05\), **p < 0.01.
5.4.3. CD11b-deficiency in Lyn^/- mice confers further impairment of survival

The analysis of 30 week old CD11b-deficient mice suggested that they were not intrinsically autoimmune-prone. Therefore to determine if deficiency of CD11b was able to modulate autoimmune disease on a susceptible genetic background, Lyn^/-CD11b^/- double knockout (DKO) mice were generated and analysed for autoimmune and inflammatory traits alongside matched Lyn-deficient mice. Previous survival studies of Lyn^/- mice on either a mixed genetic background (129Ola X C57BL/6) (85) or a BALB/c (176) background revealed that Lyn-deficiency leads to diminished survival due to autoimmune-mediated pathology. As CD11b deficiency has been attributed to promoting autoreactivity (62) and autoimmune pathology (436), median survival (days) of C57BL/6 background Lyn^/- and Lyn^/-CD11b^/- mice was quantified. Mice were aged and spontaneous deaths and ethical culls were recorded. A total of five Lyn^/- mice were culled prematurely due to birthing difficulties (n=2), fight wounds (eye and skin lesions) (n=2) or anal prolapse (n=1); these deaths were excluded from the study as they did not arise from autoimmune disease or ageing. Kaplan-Meier survival studies demonstrated that DKO mice had markedly reduced median survival of 332 days (254-458 days) compared to the survival of Lyn^/- mice, which was 414.5 days (373-528 days) (log rank p=0.0159, Gehan-Breslow-Wilcoxon p=0.0131) (Figure 5.3A). The reduced survival of Lyn^/-CD11b^/- mice suggests that these mice may develop autoimmune disease more rapidly.

During the early stages of generating the Lyn^/-CD11b^/- mouse strain, spontaneous deaths of female Lyn^/-CD11b^/- mice within 10 days of giving birth were observed. To investigate whether there was a bias against female survival, median survival of Lyn^/-CD11b^/- and Lyn^/- mouse between males and females was compared. Interestingly, while there was no difference in the median survival between female (301 days, 193.5-432.5 days) and male (342 days, 301.5-466.8 days) mice of the Lyn^/-CD11b^/- strain (log-rank p=.6030, Gehan-Breslow-Wilcoxon p=.3675) (Figure 5.3B), a strong bias against female survival (381 days, 347.5-414.5 days) compared to males (509 days, 422.5-592 days) was observed in Lyn^/- mice on the C57BL/6 genetic background (log-rank p=.0265, Gehan-Breslow-Wilcoxon p=.0244) (Figure 5.3C). This sex-bias is likely independent of genetic background, as reanalysis of previously published survival data on BALB/c Lyn^/- mice (176) demonstrated a trending difference in median survival between females (348.5 days, 268.5-371 days) and males (369.5 days, 360-394 days) (log-rank p=.1243, Gehan-Breslow-Wilcoxon p=.0764) (Figure 5.3D). While
comparison of the survival of female Lyn$^{-/-}$ and Lyn$^{-/-}$CD11b$^{-/-}$ mice revealed no significant differences (log-rank p=.9153, Gehan-Breslow-Wilcoxon p=.3758) (Figure 5.3E), male Lyn$^{-/-}$CD11b$^{-/-}$ mice had a significant reduced survival compared to Lyn$^{-/-}$ mice (log-rank p=.0017, Gehan-Breslow-Wilcoxon p=.0030) (Figure 5.3F). This indicates that CD11b-deficiency normalises the survival sex-bias in Lyn$^{-/-}$ mice.
Figure 5.3  Survival is further impaired in Lyn−/− mice deficient in CD11b
Survival of Lyn−/− and Lyn−/−CD11b−/− mice on the C57BL/6 background was determined by Kaplan-Meier survival curves for (A) Lyn−/− (n=18) vs Lyn−/−CD11b−/− (n=19) mice, (B) Male (n=10) vs Female (n=9) Lyn−/−CD11b−/− mice, (C) Male (n=9) vs Female (n=9) C57BL/6 background Lyn−/− mice, (D) Male (n=10) vs Female (n=10) BALB/c background Lyn−/− mice, (E) Female Lyn−/− (n=9) and Lyn−/−CD11b−/− (n=9) mice and (F) Male Lyn−/− (n=9) and Lyn−/−CD11b−/− (n=10) mice. Statistical significance determined by Log-rank and Gehan-Beslow-Wilcoxon tests, p value is stated in each instance, 0.05<p<0.1 is considered trending, p<0.05 is significant.
5.4.4. Inflammatory traits are accelerated in Lyn<sup>−/−</sup> mice lacking CD11b.

As enlargement of secondary lymphoid organs is a phenotype seen in both Lyn<sup>−/−</sup> mice and CD11b<sup>−/−</sup> mice and is often associated with inflammatory extramedullary haematopoiesis, we examined the spleens and lymph nodes of Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice. Splenomegaly was not evident in Lyn<sup>−/−</sup> mice at 12 or 24 weeks and did not manifest until the mice were 36 weeks old (Figure 5.4A). Similarly, mild splenomegaly was observed in 36 week old CD11b<sup>−/−</sup> mice (Figure 5.4A). In contrast, splenomegaly was evident in Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice from 24 weeks of age indicating that this phenotype is accelerated (Figure 5.4A). While lymphadenopathy has been reported in Lyn<sup>−/−</sup> mice on the BALB/c background (176), it is not commonly seen in C57BL/6 background Lyn<sup>−/−</sup> mice. By comparison, CD11b<sup>−/−</sup> mice develop mild lymphadenopathy that is apparent at 24 weeks of age and this phenotype is exacerbated by CD11b deficiency in Lyn<sup>−/−</sup> mice (Figure 5.4B).

In Lyn<sup>−/−</sup> mice, splenomegaly is driven in part by extramedullary erythropoiesis and expansion of erythroid cells (180). To see whether erythroid expansion is further exacerbated in double deficient mice, splenic erythroblasts (Ter119<sup>+</sup> and/or CD71<sup>+</sup>) were examined by flow cytometry. Both Lyn<sup>−/−</sup> and Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice exhibited similarly expanded proportions of erythroblasts at all ages examined (Figure 5.4C), indicating that while increased proportions of erythroblasts are contributing to spleen enlargement in Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice, this does not account for their more rapid onset of splenomegaly. Interestingly, mild erythrocytosis was observed in CD11b<sup>−/−</sup> mice at all ages examined (Figure 5.4C).

Concurrent with extramedullary erythropoiesis, inflammation-driven splenic myelopoiesis also contributes to splenomegaly in Lyn<sup>−/−</sup> mice (180). To determine whether this process was further exacerbated in Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice, myeloid cell populations were quantified using flow cytometry; defining neutrophils as Ly6G<sup>+</sup>c-fms<sup>−</sup> and macrophages as Ly6G c-fms<sup>+</sup>. No changes in neutrophil numbers were observed in young mice or in 24 week old Lyn<sup>−/−</sup> mice. However, a marked expansion of neutrophils was observed in 24 week old CD11b<sup>−/−</sup> mice and Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice, although the numbers in Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice were significantly elevated over those in CD11b<sup>−/−</sup> mice (Figure 5.4D, 5.4E). By 36 weeks of age, all mutant mice exhibited increased numbers of neutrophils compared to control mice (Figure 5.4B). Splenic macrophage expansion was evident in Lyn<sup>−/−</sup>/CD11b<sup>−/−</sup> mice at 12 weeks of age and further increased as the mice aged, whereas increased numbers were not present in CD11b<sup>−/−</sup> mice or
Lyn⁻/⁻ mice until 24 weeks of age (Figure 5.4C). These studies suggest that inflammation-driven myeloid cell expansion is enhanced in Lyn⁻/⁻CD11b⁻/⁻ mice and contributes to their earlier onset development of splenomegaly.
Figure 5.4  Loss of CD11b on a Lyn-deficient background impairs survival and exacerbates inflammatory splenomegaly and lymphadenopathy by exacerbating splenic haematopoietic expansion

C57BL/6 (C57), Lyn−/− (L−/−), CD11b−/− (11b−/−) and Lyn−/−CD11b−/− (DKO) mice were evaluated over time for (A) splenomegaly, (B) lymphadenopathy, (C) splenic erythrocytosis, (D) myeloid cell expansion, (E) neutrophil numbers and (F) macrophage numbers. For (A,C,E,F), data are from 3-4 experiments per timepoint, n=1-6 mice per genotype per experiment. For (B,D), image is representative of 3 experiments, n=1-6 mice per genotype. For all data, ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
5.4.5. Minor restoration of B cell lymphopenia in Lyn\(^{-/-}\)CD11b\(^{-/-}\) mice and mild enhancement of autoantibody production

Lyn\(^{-/-}\) mice exhibit B cell compartment defects which underline their inflammatory and autoimmune pathology (85, 88), therefore the influence of CD11b deficiency on the B cell compartment in Lyn\(^{-/-}\) mice was examined by flow cytometry. Lyn\(^{-/-}\) mice exhibited typical B cell lymphopenia and plasmacytosis across all time points examined (Figure 5.5A-5.5C), and while Lyn\(^{-/-}\)CD11b\(^{-/-}\) mice also showed markedly reduced numbers of B cells compared to control mice, a mild recovery of B cell numbers was observed (Figure 5.5A, 5.5B). Lyn\(^{-/-}\)CD11b\(^{-/-}\) mice also exhibited plasmacytosis, which was strikingly similar to that observed in Lyn\(^{-/-}\) mice (Figure 5.5A, 5.5C). While there were no B cell compartment differences observed in CD11b\(^{-/-}\) mice, at 36 weeks of age they showed a significant increase in numbers of plasma cells (Figure 5.5C).

To determine whether changes in the immune cell compartment of Lyn\(^{-/-}\)CD11b\(^{-/-}\) influenced pathogenic autoantibody production, anti-dsDNA IgG antibodies were measured by ELISA. Lyn\(^{-/-}\)CD11b\(^{-/-}\) mice developed significant autoantibody titres, increasing with age and disease development similar to Lyn\(^{-/-}\) mice, however they had further enhanced autoantibody titres at 24 weeks compared to Lyn\(^{-/-}\) mice, suggesting that autoantibody production is more rapid in these mice (Figure 5.5D). Interestingly, CD11b\(^{-/-}\) mice exhibited a slight increase in plasma cell numbers and mildly elevated anti-dsDNA IgG titres at 36 weeks (Figure 5.5C, 5.5D), indicating that these mice have the potential to develop mild autoimmunity with age.
A

B

C

D

123
Figure 5.5  B cell changes and exacerbated development of autoantibodies in Lyn-deficient mice lacking CD11b

(A) Representative flow cytometry of spleen cells from 36 week old mice stained with antibodies against B220 and CD138 to delineate proportions of B cells (B220^+CD138^{low}) and plasmablasts and plasma cells (B220^{+/−}CD138^{high}). Absolute numbers of (B) B cells and (C) plasma cells in the spleen of mice of the indicated ages and genotype defined by flow cytometry and cell counting. (D) Relative anti-ds DNA titres in 12, 24 and 36 week old mice by ELISA calculated using a reference sample. C57 = C57BL/6, L^{-/-} = Lyn^{-/-}; 11b^{-/-} = CD11b^{-/-}; DKO = Lyn^{-/-}CD11b^{-/-}. For (A-C), data are from 3-4 experiments per timepoint, n=1-6 mice per genotype per experiment. For all data, ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
5.4.6. T cell lymphopenia is recovered while T cell hyper-activation is partially enhanced in CD11b-deficient Lyn−/− mice

T cells which do not express Lyn, are major contributors to autoimmune pathology in Lyn−/− mice by driving pathogenic isotype-switched ANA production (198). A hallmark of chronic inflammation in aged Lyn−/− mice is the shift to a hyper-activated T cell phenotype through the upregulation of cell surface CD44 and CD69 and downregulation of CD62L, which is driven by the inflammatory environment (88, 183). Therefore, to examine the impact of CD11b deficiency on this compartment in Lyn−/− mice, T cells were analysed by flow cytometry at 12, 24 and 36 weeks of age. Lyn−/− mice consistently exhibited both CD4+ and CD8+ T cell lymphopenia at all of the time-points examined (Figure 5.6A-C). Interestingly, as previously shown (Figure 5.2D-E), CD11b−/− mice had unperturbed T cell numbers in 24 and 36 week old mice, but a slight reduction in both CD4+ and CD8+ T cell numbers was evident in young mice (Figure 5.6A-C). CD4+ T cell numbers were normal in Lyn−/−CD11b−/− mice; and while CD8+ T cell lymphopenia persisted in young and mid-aged mice, a recovery of CD8+ T cells was evident in aged Lyn−/−CD11b−/− mice (Figure 5.6A-C). At 24 weeks of age, CD4+ T cells from Lyn−/−CD11b−/− mice showed a hyper-activated phenotype that was enhanced compared to Lyn−/− CD4+ T cells, evidenced by a greater proportion of cells downregulating CD62L expression, although CD4+ T cells from both Lyn−/− mice and Lyn−/−CD11b−/− mice showed similar upregulation of CD44 and CD69 (Figure 5.6D). The CD8+ T cell compartment of 24 week old Lyn−/−CD11b−/− mice exhibited a significant increase in CD62L- cells compared to CD8+ T cells from Lyn−/− mice but no apparent change in CD44 or CD69 (Figure 5.6E). The finding of enhanced T cell activation in Lyn−/−CD11b−/− mice suggests that the inflammatory environment is augmented in these mice compared to Lyn−/− mice, which is leading to these accelerated effects.
Figure 5.6  Spleen T cell numbers are partially rescued and hyperactivation enhanced in Lyn-deficient mice lacking CD11b

(A) Representative flow cytometry of spleen cells from 36 week old mice stained with antibodies against CD4 and CD8 to examine the T cell compartment. Absolute numbers of (B) CD4 T cells and (C) CD8 T cells in the spleen of mice of the indicated ages and genotype defined by flow cytometry and cell counting. Proportions of activated (D) CD4+ and (E) CD8+ cells as determined by expression of activation markers CD44 and CD69 and downregulation of CD62L by flow cytometry. C57 = C57BL/6, L/- = Lyn⁻/⁻, 11b⁻/⁻ = CD11b⁻/⁻, DKO = Lyn⁻/⁻CD11b⁻/⁻. Data are from 3-4 experiments per timepoint, n=1-6 mice per genotype per experiment. For all data, ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
5.4.7. Severity of kidney pathology is accelerated in CD11b-deficient Lyn^−/− mice due to enhanced immune cell infiltration

To determine the impact of CD11b-deficiency on kidney pathology in Lyn^−/− mice, glomerular expansion was quantified. While mild glomerulonephritis was observed in 24 week old Lyn^−/− mice (3556 µm², 3441-3814 µm²), this phenotype had already progressed significantly in Lyn^−/−CD11b^−/− mice (4952 µm², 4188-5349 µm²) (Figure 5.7A), however by 36 weeks of age, both Lyn^−/− and Lyn^−/−CD11b^−/− mice exhibited substantial disease with no significant differences observed in glomerular size: Lyn^−/− (4913 µm², 4632-5698 µm²) and Lyn^−/−CD11b^−/− (5408 µm², 4591-6852 µm²) (Figure 5.7A). As previously observed in 30 week old mice (Figure 5.2I), CD11b^−/− mice do not show evidence of glomerulonephritis at 24 weeks (C57: 2882 µm², 2627-3356 µm²; CD11b^−/−: 2614 µm², 2486-3056 µm²) or 36 weeks (C57: 3081 µm², 2930-3246 µm²; CD11b^−/−: 3399 µm², 2966-3554 µm²) of age (Figure 5.7A). Collectively this data indicates that deficiency of CD11b exacerbates the development of kidney pathology in Lyn^−/− mice.

To further explore the more rapid onset of pathology in Lyn^−/−CD11b^−/− mice, we examined immune cell infiltration into the kidney using immunohistochemistry. Kidney sections from 24 week old mice were stained with antibodies targeting CD45 (pan leukocyte marker) or F4/80 (macrophage marker), the kidney cortex imaged and enzyme-mediated colour change quantified. While leukocyte infiltration into the glomeruli was negligible in C57BL/6 (0.49%, 0.27-0.73%) and CD11b^−/− (0.74%, 0.40-0.91%) mice, Lyn^−/− mice had moderate leukocyte infiltration (2.4%, 1.1-4.6%) which was further exacerbated by CD11b-deficiency (Lyn^−/−CD11b^−/−: 4.7%, 2.5-7.5%) (Figure 5.7B). Macrophages were largely excluded from the glomeruli; in the C57BL/6 and CD11b^−/− groups, F4/80 staining appeared diffuse throughout the tissue while in Lyn^−/− and Lyn^−/−CD11b^−/− groups, F4/80 staining was more focussed to perilobular areas (Figure 5.7B). Overall, while 24 week old Lyn^−/− mice did not show significantly elevated macrophage infiltration of the kidney (C57: 1.8%, 1.4-2.2%; CD11b^−/−: 1.4%, 0.95-1.9%; Lyn^−/−: 2.1%, 1.4-2.7%) macrophage infiltration was significant in Lyn^−/−CD11b^−/− mice at this age (3.8%, 2.7-5.5%) (Figure 5.7B). This data suggests that immune cell infiltration into the kidney occurs more rapidly in Lyn^−/−CD11b^−/− mice and that this may account for the exacerbation in kidney pathology in these mice.
Figure 5.7  Exacerbated development of glomerulonephritis in Lyn-deficient mice lacking CD11b

(A) Representative photomicrographs of H&E-stained kidney sections from 24 and 36 week old C57BL/6, Lyn−/−, CD11b−/− and Lyn−/−CD11b−/− (DKO) mice, and corresponding measurement of the glomerular area. Each data point represents a single mouse with 20-30 glomeruli measured per mouse. (B) Representative immunohistochemistry analysis of kidney sections from the 24 week old mice in (A), staining for leukocytes using anti-CD45 and macrophages with antibodies recognising F4/80, and corresponding quantitation of stained area. C57 = C57BL/6, L−/- = Lyn−/−, 11b−/- = CD11b−/−, DKO = Lyn−/−CD11b−/−. For all data, ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
5.5. Discussion
In this study, we have demonstrated that the leukocyte integrin CD11b influences the development of inflammation and autoimmune pathology in experimental lupus and we have now established its status as an autoimmune susceptibility gene in mice. Deficiency of CD11b in Lyn−/− mice further promoted inflammatory phenotypes including splenomegaly and lymphadenopathy, driven largely by the expansion of macrophages and increases in activated T cells, which altogether resulted in elevated autoantibody production and more rapid onset glomerulonephritis.

CD11b is highly expressed on macrophages, neutrophils and dendritic cells, with B and T cells exhibiting lower expression, and it is accredited with both pro-inflammatory and regulatory functions. For example, on macrophages, CD11b promotes the clearance of apoptotic cells and complement bound immune complexes (437) as well as restricts TLR-mediated pro-inflammatory cytokine production via regulation of MyD88 and TRIF (61), protecting against aberrant inflammatory responses and development of kidney pathology. Conversely, on neutrophils, CD11b promotes pathogenic processes including inflammation-induced tissue infiltration, production of radical oxygen species and the release of nuclear antigens and granular products through NETosis (367, 431). In autoimmune-prone Lyn−/− mice, we observed differential CD11b expression on leukocytes: neutrophils and B cells expressed relatively higher levels whilst macrophage expression was lower. Increased expression of CD11b on immune cells is reported to occur upon cellular activation (434, 438, 439). In Lyn−/− mice, modulated CD11b expression preceded onset of overt disease suggesting that the loss of Lyn is central to this phenotype. It is well known that Lyn-deficient mice have hyperactive B cells and altered signalling pathways (86) and neutrophils from Lyn−/− mice are also reported to exhibit hyper-responsiveness (425). Thus, their increased CD11b expression may be reflective of their activation status. The observed downregulation of CD11b on Lyn−/− macrophages is likely to promote inflammation by impairing its regulatory effect on cytokine production, while the upregulation of CD11b on Lyn−/− neutrophils may stimulate the aforementioned pathogenic functions. Indeed, an earlier study reported that Lyn−/− neutrophils exhibit enhanced adhesion-dependent integrin signalling responses including disease related processes such as degranulation and superoxide release (425). While this was not attributed to enhanced CD11a integrin expression, which was shown to be stable, levels of CD11b were
not assessed (425). Thus, it is possible that dysregulation of CD11b expression in Lyn-deficient myeloid cells may promote their pathogenic nature, therefore contributing to disease in these animals. Lyn is well-characterized as a major regulator of B cell function (86), with B cells central to the development of inflammation and autoimmune pathology in Lyn<sup>−/−</sup> mice (88, 180, 185). B cell expression of CD11b is implicated in promoting Lyn-mediated regulation of BCR signalling, especially in the context of autoimmunity (62). Given that Lyn and CD11b are both tightly involved in regulation of B cell responses, it is possible that in B cells, loss of Lyn triggers upregulation of CD11b as a compensatory mechanism in an effort to regain control. Interestingly a small subset of autoreactive age-associated B cells, which express integrins CD11b and CD11c and are highly responsive to innate stimuli, are found predominantly in aged and autoimmune-prone mice (440), which may also account for the expansion of CD11b expression on Lyn<sup>−/−</sup> B cells, especially as the mice age. Determining the expression profile of CD11b on B cell subsets from Lyn<sup>−/−</sup> mice may help elucidate the function and nature of CD11b expression on Lyn<sup>−/−</sup> B cells.

GWAS studies in SLE have led to the identification of SNPs in ITGAM, the gene encoding human CD11b, which are strongly linked with SLE (441), and in vitro and ex vivo studies have determined that these SNPs result in impaired CD11b function without affecting expression or activation of the CD11b protein. Therefore, it seems likely that CD11b is protective against development of autoimmune pathology. We demonstrate in mice, that deficiency of CD11b alone drives some inflammatory phenotypes including secondary lymphoid organ enlargement and expansion of splenic myeloid cells and erythroblasts, yet the mice only show mild autoimmune traits when 36 weeks of age (mild plasmacytosis and minor increases in anti-dsDNA antibodies), which is insufficient to manifest into typical autoimmune pathology. We did not examine older animals, so it remains possible that they may develop a mild form of autoimmune-mediated glomerulonephritis late in life. Nonetheless, when coupled with Lyn deficiency, CD11b deficiency severely exacerbated inflammation and autoimmune pathology, indicating that loss of CD11b function is deleterious in inflammatory autoimmune disease settings. SLE is a notoriously heterogeneous disease, and, with the possible exception of defects in C1q (54), requires the accumulation of multiple genetic defects combined with environmental influences for disease to develop. To this effect, and supported by our analysis of CD11b-deficient mice, SNPs in the ITGAM locus that lead to loss of function of CD11b, while
conferring elevated susceptibility to development of autoimmune disease, are unlikely to drive disease alone in humans.

One of the major phenotypes of Lyn^{-/-} mice is severe B cell lymphopenia, a trait that is intrinsically linked to Lyn-deficiency, accompanied by expansion of plasma cells (85, 88). Interestingly, while these phenotypes persisted in Lyn^{-/-}/CD11b^{-/-} mice, a mild recovery in the splenic B cell compartment was observed. This coincided with plasmacytosis and a significant increase in autoantibody production, suggesting that CD11b-deficiency may be selectively rescuing autoreactive B cells in Lyn^{-/-} mice and promoting autoantibody responses. Interestingly, a modest expansion in plasma cell numbers coincided with a very mild yet significant increase in autoantibody production in aged CD11b^{-/-} mice. This is not surprising given that CD11b is implicated in moderating autoreactive BCR responses, a protective mechanism utilizing the Lyn-CD22-SHP-1 axis (62). Whilst loss of either CD11b or Lyn alone would impair this axis, our data suggests that compound deficiency of both Lyn and CD11b has altogether ablated this regulatory response, driving enhanced class-switched autoantibody production and retention of autoreactive B cells. Similarly, activated T cells have been shown to upregulate expression of CD11b which has a regulatory role in restricting proliferation (442). While T cells do not express Lyn, systemic inflammation promotes an age-associated hyper-activated phenotype which is essential for autoantibody isotype switching in Lyn^{-/-} mice (183). It is possible that in combination with the elevated inflammation, the loss of this CD11b mediated regulation in Lyn^{-/-}/CD11b^{-/-} mice drives CD4+ T cell expansion and further exacerbation of T cell hyperactivation.

Examination of the kidneys from Lyn^{-/-}/CD11b^{-/-} mice revealed enhanced and more rapid onset of glomerulonephritis and immune cell infiltration compared to Lyn^{-/-} mice. Interestingly, while the majority of the infiltrating CD45+ leukocytes were found in the glomerulus, in chronically inflamed kidneys from Lyn^{-/-} and Lyn^{-/-}/CD11b^{-/-} mice, macrophages were found to be localized to the space surrounding the glomeruli. A study investigating induced glomerulonephritis in CD11b-deficient mice ruled out a contribution for macrophages in generating kidney disease, as macrophage depletion in the induced disease model did not rescue these mice from kidney pathology (436). In our model, macrophages did not infiltrate into the glomeruli, suggesting they may not directly contribute to glomerular damage; but similar to observations in a CD11b-deficient model of tubulointerstitial nephritis (443), in
kidneys from Lyn−/−CD11b−/− mice, macrophages localized to the peri-glomerular space which correlated with enhanced kidney pathology, suggesting that these cells may play a supportive role. Neutrophils are notoriously difficult to stain for histologically; and while we have not been able to specifically stain for neutrophils in this study, we predict that the enhanced CD45 glomerular staining in Lyn−/−CD11b−/− mice is most likely representative of infiltrating neutrophils. In support of this, enhanced neutrophilic infiltration is a common hallmark of CD11b-deficient disease models (367, 436, 444), however, the mechanism supporting accumulation of neutrophils at sites of disease in these models is still not well understood.

CD11b deficiency can promote neutrophil extravasation and influx to sites of inflammation and damage (436, 445) which may account for the increased CD45 immunostaining observed in Lyn−/−CD11b−/− glomeruli. Our study suggests distinct roles for macrophages and neutrophils in driving kidney damage in Lyn−/−CD11b−/− mice. Macrophages, which are excluded from the glomerulus, are likely to be promoting inflammation and cellular infiltration by dysregulated overproduction of cytokines and chemokines; which in turn attracts and activates neutrophils in the glomeruli, where they can elicit their pathogenic functions.

Surprisingly, while we observed expanded splenic macrophages in Lyn−/−CD11b−/− mice compared to Lyn−/− mice, neutrophils numbers in spleen were increased to a similar extent. Whilst the further dysregulated inflammatory environment in Lyn−/−CD11b−/− mice would likely promote myelopoiesis and myeloid cell survival, CD11b also plays a dichotomous role in neutrophil survival. Adhesion-mediated CD11b ligation promotes neutrophil survival (446); the loss of these signals may increase neutrophil turnover, stabilizing peripheral cell numbers in Lyn−/−CD11b−/− mice. Conversely, extravasated CD11b-deficient neutrophils are resistant to apoptosis (367), promoting their accumulation at sites of inflammation. Further studies on neutrophils from Lyn−/−CD11b−/− mice will help to answer these remaining questions.

A previous study examined the role of leukocyte integrins on pathology and survival in the naturally occurring lupus-prone mouse model, MRL/MpJ-FasIpr (447). Contrary to the exacerbation of kidney pathology and impaired survival we observed in Lyn−/−CD11b−/− mice, deficiency of CD11b in MRL/MpJ-FasIpr mice conferred no pathological or clinical differences (447). Our studies have shown that CD11b-deficiency is in itself unlikely to confer significant autoimmune susceptibility but can work synergistically with other autoimmune susceptibility mutations to exacerbate disease. Given that the progression of disease and survival in
MRL/MpJ-Fas\textsuperscript{lpr} mice already progress quite rapidly (447) when compared to Lyn\textsuperscript{−/−} mice, it is possible that concurrent CD11b-deficiency in this model may not further contribute to autoimmune pathogenesis and mortality. Furthermore, CD11b-mediated regulation of macrophage inflammation and autoreactive B cell receptor responses are both reliant on Src family kinases, primarily Lyn, to promote intracellular signals (61-63). Therefore it is likely that the “double-hit” concomitant loss of CD11b and Lyn to these immunoregulatory processes is promoting inflammation and autoimmune pathology in our model. This suggests that CD11b-deficiency and Lyn-deficiency are, at least in part, working synergistically to drive autoimmunity, while the Fas\textsuperscript{lpr} mutation is acting independently of CD11b-deficiency.

However, given the more rapid onset of disease in the Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} mice, CD11b-deficiency may have additional Lyn-independent inflammation-promoting effects. This highlights the importance of interactions between specific disease susceptibility genes being more pathogenic than the mere accumulation of genetic mutations in human SLE. While GWAS have shown that Lyn has a minor association with SLE (94), it is likely that the impact of other susceptibility genes influences Lyn activity, as Lyn is central to many immunoregulatory pathways (87). The regulatory pathway involving CD11b and Src family kinases in macrophages has been well characterized (61) and preliminary studies have demonstrated that therapeutic ligation of CD11b on macrophages using agonistic leukadherin LA1, restricts the activation of the NF-κB (nuclear factor kappa light chain enhancer of B cells) and IRF (interferon regulatory factor) pro-inflammatory cytokine synthesis pathways, rescuing lupus prone mice from disease (63). This highlights a relatively novel therapeutic avenue for treatments in SLE.

In summary, in this study we have determined that CD11b deficiency can act pathogenically and promote inflammatory autoimmune disease in a susceptible genetic background. While CD11b has both pro-inflammatory and regulatory functions, in the context of the Lyn\textsuperscript{−/−} mouse model of lupus, CD11b expression was modulated on B cells and myeloid cells, which likely influences their function. Compound Lyn\textsuperscript{−/−}CD11b\textsuperscript{−/−} mice exhibited exacerbation of inflammatory phenotypes culminating in enhanced, early onset glomerulonephritis, indicating that CD11b is protective in Lyn\textsuperscript{−/−} mice. CD11b deficiency on a Lyn\textsuperscript{−/−} background promoted infiltration and accumulation of myeloid cells into the kidney; macrophages localizing to the peri-glomerular space and recruitment of leukocytes, most likely neutrophils,
into the glomeruli where they propagate kidney damage. This supports boosting of the CD11b regulatory axis as a novel therapeutic avenue in chronic inflammatory autoimmune disease such as SLE.
Chapter 6
Final Discussion and Concluding Remarks
Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease which largely affects women of child bearing age which can manifest as an array of immunopathologies, with renal disease being the biggest contributor to morbidity and mortality (3). While standard treatments for SLE are generally efficacious, the reliance on broad and potent immunosuppressive and anti-inflammatory medications can result in many off-target effects and can be toxic at higher doses, with some patients unable to be managed effectively due to these limitations. This highlights the need for novel targeted therapeutics which can safely control disease, especially in patients who fail to respond to the current standard therapy.

One of the obstacles to this is the lack of understanding and appreciation of the diversity and intricacy of the pathogenic mechanisms involved in SLE. Given that SLE is a multifactorial, heterogeneous disease with numerous disease processes and clinical manifestations, it is unlikely that a single targeted therapeutic will be efficacious in all patients, indicating the need to correlate therapeutic efficacy with disease patterns to provide the best therapeutic solution for the individual patient. This partially accounts for the lack of success in novel therapeutic trials for SLE; the majority of which have historically focussed on targeting and depleting B cells. While B cells are important pathological mediators of disease, data from human patients, which are supported by numerous studies conducted in animal models have concluded that systemic inflammation and its influence on myeloid cell activation are also essential key players in SLE pathogenesis (178). These studies also highlight inflammatory processes and mediators as viable therapeutic targets to improve disease outcomes in SLE.

The studies conducted in this thesis provide new insight into the pathogenic mechanisms of inflammation using mice deficient in the immunoregulatory tyrosine kinase, Lyn (Lyn^−/−), which exhibit widespread hyperactivation of the immune system and systemic inflammation as well as B cell mediated autoimmune kidney pathology, making it a robust experimental model of human SLE (87). These studies assessed the impact of inflammation in pathogenic processes as well as the effect of modulating inflammation on disease outcomes in these mice. Specifically, this thesis has examined the effect of inhibiting the pro-inflammatory cytokine IL-6 therapeutically using a monoclonal antibody, assessed the impact of the gut microbiome and by inference, short chain fatty acid metabolites by feeding of a high fibre diet and investigated the dichotomous role of integrin CD11b on inflammatory processes and disease outcomes in lupus-like disease. Collectively, these studies demonstrate proof of principle and
support the targeting of inflammation, either therapeutically or metabolically, for treatment of SLE.

**Modulating inflammation in lupus**

Myeloid cells are key drivers of inflammatory responses and are implicated in perpetuating aberrant systemic inflammation in autoimmune disease. In lupus, myeloid cells are hyperactivated and over-produce pro-inflammatory cytokines and growth factors which promote leukocyte activation and self-sustaining myeloid cell expansion, as well as infiltrating into the kidney to promote local inflammation and tissue destruction (Figure 1.1) (178, 181). Similarly, hyperactivated CD4+ T cells have been implicated in driving autoantibody responses in Lyn−/− mice (88, 183, 198). A number of studies have shown that genetic modulation of inflammation, which moderates the expansion of systemic myeloid cell populations and restores normal expression of T cell activation markers, correlates with significant improvements in renal disease outcomes in Lyn−/− mice (88, 183, 202). Our studies highlight that improvements in kidney pathology can be potentiated by therapeutic interventions that largely moderate inflammatory phenotypes, especially myeloid cell expansion and activation and T cell hyperactivation; although the impact of this can be variable and the underlying mechanisms have not been fully discerned.

Significant changes to the B cell compartment and subsequent autoantibody production are not required to improve kidney pathology in Lyn−/− mice, further dissociating B cell defects from autoimmune pathology (88, 190), and this is supported by findings in Chapter 4. It would be expected that autoantibody production would be significantly dampened when T cell activation is suppressed, yet our findings in Chapter 4 indicate that even partial T cell activation is sufficient to drive autoantibody production and suppression of this response can occur independently of suppression of T cell hyperactivation. One could argue that, in contrast to genetic deletion of an inflammatory mediator which generates an ‘inflammation naïve’ system from conception, therapeutic modulation of T cell hyperactivation may be unachievable if treatment is commenced from disease onset and T cells are already primed for activation. This may underpin the persistence of autoantibody responses in Lyn−/− mice that received anti-IL-6 mAb treatment or switched to a high fibre diet from when disease has been established. The suppression of pathogenic autoantibodies during early-mid stages of
treatment in these studies may be due to transient moderation of T cell hyperactivation however this was unable to be assessed in these longitudinal study. The resurgence of autoantibodies at the endpoint suggests that some disease mechanisms have become refractory to treatment. This hypothesis, however, doesn’t account for the persistence of autoantibodies in mice that were weaned onto a high fibre diet from a young age and achieved suppression of T cell hyperactivation, counteracting the dogma that activated T cells are central to pathogenic autoantibody responses and suggests that other influences may also be involved, such as hyperactivated myeloid cells.

The studies in this thesis have implicated myeloid cells as key drivers of inflammation and pathology in Lyn−/− mice. Altered expression of the leukocyte integrin CD11b on neutrophils, macrophages and also B cells in Lyn−/− mice has been identified for the first time, and in previous studies by other groups, increased CD11b expression has been shown to be a marker of activation (437, 438). The implications of this, which have been previously discussed, suggest that the increased expression on neutrophils promotes their pathogenic functions, including enhanced survival and infiltration into sites of inflammation, while the reduced expression on macrophages impairs their immune complex clearing capabilities and regulation of inflammatory responses. The altered expression of CD11b was evident in young mice and preceded the onset of disease, indicating that this may be a Lyn-intrinsic defect. Interestingly, acute therapeutic modulation of inflammation, in the instance of anti-IL-6 mAb treatment and high fibre diet feeding from disease onset, demonstrated partial resolution of CD11b overexpression on neutrophils, indicating that this is in fact likely influenced by the inflammatory environment. Conversely, macrophages were refractory to changes in CD11b expression in response to genetic and therapeutic modulation of inflammation. The discrepancy between the responses of these two cell types may lie in their localisation; neutrophils which reside in the bone marrow and circulation can be directly and acutely influenced by systemic factors, yet macrophages, which originate from the bone marrow then migrate to peripheral tissues may be primed by early inflammatory signals but then are less exposed to changes from the systemic environment. Nevertheless, our studies indicate that expression of CD11b can act as marker of myeloid cell activation and suggest that CD11b may directly regulate their pathogenic capacity. The localisation of myeloid cells within the inflamed kidney also prompted consideration of differing roles for macrophages and
neutrophils in autoimmune glomerulonephritis; peri-glomerular macrophages may be perpetuating local inflammation via uncontrolled cytokine and chemokine production, promoting infiltration and activation of neutrophils into the glomeruli to exert tissue damaging effector functions. Further studies exploring the activation and dysregulated effector functions of myeloid cells in Lyn\(^{-/-}\) mice would be of benefit to fully delineate their role in autoimmune kidney pathology.

**Sex bias and environmental influences in Lyn\(^{-/-}\) mice**

This thesis also uncovered environmental influences and sex biases which are well known contributors to autoimmune disease, but thus far have not been evaluated extensively in the Lyn\(^{-/-}\) mouse model. The incidence and burden of autoimmune disease disproportionally affects women, with 90% of SLE cases occurring in females. This has prompted investigation into the contribution of sex hormones on the development of autoimmune disease which has implications for puberty, pregnancy, menopause and even transsexuality (448, 449). It has been noted that the magnitude of immune responses are greater in females (450), and indeed, traits of systemic inflammation such as splenic erythrocytosis are enhanced in female mice compared to males, under the control of oestrogen (451, 452). While it has been observed that Lyn activity can influence erythropoiesis (451, 453), female Lyn\(^{-/-}\) mice often present with further elevated erythrocytosis compared to their male counterparts (unpublished data). Furthermore, female Lyn\(^{-/-}\) mice had significantly impaired survival compared to male mice, which suggests that autoimmune pathology is also biased toward female Lyn\(^{-/-}\) mice, which interestingly was normalised by concomitant deficiency of CD11b. Therefore it would be prudent to investigate the immunological and pathological differences influenced by sex in Lyn\(^{-/-}\) mice to improve relevance of our mouse studies to human disease.

On a similar note, environmental factors can heavily influence autoimmune disease induction and severity. While Lyn-deficient mice on the BALB/c and C57BL/6 genetic backgrounds have not been studied alongside each other, it has been reported that, within the same housing facility (Ludwig Institute of Cancer Research, LICR), C57BL/6 background Lyn\(^{-/-}\) mice were found to have accelerated autoimmune pathology and impaired survival compared to those on the BALB/c background (87). Since housing mice in the Monash ICU facility, it has been observed that pathology in the C57BL/6 background Lyn\(^{-/-}\) mice develops much later than
previously reported (significant glomerulonephritis at 36-42 wks vs. 26-30 wks in previous studies) (85, 88, 202). Indeed, the survival of C57BL/6 Lyn−/− mice housed at M.ICU (414.5 days, 373-528 days) (Figure 5.3.) was improved over that of BALB/c Lyn−/− mice housed at LICR, which our lab previously reported to be 360.5 days (176). While survival of BALB/c Lyn−/− mice housed at M.ICU has not been formally assessed, these mice are very robust and are likely to have a far better survival to that originally reported. This indicates that animal housing facility environments can have a drastic impact on disease development in autoimmune models, but also signifies that environmental influences of disease can be assessed in Lyn−/− mice. Indeed previous studies have shown the Lyn−/− mice lacking environmental pathogen recognition messages through the deletion of TLR signal mediator MyD88 (Lyn−/−MyD88−/−), are rescued from disease (182).

Study Limitations
There is much criticism of studies which utilise mouse models of disease as they often do not faithfully recapitulate human disease and the findings may not translate to human settings. Indeed, SLE is an incredibly complex, multifactorial, heterogenic disease which has many clinical presentations and therefore it would be impossible for a single mutant mouse strain to recapitulate the complexity of this disease. Through the utilisation of the Lyn−/− mouse model, we are able to investigate both the systemic inflammatory and the autoimmune pathology arms of lupus. Not only is the pathology of lupus well recapitulated in Lyn−/− mice, Lyn-deficiency itself is both physiologically and genetically relevant to human disease, making it a robust pre-clinical model of SLE and more specifically Lupus Nephritis. That being said, testing therapeutics in multiple different pre-clinical models which exhibit similar disease under the influence of differing genetic mutations and different pathogenesis may have superior indication of efficacy in human settings. As has been discussed previously, environmental influences have a significant impact on immune responses and disease. Animal models that are housed in artificial pathogen-free environments lack the immunological maturity and memory that is present in animals and humans in the natural environment. Recent reports have shown that ‘microbial experience’ in laboratory mice which could be achieved through transient infections or shifts in the gut microbiome from co-housing with ‘dirty’ mice, can promote a more realistic activation of the immune system, with the implication that utilisation of these ‘humanised’ models will better recapitulate human
autoimmune disease and responses to therapeutics (454, 455). It is important to acknowledge that this has yet to be assessed in disease models and that manipulation of the gut microbiome by co-housing may not be suitable for diet and microbiota studies.

Future Directions
These studies, among others, indicate that the therapeutic targeting of inflammation may be a promising alternative approach in SLE disease management (178). While pan-IL-6 inhibiting therapies have shown efficacy in Lyn−/− mice and other models of lupus as well as in clinical trials of human lupus, broad inhibition of IL-6 can lead to immunosuppression and neutropenia which increases the susceptibility of infection (353, 357). Given the pleiotropic nature of IL-6, with evidence suggesting that anti-inflammatory or infection resolving effects of IL-6 are mediated by the classical signalling pathway, whilst pro-inflammatory and disease propagating effects are mediated via the trans-signalling pathway (Figure 1.5) (328), selective blockade of the IL-6 trans-signalling pathway may be a superior therapeutic approach than pan-IL-6 inhibition. A fusion protein, sgp130Fc which inhibits circulating IL-6/IL-6R complexes has demonstrated improvements to kidney pathology via moderation of kidney complement deposition when introduced genetically to Lyn−/− mice (Lyn−/−sgp130FcTg) (202) and has shown therapeutic efficacy in animal models of RA (359, 360), atherosclerosis (361), Crohn’s disease (329), Emphysema (456) and Nephrotoxic nephritis (457). Safety and efficacy of sgp130Fc (trade name Olamkicept) has been shown in humans as it has successfully completed phase I clinical trials and phase II trials are currently underway for inflammatory bowel disease (458), therefore this may be a promising therapeutic avenue for SLE.

The pathogenesis of SLE is dependent on both dysregulation and loss of tolerance in B cells in conjunction with a systemic inflammatory environment. The lack of efficacy of targeted therapies in SLE may lie in that only one of these pathogenic arms is targeted by a specific agent. As in the successful early IL-6 blocking studies in NZBxNZW F1 mice, which incidentally recapitulated “inflammation naïve” CD4 T cells via initial depletion by a tolerising dose of anti-CD4 mAb; combination therapy targeting both aberrantly activated lymphocytes and inflammation may be a superior therapeutic approach for treating SLE. More recent studies have shown greater therapeutic efficacy in NZBxNZW F1 mice treated in combination with a B cell depleting agent (anti-CD20 mAb) and inhibition of inflammatory B cell activation and
survival (anti-BAFF mAb) (459), illustrating that compound therapy is likely to be a superior therapeutic approach. As SLE patients would require long term treatment, continued dual therapy would likely lead to severe immunosuppression and increase susceptibility to infection. Therefore combination therapy could be conducted in phases; the first of which depletes autoreactive lymphocytes and suppresses inflammation, while continued treatment would focus on suppressing inflammation, allowing lymphocytes to regenerate in a suppressed inflammatory environment.

The findings of Chapter 4 revealed a profound impact of dietary fibre on moderating systemic inflammatory phenotypes and protecting against development of severe glomerulonephritis in mice. As previously detailed, dietary fibre can be fermented by the gut microbiome to form SCFAs which can act systemically to dampen inflammatory responses (132). While the extensive gut microbiome composition analyses undertaken demonstrated that a high fibre diet led to the expansion of SCFA-forming bacteria, a direct analysis of these metabolites has yet to be conducted. Systemic metabolites in the serum can be measured using nuclear magnetic resonance spectroscopy and detailed metabolite profiles from Lyn−/− mice reared on a high fibre or control diet could be compared to determine protective and disease related metabolite patterns. Long term studies should determine the mechanism by which SCFA and other potentially protective metabolites elicit their effects on the immune system, particularly in myeloid cells and on inflammatory processes. While it is known that SCFAs can signal through cell surface G-protein coupled receptors (FFAR2, FFAR3), currently it is not completely clear which signalling pathways, transcription factors and intracellular processes are modulated downstream of FFAR ligation, and how these contribute to disease. High fibre meal plan or supplement studies could be conducted in SLE patients to determine whether the findings of the experimental diet studies in this thesis translate in a human setting. It is important to consider that translation of diet studies in humans can prove difficult due to variability in human gut microbial compositions, the specific dietary requirements of individuals and lack of compliance to limited or restricted dietary options. To overcome this, preliminary studies could profile the gut bacterial communities from stool samples and systemic metabolite patterns from serum samples in SLE patients and compare to healthy controls to discern patterns which correlate with disease state, similar to the studies proposed in Lyn−/− mice.
The pathogenic mechanisms of myeloid cells in glomerulonephritis have been briefly explored in this thesis. The studies in Chapter 5 have implicated the leukocyte integrin CD11b in controlling leukocyte migration into the kidney, while a previous study has shown that CD11a promotes autoimmune disease and myeloid cell infiltration into the kidney of lupus-prone MRL<sup>lpr/lpr</sup> mice (447), indicating non-redundant functions of integrins in leukocyte migration and lupus nephritis. It has been speculated that CD11b-deficiency promotes neutrophil extravasation and infiltration into the inflamed kidney where they mediate tissue pathology through enhanced effector functions, likely under the influence of dysregulated inflammatory signals from macrophages in Lyn<sup>−/−</sup> mice. Although the detection of neutrophils in processed, <i>ex vivo</i> tissue is notoriously difficult, visualisation of neutrophil trafficking can be achieved <i>in situ</i> using intravital microscopy in transparent surface tissues such as the cremaster muscle, or even in the glomeruli using multiphoton microscopy (460). On a similar note, while Lyn-deficient neutrophils are shown to be hyperactive and exhibit enhanced adhesion-dependent integrin functions, the pathogenic mechanisms of neutrophils in glomerulonephritis is still undetermined in Lyn<sup>−/−</sup> mice. A recent study has demonstrated that neutrophil extracellular traps (NETs) are detectable in inflamed glomeruli using multiphoton microscopy (461), revealing a potential pathogenic function of neutrophils in lupus nephritis. Utilisation of this imaging technology to observe neutrophils and leukocyte trafficking and infiltration into the kidney and detection of NETs in the glomeruli in both Lyn<sup>−/−</sup> and Lyn<sup>−/−</sup>CD11b<sup>−/−</sup> mouse models will provide valuable insight into the migratory behaviour of these cells as well as the influence of CD11b in these pathogenic processes.

**Final Conclusion**

Standard therapy for SLE has remained relatively unchanged over the past 50 years, and novel targeted therapies directed against the B cell compartment have, by and large, failed to achieve clinical efficacy. Collectively the studies within this thesis have provided compelling evidence for the modulation of inflammation as an effective therapeutic strategy for controlling disease manifestations in lupus. These studies have identified an underappreciated contribution of myeloid cells as both perpetuators of inflammation and effectors of tissue damage and the balance of dietary metabolites and the gut microbiome as potential alleviators of disease in Lyn<sup>−/−</sup> mice. The implications of these studies will help shape novel therapeutic strategies to effectively control disease in human SLE.


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