

The New Pathology: Dissecting Cellular Responses in Lupus Nephritis



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Abstract

SLE is a prototypic chronic autoimmune disease characterised by the production of wide spectrum of autoantibodies to ubiquitous antigens such as anti-dsDNA, targeting multiple organs. Lupus Nephritis (LN) is serious complication of SLE impacting ~50% of people with SLE and characterised by renal immune-complex (IC) deposition. Current methods of classifying LN are reliant on histological and clinical evidence, and do not reliably predict disease progression, while understanding of the cellular mechanisms of pathology is incomplete.

Mouse lupus models share clinical features of human disease including renal involvement and represent a way to manipulate and study early active disease on a homogenous background. Using two murine models of LN this thesis aims to understand identify disease drivers and potential therapeutic targets, and to establish an approach applicable to human kidney in the future.

To establish a platform to characterise LN in vivo, I phenotypically characterised two murine models of early LN, an inducible model using the TLR7 agonist imiquimod, and MRL/lpr, deficient in apoptotic mediator *Fas*. Immune phenotyping of both models shows systemic disease consistent with SLE. IMQ kidneys showed IC deposition, mesangial expansion, and endothelial proliferation consistent with class-II LN and MRL/lpr mice additionally developed crescents, and disease consistent with class-III LN.

Single cell transcriptomic data from the kidneys of imiquimod treated mice identified alterations in mesangial cells suggestive of de-differentiation and adoption of a fibroblast-like phenotype, associated with downregulation of *Itga8*. In the kidneys of MRL/lpr mice, early S1/S2 proximal tubule segments were identified as interferon (IFN) susceptible targets. Integrated analysis across the models identified enriched intrarenal, T, B, and myeloid populations in LN. MRL/lpr mice had the largest immune infiltrate, including NK

and CD8 T cells expressing markers of cytotoxicity, exhaustion, and degranulation. Shared innate immune renal phenotypes across models included increased CD11b^{hi} resident macrophage populations, expressing genes implicated in phagocytosis and efferocytosis, with trajectory analysis suggesting these macrophages may arise from increased patrolling non-classical monocytes. Altered pan immune signatures included differentially enhanced expression of homing receptor *Itga4*, which was validated at the protein level in the kidneys of MRL/lpr mice, identifying it as a potential target for *in vivo* assessment.

Taking forward transcriptomically identified targets, blockade of *Itga4* in MRL/lpr mice did not have any impact on disease progression but did reduce proportions of non-classical monocyte infiltration in the kidney. To test the role of ITGA8 in mesangial cells, *ITGA8* was knocked down in vitro, resulting in a more fibroblast like phenotype, akin to that seen in the kidneys of imiquimod treated mice. Restoration of expression in knockdown cell lines rescued the phenotype, confirming a gene specific effect and suggesting a functional role for this ITGA8 in mesangial fibrosis, suggesting both a potential therapeutic target and an in vitro model for future screens.

Integrated kidney snRNA-seq in two LN models delineates individual renal cell signatures and a shared immune signature, which may be important in early active disease. Our comprehensive kidney single nuclei approach is generalisable and highlights candidate genes for functional validation. A similar integrated approach will be directly applicable to human pathology

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Acronyms and Abbreviations

ABCs	Age Associated B Cells
ACR	American College of Rheumatology
ALPS	Autoimmune Lymphoproliferative Syndrome
ANOVA	Analysis of variance
APRIL	A Proliferation Inducing Ligand
ATAC-Seq	Assay for Transposase-Accessible Chromatin Sequencing
BAFF	B cell activating factor
Bcl6	B Cell Lymphoma 6
cDCs	Conventional DCs
CKD	Chronic kidney disease
CRP	C-Reactive Protein
CSF-1R	Colony Stimulating Factor 1 Receptor
CTLA4	T-Lymphocyte Associated Protein 4
CTLs	Cytotoxic T Lymphocytes
CXCL	Chemokine Ligand
CYC	Cyclophosphamide
DAMPs	Damage Associated Molecular Patterns
DC-LAMP	DC Lysosome Associated Membrane Glycoprotein
DN	Double Negative

eGFR	estimated glomerular filtration rate
ER	Oestrogen receptors
ESRD	End stage renal disease
FcRn	Neonate FC receptor
GATA	GATA binding protein
GBD	Global Burden of Disease
GBM	Glomerular basement membrane
GCs	Germinal centres
GECs	Glomerular endothelial Cells
GN	Glomerulonephritis
GWAS	Genome Wide Association Studies
HA	Hyaluronic acid
HLA	Human leukocyte antigen
HMGB1	High mobility group box protein 1
HRQOL	Health related quality of life metrics
HSPG	Heparan sulfate proteoglycans
IC	Immune complex
IFN	Interferon
IL	Interleukin

ISN/RPS	International Society of Nephrology and Renal Pathology Society
JSLE	Juvenile onset SLE
KLRK1	Killer Cell Lectin Like Receptor 1
KRMs	Kidney Resident Macrophages
LAMP1	Lysosomal associated membrane protein 1
LN	Lupus nephritis
LPAM1	Lymphocyte Peyers Patch Adhesion Molecule 1
Ly6c1	Lymphocyte Antigen 6 Family Member C1
M-CSF	Macrophage Colony Stimulating Factor
MC	Mesangial cell
MCP	Monocyte chemoattractant protein-1
MFT	Myofibroblast transdifferentiation
MHC	Major histocompatibility complex
MMF	Mycophenolate Mofetil
MMP	Matrix metalloproteinases
mTOR	Mammalian Target of Rapamycin
NETs	Neutrophil extracellular traps
NFAT	Nuclear factors of activated t cell
NHS	National Health Service

NK	Natural killer
OTUD1	Ovarian Tumor Protease Deubiquitinase 1
PBMC	Peripheral blood mononuclear cell
pDCs	Plasmacytoid Dendritic Cells
PDGFR	platelet derived growth factor receptor
PEC	Parietal epithelial cells
PML	Progressive Multifocal Leukoencephalopathy
PRR	Pattern recognition receptor
PTX3	Pentraxin 3
QOL	Quality of Life
RA	Rheumatoid Arthritis
RAAS	Renin angiotensin aldosterone system
RCTD	Robust Decomposition of Cell Type Mixtures
RORYt	RAR-Related Orphan Receptor Gamma T
ROS	Reactive oxygen species
RRT	Renal replacement therapies
RTECs	Renal Tubular Epithelial Cells
SAMHD1	Sterile Alpha Motif and HD Domain Containing Protein 1
SAP	Serum Amyloid P Component
scRNA-seq	Single-cell RNA sequencing

SDC1	Syndecan 1
SIGLEC	Sialic Acid-Binding LG-Like Lectin 1
SLAMF	Signalling Lymphocytic Activation Molecule Family Member
SLE	Systemic lupus erythematosus
snRNA-seq	Single-nuclei RNA sequencing
STAT1	Signal Transducer and Activator of Transcription 1
STING	Stimulator of Interferon Genes
Syk	Spleen Tyrosine Kinase
TCR	T Cell Receptor
Tfh	T Follicular Helper Cells
TGFB	Transforming Growth Factor B
TLR	Toll Like Receptor
TNF-a	Tumour necrosis factor a
Tregs	Regulatory T cells
TRMs	Tissue resident macrophage
UKRR	UK Renal Registry
VEGF	Vascular endothelial growth factor
VLA4	Very Late Antigen 4
XCI	X Chromosome Inactivation
ZAP70	Zeta Chain Associated Protein Kinase 70

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Chapter 1 : Introduction

1.1 Overview: Burden of renal disease

Renal disease is estimated to affect >10% of the population worldwide accounting for 5-10 million deaths annually(7, 8). The Global Burden of Disease, Injuries and Risk Factors Study (GBD) ranked kidney disease as the 12th leading cause of death worldwide in 2017, predicted to become the 5th leading cause by 2040(9, 10). Outcomes of kidney disease such as end stage renal disease (ESRD), are managed with expensive renal replacement therapies (RRT), either dialysis or transplantation(11). In the UK alone between 2009-2010 the estimated spending associated with chronic kidney disease (CKD) was £1.5 billion pounds, accounting for 1.3% of the National Health Service (NHS) budget(12, 13). The costs of RRT remain high making these therapies unaffordable to many, especially in lower- and middle-income countries, resulting in the death of >1 million people annually from untreated renal failure(14).

Early intervention coupled with more targeted therapies is key to lessening this burden, but both are reliant on accurate and clinically relevant diagnostics. Renal pathology forms the cornerstone for the diagnosis and treatment of kidney disease in current clinical practice(14, 15). This depends on empirically derived evidence obtained from tissue and clinical parameters, often without a robust aetiologic basis, limiting its prognostic and therapeutic value. This lack of mechanistic understanding represents a major limitation for the advancement of disease classification and the development of new treatments for renal disease(15).

This thesis focuses on renal involvement in systemic lupus erythematosus (SLE), known as lupus nephritis (LN), for which better understanding of cellular pathology is needed. This is exemplified by the current disease classification which does not reliably predict disease outcomes in LN patients, with current treatments focusing on broad spectrum systemic

immune suppression and associated with unfavourable side effects(6, 16). Deconvoluting dysregulated disease pathways from protective tissue responses to injury remains a major challenge, even with the advent of newer molecular technologies such as single cell/nuclei RNA sequencing (scRNA-seq/snRNA-seq)(17). With disease progression non-specific signals of fibrosis and tissue damage may dominate, making it increasingly difficult to unpick disease causative mechanisms. Animal models therefore provide an important tool to interrogate and manipulate early disease on a homogenous background, and to extrapolate findings to human disease. Therefore, this thesis aims to phenotype and characterise mouse models of LN at a cellular level, to identify and define disease associated and potentially therapeutically targetable pathways, to explore the functional basis of some of these findings using in vitro and in vivo experiments and compare these to existing human LN data.

1.2 Introduction to the kidney

1.2.1 Function of the kidney and introduction to the nephron

The kidneys perform essential homeostatic functions within the body, including acid base regulation, salt balancing, filtering the blood, bone metabolism, regulation of blood pressure and erythropoiesis(1). The roles of the kidneys are summarised in Figure 1.1.

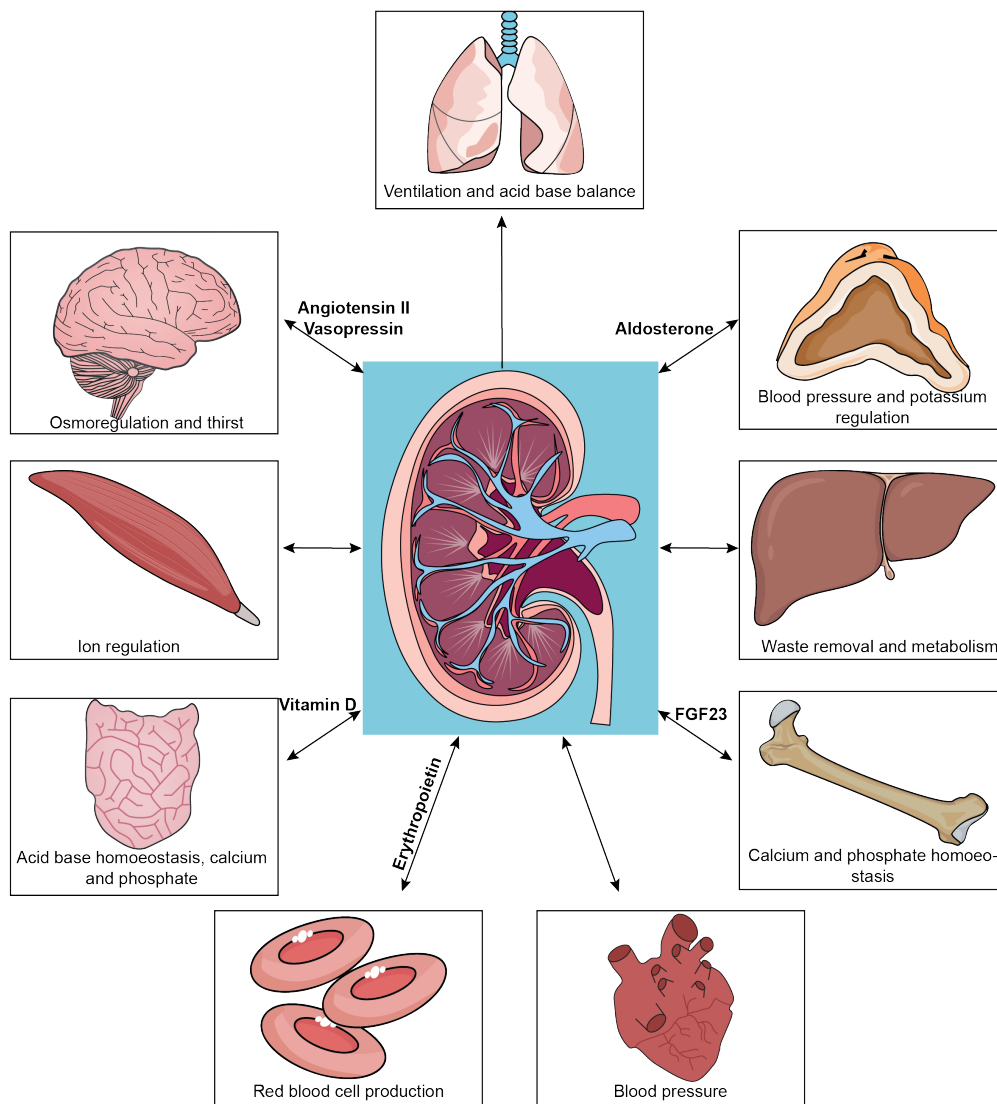


Figure 1.1: Homoeostatic roles of the kidney.

Role of the kidney for maintaining body function. Adapted from(1).

The functional unit of the kidney is the nephron (Figure 1.2A), each human kidney contains between 800,000 to 1,000,000 nephrons, which do not regenerate, therefore the numbers gradually decline with age, disease, or renal injury(18). The nephron is comprised of a tuft of capillaries known as the glomerulus (Figure 1.2B) which sits in the Bowmans capsule, followed by a long tubular section(18). Glomeruli filter approximately 180 litres of blood per day, which is concentrated and reabsorbed during its passage through the tubule, producing 1-2 litres of urine to be excreted(1, 18, 19).

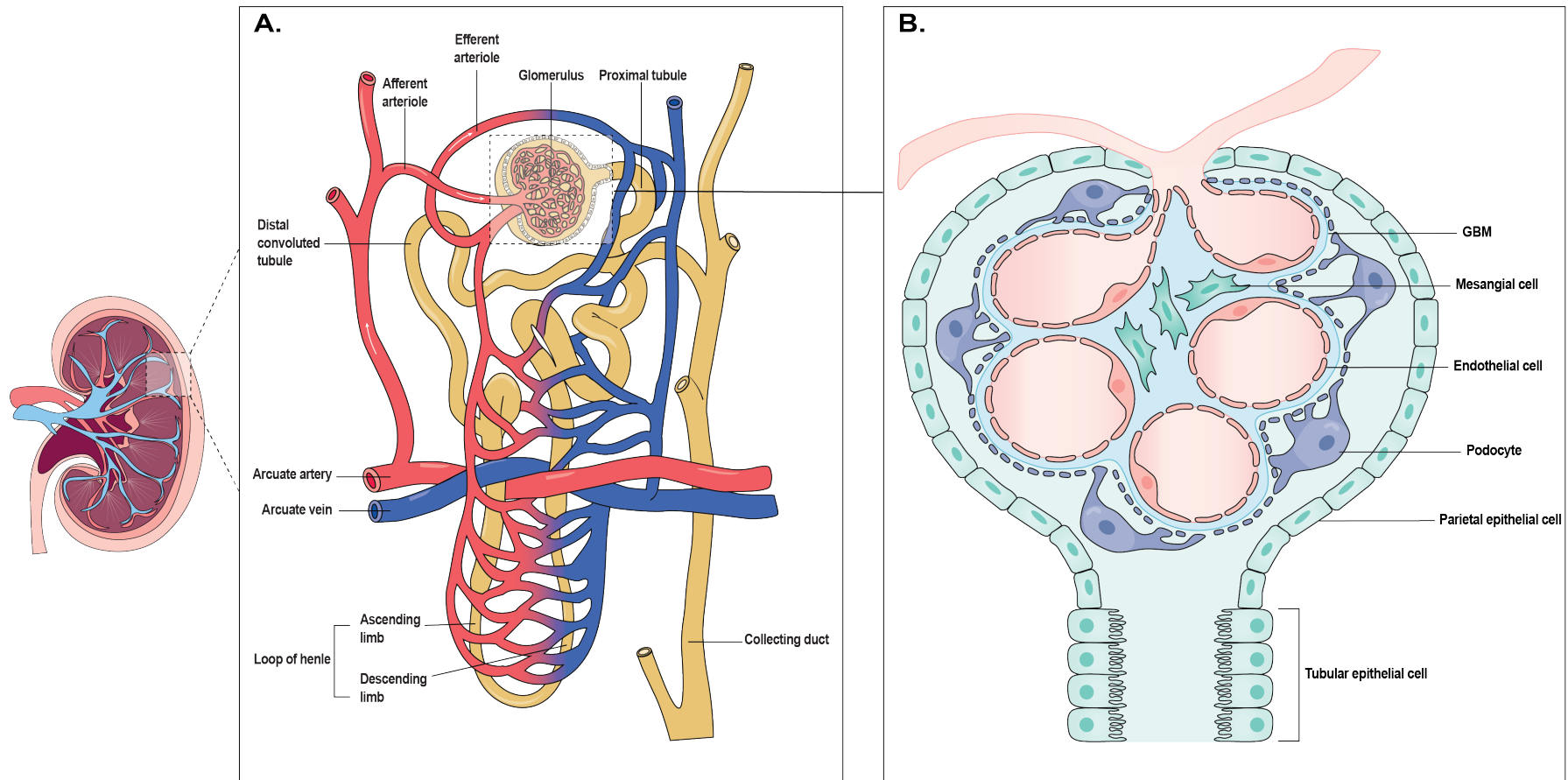


Figure 1.2: The structure of the nephron and glomerulus.

The functional unit of the kidney is the **(A)** nephron which is composed of the tubule and the **(B)** glomerulus, which sits surrounded by the Bowmans capsule. The main cell types in the glomerulus are visceral epithelial cells known as podocytes, stromal cells which sit in the middle of the tuft known as mesangial cells, endothelial cells which line the capillary lumen and parietal epithelial cells which line Bowman's capsule. Adapted from(2).

Within the renal glomerulus, the glomerular filtration barrier is the interface between the blood and urinary filtrate. It is formed of specialised fenestrated endothelial cells, an extra-cellular basement membrane and highly specialised epithelial cells called podocytes (Figure 1.3A-C)(1, 18, 20). This barrier is selectively permeable allowing passage of salts, water, and proteins with a molecular weight less than albumin (67 KDa), while red blood cells and larger plasma proteins, particularly those with a negative charge, cannot cross(1, 18). Damage to the glomerular filtration barrier affects both the glomerular filtration rate and selectivity. Thus, detection of proteins such as albumin in the urine, proteinuria, is indicative of glomerular renal disease(1, 18). After passing through the glomerulus, the filtrate enters the tubules which comprise several distinct segments with varying functions(18). Firstly, the proximal tubule reabsorbs up to two thirds of water and solutes and secretes waste and drug metabolites and toxins(18). The loop of Henle is important for generating a medullary sodium gradient to facilitate urinary concentration. Finally, the distal tubule, connecting tubule and the collecting duct are responsive to aldosterone and vasopressin and fine tune the urinary concentration and composition(1, 18).

1.2.2 The glomerulus and glomerular cells

The spherical glomerulus is comprised of a specialised tuft of capillaries, sitting in Bowman's capsule, specialised glomerular parietal epithelial cells (PECs) line the capsule basement membrane(21). Within the glomerular capillary tufts are mesangial cells, endothelial cells and podocytes, interactions between these highly specialised cells are essential to glomerular functionality(21).

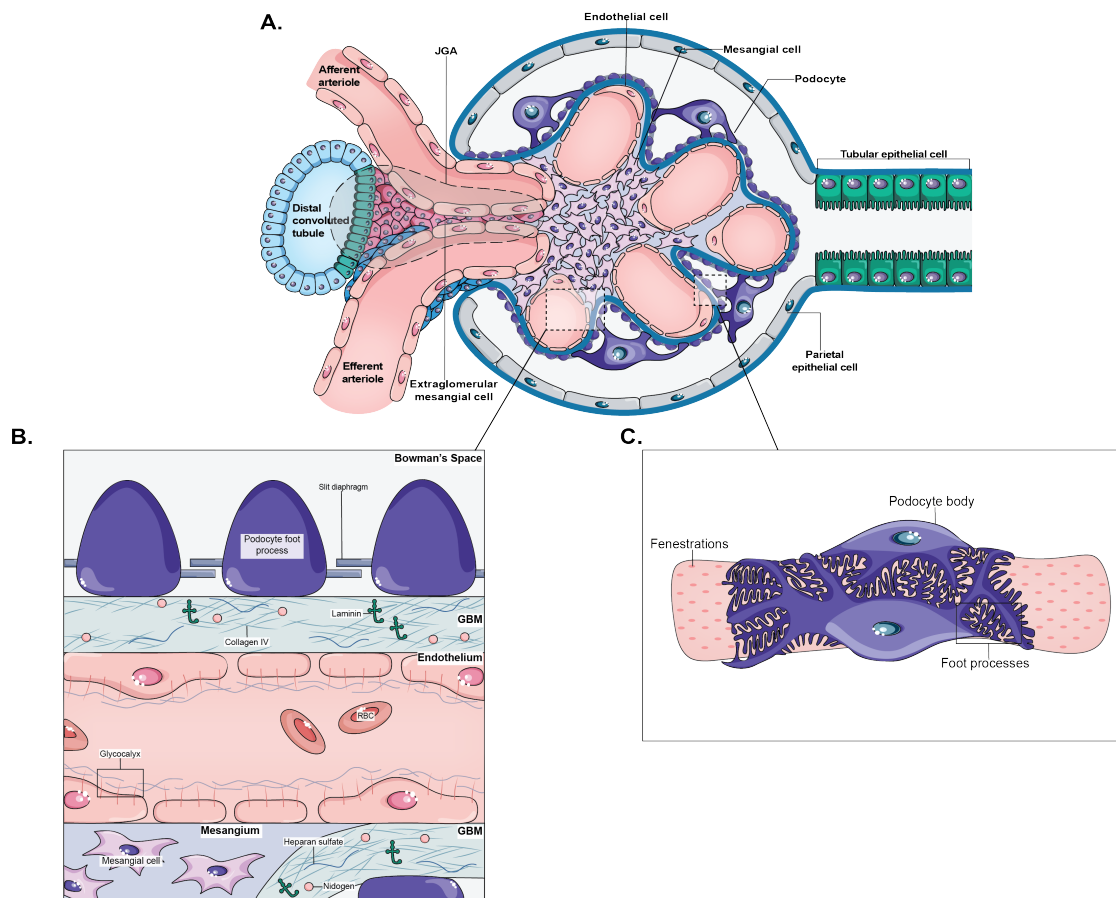


Figure 1.3: The structure of the glomerulus.

The filtration unit of the kidney is (A) the glomerulus and associated resident cells as in **Figure 1.2B**. At the interface between the blood and urinary filtrate is (B) glomerular filtration barrier, which on the luminal aspect consists of fenestrated endothelial cells which are covered in negatively charged glycocalyx. Followed by the glomerular basement membrane (GBM) which is composed of various extracellular matrix proteins such as laminins, collagens, nidogen and heparan sulfate. On the outer aspect of the GBM are podocytes which are specialised visceral epithelial cells. Podocytes are terminally differentiated cells with (C) a main body and specialised interdigitating foot processes which wrap around capillaries and are essential for the filtering functions of the glomerulus. Adapted from(2-4)

1.2.2.1 Endothelial cells

Glomerular endothelial cells (GECs) are fenestrated, and covered in glycocalyx, a negatively charged mesh-like structure composed of proteoglycans, glycoproteins, and glycolipids(1, 18, 20). The glycocalyx restricts the passage of negatively charged plasma proteins into the filtrate(20, 21). Endothelial cell signalling is required for glomerular development. For example, endothelial platelet derived growth factor beta (PDGFB), is required for the migration of mesangial cells into the capillary tufts via expression of its

receptor platelet derived growth factor receptor beta (PDGFRB)(22-24). The glomerular basement membrane (GBM) is synthesised by and separates the endothelial cells and the podocytes(25). This mesh of extracellular matrix proteins is important in permselectivity and provides structural support for the glomerular capillaries(26). The GBM is mainly composed of laminins, collagens, nidogen and heparan sulphate proteoglycans, and mutations in genes encoding GBM components result in kidney diseases, for example Alport syndrome due to defects in type IV collagens(26).

1.2.2.2 Podocytes

Covering the outer aspect of the GBM are podocytes, these are terminally differentiated visceral epithelial cells, with long interdigitated foot processes that wrap around the glomerular capillaries, forming the final layer of the glomerular filtration barrier(27, 28). Podocyte injury results in structural alterations and retraction of the foot processes known as effacement, and podocyte loss, clinically evident as proteinuria(29). Foot processes of adjacent podocytes are connected to each other by specialised junctions known as slit diaphragms, crucial for GBM size selectivity(27, 28). The slit diaphragm is composed of tight junction proteins such as Zonula Occludens-1 (ZO-1), as well as podocyte specific transmembrane proteins nephrin, nephrin-like-1 (NEPH1) and podocin(30-32). Nephrin and NEPH1 are the major components of the extracellular portion of the slit diaphragm, forming the mesh like sieve(32). The importance of slit diaphragm formation is demonstrated by the association of nephrin with congenital nephrotic syndrome, characterised by severe proteinuria(33). Podocyte foot processes are the main anchor to the GBM via a complex network of adhesion receptors such as integrins, which interact with actin cytoskeletal components enabling podocytes to adapt to and withstand the mechanical forces within the glomerulus(28, 34). Mutations in laminin binding to integrin $\alpha 3$ result in nephrotic syndrome in humans, and impaired foot process and GBM development in mice(28, 35, 36). Loss of podocytes initially results in compensation by glomerular hypertrophy; however, this

hinders the ability of podocytes to maintain slit diaphragms and results in localised exposure to increased shear stress, which ultimately contributes to further podocyte loss by detachment(37, 38). Once a critical number of podocytes are lost (approximately 40%), hypertrophic compensation is insufficient, resulting in glomerular sclerosis and scarring(38, 39).

Crosstalk between podocytes and other resident cells is required for the development and maintenance of glomerular structures. Podocyte secretion of vascular endothelial growth factor A (VEGFA) initiates endothelial cell migration and capillary loop formation and supports subsequent maintenance of the glomerular endothelium and capillaries(24, 40, 41).

1.2.2.3 Mesangial cells

Mesangial cells (MCs) are a pericyte like cell and the most abundant cell type in the glomerulus comprising 30-40% of the total cells, surrounded by a self-produced extracellular mesangial matrix. Together the MCs and their matrix form a structural stalk that holds together the capillary loops(42). MCs have contractile properties like smooth muscle cells, are in direct contact with the capillary lumen and can clear macromolecules that transverse the endothelial barrier via phagocytosis, receptor independent pinocytosis and receptor dependent uptake pathways(43-45).

Another housekeeping function of MCs is clearance of apoptotic debris, which is particularly important in preventing the further propagation of inflammatory pathways within the kidney(46). The mesangial matrix is mainly composed of type IV and V collagen, laminins, fibronectin and heparan sulfate proteoglycans (HSPGs), turnover of this matrix is tightly regulated via mesangial expression of extracellular matrix degrading enzymes such as metalloproteinases (MMPs)(47, 48). Fibronectin promotes matrix adhesion via binding to integrins (e.g., integrin $\alpha 8$) expressed by mesangial cells(47, 48).

MC dysregulation results in aberrant proliferation and mesangial matrix expansion, a hallmark of several glomerular diseases (e.g., IgA nephropathy, diabetic kidney disease) (49). Upon activation, MCs can produce growth factors, cytokines, and chemokines. MC production of transforming growth factor beta (TGF- β), a mediator of fibrosis, has been shown in vitro to increase production of matrix components and concurrently inhibit MMPs resulting in matrix expansion(50, 51). In mouse models of renal disease, MCs express proinflammatory cytokines such as tumour necrosis factor alpha (TNF- α) and interleukin 6 (IL-6) and chemokines such as monocyte chemoattractant protein-1 (MCP-1/CCL2) and chemokine ligand 1 (CXCL1), involved in recruitment of immune cells(42, 52).

1.2.3 CKD and glomerular immune complex disease in the kidney

CKD accounts for 4.6% all deaths annually, and while global mortality from most communicable and non-communicable diseases has been declining, CKD deaths are increasing(8, 10, 53). There are many causes of CKD, including diabetes, hypertension, genetic disease, urologic disease, glomerulonephritis (GN) and unknown or idiopathic causes, with the first two being the largest contributors(8, 13). As of the 25th UK Renal registry (UKRR) annual report, GN accounts for ~13.3% of ESRD cases(54). GN can be categorised based on histopathological patterns on renal biopsy, immune complex (IC) GN represents a heterogenous group of diseases, with positivity for IC in the kidney(55). Autoimmune GN diseases can be a result of autoantibodies targeting specific renal antigens (anti-GBM disease or membranous nephropathy) or downstream of IC deposition characterised by the presence of autoantibodies, often in conjunction with extrarenal manifestations, with the archetypal example of the latter being systemic lupus erythematosus (SLE)(3, 56). SLE is a prototypic chronic autoimmune disease characterised by the production of wide spectrum of autoantibodies to ubiquitous antigens such as anti-dsDNA, which can result in tissue damage due to IC deposition, including in kidneys, brain, and skin(6).

LN represents a serious complication of SLE affecting the kidney, associated with greater risk of mortality among SLE patients(57, 58). The International Society of Nephrology and Renal Pathology Society (ISN/RPS) system classifies LN into 6 classes, with further histologically based subclassifications(59). The ISN/RPS system is superior to previous classification systems, particularly when comparing non-proliferative (classes I, II and V) and proliferative (classes III and IV) disease, however despite histological and serological differences within subgroups there is no difference in prognosis between class III-IV subclasses, and since class III-IV disease is most aggressively treated with immunosuppression this lack of stratification in predictive value is problematic(59, 60). Clinical and histological parameters are also limited in predictive power for risk of renal flare and there is a discordance between histological and clinical remission(61, 62). This highlights the need for a better understanding of the underlying molecular mechanisms of LN, and a new approach to tissue pathology, to stratify disease, identify new therapeutic targets for treatments and to better predict disease progression.

1.3 Systemic Lupus Erythematosus (SLE)

SLE can serve as an exemplar for developing a more cellular approach towards renal pathology. A hallmark of LN is IC deposition in the glomerular space, ICs are aggregates comprised of antibody bound to foreign or self-antigens, which circulate in the blood eventually depositing into tissue space(63). However, not all those with SLE develop LN, nor do all SLE patients with IC deposition at the time of biopsy develop renal impairment, with the drivers for patients who do progress to LN and CKD not well understood(64, 65)

1.3.1 Background and epidemiology

SLE is characterised by production of autoantibodies to self-components (e.g., DNA), aberrant adaptive and innate immune responses and tissue immune complex deposition and damage(6, 66). SLE can vary in severity from mild (e.g., rashes and joint pain) to potentially life threatening (e.g., ESRD or severe neurological disease)(6). The

heterogenous presentation of this disease is illustrated by the American College of Rheumatology (ACR) classification, with 11 criteria, and 4 required for diagnosis(6, 67). Renal complications affect up to 50% of adults and up to 80% of children with SLE, with 10-30% going on to develop ESRD(58). The five-year survival rate with SLE has improved from 64-87% in the 1980s to >90%, however the mortality rate in people with SLE remains 2-5 times greater than the general population(57). Renal disease confers the highest risk of mortality, 8 times higher than the general population(57).

Women, particularly those of reproductive age, are more likely than men to develop SLE, with a female: male ratio of between 6-13:1(68-70). Global SLE incidence is estimated to be 5.14 per 100,000 person years and prevalence 43.7 per 100,000 persons or 3.41 million people(71). SLE varies significantly by ethnicity, with non-white populations having a higher incidence and prevalence in comparison to white populations(72). The highest prevalence is in Afro Caribbeans (5-10-fold), followed by South Asian, Hispanic and Asian/Pacific Islander populations as compared to white populations(73, 74).

Severity of disease manifestations has also been linked to ethnicity, US studies report greater prevalence of renal, neurological, and haematological abnormalities in African Americans, Asian/Pacific Islander and Hispanic patients when compared to white populations(74). These groups were also more likely to develop severe manifestations (e.g., LN) earlier on following an SLE diagnosis, with more frequent accumulation of renal damage and progression to ESRD(73, 74). Similar patterns of renal disease are described in UK Afro-Caribbeans and Asians with SLE(68, 73, 75, 76). Age is also linked to disease severity, with juvenile onset SLE (age <16) (JSLE) patients more likely to have severe complications including renal disease, in comparison to adult SLE(77-79).

In addition to increased mortality, SLE carries a significant economic burden and impact on health-related quality of life metrics (HRQOL)(70). The annual mean cost to the NHS in the first year after diagnosis per SLE patient is estimated at £7532(80). In the US health care

costs for patients with LN are 1.2-3 times higher when compared to people with SLE without renal involvement and 5.1-6.3 times higher than the general population(81, 82). Costs for those with ESRD are higher still, with the cost for dialysis alone at £23,737 in the UK(83). Quality of life (QOL) metrics, including disease activity, sleep quality and fatigue are significantly reduced in people with SLE in contrast to the general population (64, 84). HRQOL is particularly poor in those with renal involvement, with active LN having the greatest impact on physical and emotional functionality(85).

1.3.2 Genetic aetiology of SLE

Mechanisms underlying SLE pathogenesis are not well understood, but a complex interplay between environmental, genetic, and immunological factors contribute towards disease onset and development(6, 86). SLE concordance is 24% in monozygotic twins as compared to 2% for dizygotic twins, and familial aggregation in large cohort studies and the discovery of monogenic genetic variants driving SLE-like disease point to a strong genetic component(87-89).

1.3.2.1 Polygenic SLE susceptibility loci

The genetic contribution to SLE risk is in most cases due to a mix of variants with individually modest effect size(90-92). Genome wide association studies (GWAS) have identified >100 loci associated with SLE(92, 93). Within the highly polymorphic Major Histocompatibility complex (MHC), known as the human leukocyte antigen (HLA) region in humans, several susceptibility loci account for ~2.6% of SLE genetic heritability(94). The primary signal is in the MHC class II region, with risk haplotypes in *HLA-DRB1*, *HLA-DQB1* and *HLA-DQA1* alleles(91, 94-96). Other GWAS risk alleles are associated with genes involved in interferon (IFN) signalling and production (*IRF5*, *TLR7* and *STAT4*), clearance of immune complexes (*ITGAM*, *FCGR2A* and *FCGR2B*), regulation of inflammation (*IRAK1* and *TNFAIP3*) and B and T cell activation and tolerance (*ETS1*, *PTPN22* and *IKZF1*) have all been linked with polygenic SLE(97-103).

Epigenetic DNA modifications including hypomethylation in genes linked with autoreactivity(104, 105). For example enhanced expression of *PRF1* encoding cytolytic protein perforin, due to hypomethylation has been observed in CD4 T cells from SLE patients with active disease and is suggested to contribute towards aberrant macrophage/monocyte killing, enhancing the burden of apoptotic debris whilst reducing the cells able to clear such debris thereby perpetuating disease pathogenesis(105). Likewise hypomethylation induced enhanced expression of *ITGAL* encoding CD11a, which forms one half of the lymphocyte function associated antigen-1 (LFA-1) and is important for tissue infiltration and co-stimulation, has been observed on CD4 T cells from patients with SLE with expression shown be positively correlated with SLE disease activity index(104). Conversely, repression of IL-2 expression due to hypermethylation and decreased deacetylation has been reported in CD4 T cells from SLE patients, with this cytokine important for the generation of regulatory T cells (Tregs)(106).

1.3.2.2 Monogenic causes of SLE

Monogenic SLE is rare, but several highly penetrant mutations have been identified. Amongst the first to be described were defects in early complement proteins (C1q, C1r, C1s, C4), critical for clearance of apoptotic debris, immune complexes, and defence against infection(89, 107, 108). Primary complement deficiencies account for less than 1% of SLE cases, with susceptibility to SLE development linked to the position of the protein in the complement cascade(89, 108, 109). Increased IFN signalling, particularly a type 1 IFN signature, is seen in the serum of lupus patients, and mutations disrupting type 1 IFN signalling via nucleic acid sensing and degradation, IFN regulation and self-tolerance are associated with several autoimmune diseases including SLE(110, 111). Nucleic acid sensing pathway variants include loss of function mutations in genes encoding deoxyribonuclease (DNase) enzymes (e.g., *DNASE1*, *DNASE1L3*), which are central to the degradation of DNA molecules in apoptotic cells, have been reported in patients with early

onset SLE(89, 108). An SLE-like phenotype is also seen in *Dnase1* and *Dnase1/3* deficient mice(112, 113). Mutations in other nucleic acid degradation enzymes such as *TREX1*, the main cytosolic 3'-5' DNA exonuclease, *SAMHD1* (sterile alpha motif and HD domain containing protein 1), *RNASEH2A*, *RNASEH2B* and *RNASEH2C* have additionally been linked with development of SLE and the SLE-like disease Aicardi Goutières Syndrome(108, 114). Polymorphisms in the TLR7 gene have been linked with SLE, and recently a monogenic gain of function variant in toll-like receptor 7 (TLR7), was reported in a patient with early onset SLE(115, 116). TLRs are a class of pattern recognition receptors (PRR) that recognise distinct sets of microbial associated molecular patterns (e.g., lipopolysaccharide), TLR7 and TLR9 can recognise and bind endosomal ssRNA and DNA respectively(117). Similarly, TLR7 activation or gain of function is the main driver of disease in well-established murine models of SLE and LN including BXSB/*Yaa* mice in which disease is attributed to a duplicated copy of TLR7 gene, and mice treated with the TLR7 agonist Imiquimod(118).

Mutations in IFN regulation genes such as negative regulator ovarian tumour protease deubiquitinase 1 (OTUD1), and gain of function mutations in *TMEM173*, encoding stimulator of interferon genes (STING), have been described in monogenic SLE(119, 120). Tartrate resistant acid phosphatase (TRAP) encoded by acid phosphatase 5 (*ACP5*), primarily acts on osteopontin (OPN), which promotes type 1 IFN responses via interaction with TLR9, and TRAP mutations are associated with autoimmunity and SLE(89, 108). Defects in genes associated with self-tolerance lead to autoimmunity. Protein kinase C δ (*PRKCD*) regulates lymphocyte survival, proliferation, and apoptosis, and *PRCKD* mutations have been associated with JSLE, mirrored in *Prkcd*^{-/-} mice(121, 122). Variants in apoptotic regulatory genes *FAS* and *FASL*, which are key to the removal of self-reactive lymphocytes, cause autoimmune lymphoproliferative syndrome (ALPS) with two cases additionally reported to have SLE, and loss of Fas function is the basis of the MRL/lpr murine phenotype(123, 124).

1.3.3 Sex biases in SLE susceptibility

The 10:1 SLE female to male ratio has been linked to both the effect of sex hormones and altered X chromosome inactivation (XCI).

1.3.3.1 Sex hormones and SLE

Sex hormones play a role in immune system homeostasis and regulation, and SLE disease activity has been reported to vary with menstrual cycle, menopause, and pregnancy(125). Oestrogen receptors (ER) consist of two subtypes ER α and ER β , expressed by a variety of immune cell types and implicated in regulation of immune responses(126). In peripheral blood monocytes (PBMCs) from SLE patients, ER α mRNA expression is elevated and ER β decreased compared to healthy controls, while depletion of ER α ameliorates disease in the spontaneous SLE murine model, New Zealand black (NZB) x NZ white F1 mice(127, 128). Oestrogen may influence B cell maturation via Bcl2, skewing B cells to an autoimmune phenotype, and oestradiol treatment of PBMCs from people with active SLE increased production of anti-dsDNA antibodies and IgG in comparison to inactive SLE or healthy controls(129, 130).

Within T cell subsets, oestrogen induces in vitro expansion of CD4⁺CD25⁺FoxP3⁺ Tregs in healthy females but not in females with SLE, suggesting differential responses to oestrogen stimulation by PBMCs depending on disease status(131). In contrast, androgens may have a protective effect. Females with SLE have lower androgen levels than controls, while male hypogonadism is associated with a higher risk of SLE(132). Treatment with androgens in SLE murine models ameliorates disease and improves survival, supporting a disease protective role for these hormones in SLE(133, 134). Androgens may inhibit transcriptional factors associated with pro-inflammatory cytokines such as tumour necrosis factor (TNF), IL-1 β and IFN γ , and promote anti-inflammatory mediators such as IL-10 and transforming growth factor β (TGF β)(134, 135). However, the interplay between sex hormones and immune homeostasis can only partially explain the SLE gender disparity.

1.3.3.2 X linked drivers of SLE

X chromosome inactivation is a transcriptional process during female embryogenesis, to equalise the effect of two X chromosomes. Failures of inactivation leads to “escape genes” with double the expression and increased ‘dose’ of X chromosome genes has been linked to SLE(136). Men with Klinefelter syndrome (XXY), are 14 times more likely than XY men to develop SLE(137). In contrast women with Turner syndrome, in which one X chromosome is partially or completely missing, are protected (138).The X chromosome contains immune related genes (e.g. CD40I, TLR7), which may explain these observations(139). Indeed, reactivation of CD40I and expression on the inactive X chromosome occurs in female SLE CD4 T cells (140). Male BXSB mice with translocation of the *Yaa* locus (BXSB-*Yaa*), consisting of an estimated 16 genes from the X chromosome to the Y chromosome, have a twofold increase in *Tlr7* expression and develop a SLE like phenotype, while female BXSB mice with only one functional copy develop milder disease(118). In humans, biallelic expression of *TLR7* in specific immune populations such as B cells, monocytes and plasmacytoid dendritic cells (pDCs) has been demonstrated in immune cells from women and Klinefelter syndrome males as compared to healthy male controls, with biallelic B cells having a higher propensity to class switch to IgG and producing higher IgG titres(141). Thus, increase in gene dosage, including in TLR7, due to escape from X linked inactivation, might explain increased susceptibility to development of SLE in females.

1.3.4 Environmental triggers for SLE

Environmental exposures are suspected in 60% of SLE cases and might account for why only some genetically susceptible individuals develop disease(142, 143). SLE associated respiratory triggers include silicates and cigarette smoking(144-146). Ultraviolet (UV) exposure can exacerbate SLE particularly in people with skin manifestations, however the role of UV exposure in increasing risk is less well established(147). Lifestyle factors such

as alcohol consumption, exercise, diet, and body mass index have also been associated with SLE(148).

1.3.4.1 Drug induced lupus (DIL)

Exposure to drugs such as procainamide, hydralazine, and anti-TNF agents (infliximab, etanercept) have been linked with DIL, with genetic factors such as HLA variants and C4 allele copy, and epigenetic dysregulation thought to influence this susceptibility(149). Proposed mechanisms for DIL include drug metabolites binding to circulating proteins and forming immune activating haptens, hypomethylation of T cells resulting in increased CD11a expression, and drug metabolite induced disruptions of central tolerance(149, 150).

1.3.5 Immune system dysfunction in SLE

The innate and adaptive arms of the immune system work in synergy to orchestrate an immune response. In autoimmune disease, loss of self-tolerance promotes self-reactive T cells and B cells, resulting in the production of autoantibodies targeting important homeostatic mechanisms, IC formation, and aberrant activation of the innate immune system. Thus, disruptions to both the innate and adaptive arms of immunity are central to the development of SLE (Figure 1.4)

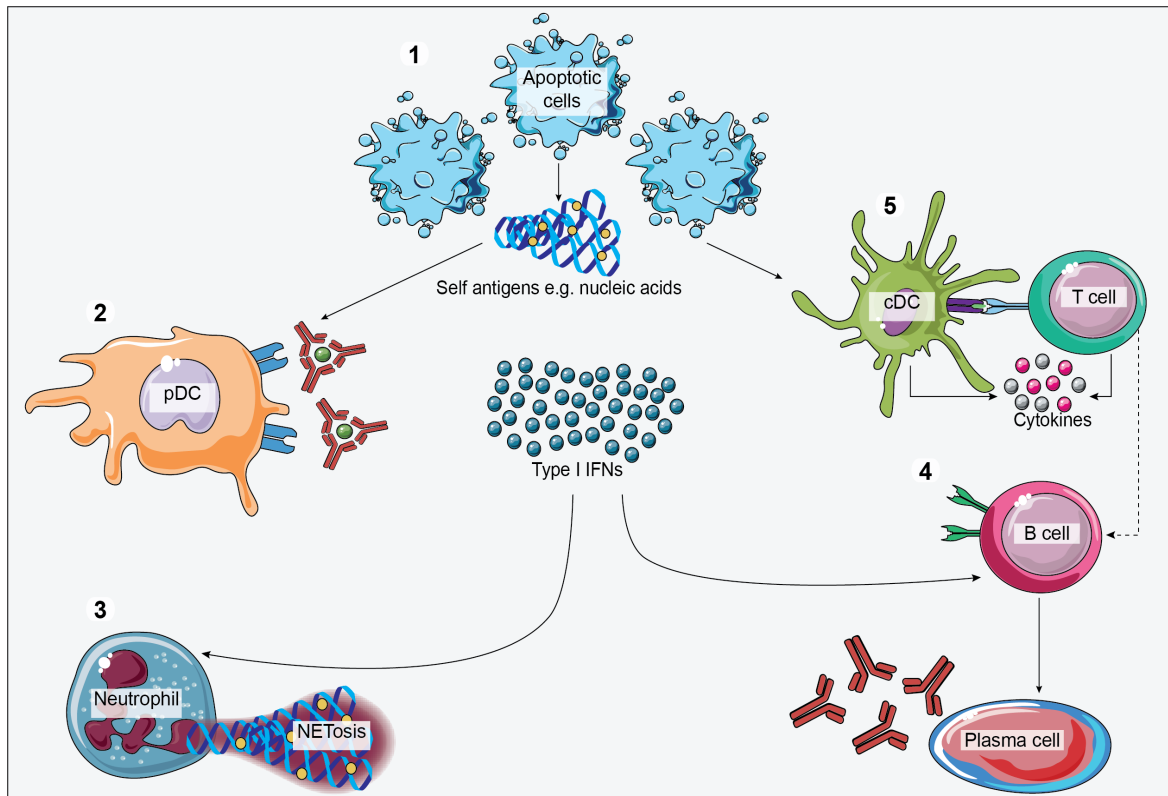


Figure 1.4: Immunological pathways implicated in SLE.

(1) Aberrant clearance of apoptotic cells and nuclear debris accumulates because of decreased phagocytosis, and this debris acts as autoantigens, triggering activation of TLRs (e.g., TLR7) and nucleic acid sensors (2) Nucleic acid activates endosomal TLR receptors in pDCs which are the main producers of type I IFNs and other proinflammatory cytokines. (3) Neutrophils in SLE are more likely to undergo a form of apoptotic death known as NETosis, IFNs stimulate NETosis, which in turn causes the release of nucleic acids and proinflammatory cytokines, thereby increasing the autoantigen burden and further stimulating the production of IFNs. (4) Nucleic acid debris activates B cells which produce more type I IFNs. B cells migrate to germinal centres and differentiate into plasma cells which produce autoantibodies, the autoantibodies bind to circulating autoantigens forming ICs. (5) Dendritic cells undergo maturation because of being stimulated by IFNs and can activate T cells via antigen presentation in the context of MHC II, resulting in further production of proinflammatory cytokines. Adapted from (5, 6)

1.3.5.1 Defective clearance of cellular debris, apoptosis, neutrophil extracellular traps, and opsonisation

Autoantibodies in SLE are typically targeted toward nuclear components, for example dsDNA and ribonucleoproteins, although autoantibodies towards cell membrane (e.g., anti-phospholipid antibodies) and cytoplasmic components are also associated with disease(67, 151). Nuclear components are typically shielded from extracellular space, raising the question of how the immune system encounters them(151). Apoptosis is a form of regulated

cell death which plays an important role in tissue homeostasis, characterised by cell shrinkage, nuclear fragmentations, chromatin condensation and exposure of the inner cytoplasmic leaflet phospholipid phosphatidylserine, however the integrity of the plasma membrane is maintained, preventing the release of nuclear material into the extracellular space(152). Typically, apoptosis is immunologically 'silent', with apoptotic bodies cleared and degraded by phagocytes (macrophages, monocytes, and neutrophils) of the innate immune system(153). If apoptotic cells are not cleared, they undergo secondary necrosis, characterised by loss of plasma membrane integrity, release of nuclear material and damage associated molecular patterns (DAMPs) such as ATP and heat shock proteins, which attract inflammatory cells(151, 154).

Both cell death and phagocytosis are altered in SLE. Increased monocyte and macrophage cell death is correlated with SLE disease activity and serum from people with SLE can induce apoptosis in immune cells from healthy controls , and in vitro monocytes from people with SLE show defective clearance(155-159). In vivo in humans with SLE, lymph node germinal centres (GCs) which are integral for differentiation of B cells into memory B cells or antibody producing plasma cells, contain multiple apoptotic cells in comparison to controls(160). Concurrently, tinged body macrophages, which ordinarily engulf apoptotic debris in germinal centres, are reduced in some SLE patients, alongside increased apoptotic debris on follicular dendritic cells, with presentation of this debris to self-reactive B cells hypothesised to promote their survival and proliferation(160).

Neutrophils undergo a specialised form of cell death known as NETosis, faster and less controlled than apoptosis(154). During NETosis, neutrophils release neutrophil extracellular traps (NETs), made up of proteins from primary granules (e.g., neutrophil elastase and myeloperoxidase), DNA and histones(161). NETs are important in ensnaring and killing pathogens such as bacteria and fungi, by delivering localised high concentrations of antimicrobial components(161, 162). However, NET components such as histones, DNA,

and high mobility group box protein 1 (HMGB1), in addition to neutrophil cytoplasmic components, may serve as a source of autoantigens(163). Neutrophils from patients with SLE show an increased predisposition for NETosis, with increased apoptotic neutrophils detected in patient sera and impaired phagocytic capacity in a distinct neutrophil population expressing low density granulocyte (LDG)(164, 165). Increased NETosis is accompanied by decreased degradation. Endonuclease DNase1 inhibitors or anti-NET antibodies may protect NETs from degradation in SLE, thereby increasing exposure to potential autoantigens (166, 167).

Clearance of apoptotic debris by phagocytes relies on alterations of surface phospholipids (e.g., phosphatidylserine) and recognition of opsonin's, which mark apoptotic cells for phagocytosis(168). Opsonizing proteins include pentraxins (e.g., C-reactive protein (CRP), serum amyloid P component (SAP), pentraxin3 (PTX3)) and complement components (e.g., C1q, C4)(169). Acquired hypocomplementemia due to complement consumption is a frequent SLE feature (30-48%)(170). Complement components promote IC clearance by maintaining their solubility and facilitating opsonisation, but SLE hypocomplementemia results in impaired IC clearance, and thus contributes to IC deposition in tissues such as the kidney, resulting in localised damage, cell death and further release of autoantigens augmenting the autoimmune response(171).

1.3.5.2 Activated dendritic cells produce type I interferon and promote T cell expansion

An increased autoantigen burden, due to increased cell death and impaired clearance, has consequences within the innate system which further promote autoimmunity. ICs formed by autoantibodies to uncleared cell debris components can be taken up by pDCs or B cells via Fc γ receptors (Fc γ R), activating TLR7 and TLR9, and resulting in the production of type I IFN (e.g., IFN α) and proinflammatory cytokines(172, 173). Although pDCs are reduced in the periphery in active SLE they accumulate in the kidneys and other sites of

inflammation(174). In mice TLR7 and TLR9 knockout in the presence of functional pDCs abrogated the generation of autoantibody producing B cells(175). Type I IFNs can prime NETosis, with uncleared NETs forming ICs with autoantibodies, which in turn activate pDCs to produce more IFN, in a positive feedback loop. Despite this strong interferon signature, IFN inhibition is not yet a proven treatment, with a clinical trial of IFN inhibitor anifrolumab failing to meet its primary endpoint(5, 176).

Conventional DCs (cDCs) are the more abundant DC subset, expressing PRRs such as TLRs, and presenting antigen to T cells(117). In SLE, increased auto antigens, and type I IFN leads to sustained activation of cDCs, initiating an inflammatory response characterised by cytokines such as IL-6(177). This activated cDC signature in the blood of people with SLE is characterised by increased expression of the co-stimulatory molecules CD86, CD80 and MHC II, important for T cell priming and activation(178). Single cell sequencing of PBMCs from SLE patients highlights changes in cDCs subsets with an increase in CD163⁺DCs, characterised by expression of pro-inflammatory markers and interferon stimulated genes(179). In co-culture these pro-inflammatory cDCs prime naïve CD4⁺ T cells towards Th2 and pro-inflammatory Th17 cells(179).

Studies in lupus prone MRL/lpr mice with a constitutive DC deletion suggest that cDCs are necessary for expansion and priming of CD8⁺ and CD4⁺ T cells but are not required for T cell activation(180). cDC specific overexpression of TLR7 in lupus prone mice promoted the development of kidney disease and identified a novel CD11b⁺ cDC renal infiltrating population localised to the glomeruli, suggesting a role for these cells in development of nephritis(181).

1.3.5.3 Monocyte and macrophage populations are skewed in SLE

Macrophages and monocytes are two major phagocytes, with impaired functionality in SLE. Imbalanced macrophage polarisation favouring classically activated M1 over alternatively activated M2 phenotypes has been associated with autoimmune disease including SLE and

pro-inflammatory M1 macrophages are found in MRL/lpr LN murine kidneys (182-184). Such M1 macrophage skewing can be induced by cytokines such as IFN- γ and activation of signal transducer and activator of transcription 1 (STAT1)(185). Pharmacological depletion of macrophages exacerbated activated lymphocyte derived DNA induced SLE in mice, particularly nephritis, and was rescued by adoptive transfer of M2 macrophages(186). Yet in another study in NZM lupus-prone mouse, increased M2b macrophages in the kidney were associated with GN, and depletion improved renal disease(187). Furthermore, evidence in humans is also complex and perhaps conflicting. Within peripheral blood from children with lupus, M1 like monocytes levels correlated with and disease severity(188). However, by immunohistochemistry, M2 like macrophages are the major infiltrate in LN renal biopsy samples and are associated with tubular damage(189). This is further complicated by immune transcriptomics findings from human LN kidneys, where a pro-inflammatory M1 like signature was most similar to patrolling blood monocytes, and trajectory analysis suggested that these monocytes may transition to a putatively more reparative M2 phenotype, characterised by increased expression of phagocytic genes and downregulation of inflammatory genes, following renal infiltration(190).

Monocytes represent blood-based macrophage precursors. In humans, monocytes in the blood can be distinguished as CD14^{high} CD16^{dim} classical monocytes and CD16^{high} CD14^{dim} non-classical monocytes. In mice classical monocytes are lymphocyte antigen 6 family member C1 Ly6c^{hi} and non-classical Ly6c^{low}(191). In homeostasis, non-classical monocytes constitute a minority of the total monocyte population (~10%), these cells patrol the vasculature acting as sentinels through clearance of dying endothelial cells and are implicated in the resolution of vascular inflammation(191). Of the two populations classical monocytes more readily develop into macrophages(192, 193). BXSB-Yaa mice have increased circulating non-classical monocytes, correlated with autoantibody titres, suggesting a role for this monocyte subset in B cell activation/modulation of antibody production(194, 195). Notably, in wild type C57B6/J mice, introduction of the Yaa locus was

insufficient to induce SLE until combined with inhibitory receptor FcγRIIB deficiency, the resulting mice (B6.FcγR11B^{-/-}.Yaa) developed expanded numbers of non-classical monocytes, with upregulated expression of genes relevant to B cell activation such as B cell activating factor (BAFF)(195, 196).

Monocytes from people with SLE have increased expression of IFN stimulated FcγR CD64 and other stimulatory FcγRs (CD16 and CD32a), with a decrease in expression of FcγRIIB (CD32b), which can promote binding to circulating ICs(197, 198). Non-classical monocytes have been shown to be expanded in the blood of patients with SLE and the frequency of sialic acid-binding Ig-like lectin 1 (SIGLEC1)⁺ non-classical monocytes has been shown to correlate with complement and anti-dsDNA antibody titres(199, 200). These cells can be recruited to tissue, and immunofluorescence staining of LN biopsies has confirmed the presence of non-classical monocytes near glomerular capillaries, where they are hypothesised to be activated by ICs, with higher grade of non-classical monocytic infiltration in LN kidneys associated with more severe renal disease (201-203). A functional role for these cells in SLE is suggested by co-culture experiments, in which non-classical monocytes from people with SLE induced selective expansion of Th1/Th2 and Th17 subsets and stimulated B cell IgG production(204). Phagocytic capacity may be inferior in non-classical monocytes in SLE, with reduced expression of phagocytic receptors (e.g., CD36 and CD163), and enhanced antigen presentation and co-stimulatory markers (e.g., CD80, CD86 and MHCII)(200, 201).

In summary alterations in macrophage subsets play a role in SLE, and damage may be attributable to both M1 and M2 macrophages as disease progresses, blurring the classical M1 vs M2 paradigm. A pathogenic role is also suggested for non-classical monocytes in SLE. The impact of macrophage/monocyte alterations and interactions with tissue resident cells within the tissue environment remains poorly understood. It is unclear whether these alterations are purely pathogenic, protective or a mix in the context of SLE.

1.3.5.4 Natural Killer cells in SLE

Natural Killer (NK) cells are a class of cytotoxic innate lymphoid cells, originating from the same progenitor as B and T cells. Unlike cytotoxic CD8 T cells, NK cells do not require priming via antigen presentation cells to carry out their killing functions(205). Numbers of peripheral NK cells are decreased in lupus prone mice and transfer of these cells delays autoimmunity(206, 207). NK cells are also reduced in blood in SLE patients but display a proinflammatory phenotype with secretion of IFN- γ and TNF- α , and enhanced cytotoxicity, and NK activation correlates with disease activity(208, 209). NK cells are detectable in the kidneys of LN patients by single cell RNA sequencing, with both putative pro-inflammatory and anti-inflammatory populations described(190). However, the functional role of NK cells in diseased tissue remains to be established.

In summary, alterations in multiple innate populations appear important for the development and perpetuation of lupus autoimmunity. Many unanswered questions remain regarding the mechanisms underlying these alterations, therefore a better understanding of the underlying innate defects, particularly within target organs, and their interactions with tissue cells are needed to better predict disease progression.

1.3.5.5 T cell dysfunction in SLE

Development of a targeted and sustained autoimmune response relies on the development of long-lasting humoral immunity mediated by B cells, which require help from T cells. Defects in both these populations are integral to the immunopathogenesis of SLE and alterations in signalling and functionality of CD4, CD8 and double negative (DN) T cell subsets can contribute to SLE.

1.3.5.5.1 Signalling defects in T cells in SLE

CD3 ξ is an integral component of the T cell receptor (TCR), which upon engagement of antigen via MHC mediates signalling through zeta chain associated protein kinase 70

(ZAP70), resulting in calcium influx and subsequent T cell activation(210). CD3 ξ signalling appears critical for T cell tolerance, as CD3 $\xi^{-/-}$ mice develop multiorgan inflammation and damage(211). CD3 ξ is downregulated in T cells from people with SLE and replaced by homologous Fc γ R in the TCR complex, which signals via recruitment of spleen tyrosine kinase (Syk), resulting in higher calcium influx and hyperactivation of T cell signalling(210, 212). Restoration of CD3 ξ functionality in vitro normalises intracellular calcium levels and signalling(213).

Increased intracellular calcium in stimulated T cells from people with SLE, as compared to controls, has been linked with enhanced NFAT transcriptional activity(214). This results in increased expression of several key inflammatory genes implicated in SLE pathogenesis (e.g., CD40L, TNF and IL17)(214, 215) .

1.3.5.5.2 CD4 T cells in SLE

Intrinsic and extrinsic factors both contribute to irregularities in CD4 T cell polarisation, causing imbalances to immune homeostasis in SLE. Polarisation of CD4 T cells can be divided into several helper subsets including Th1,Th2, Th17, follicular helper T cells (Tfh) and Tregs(216).

Th1

Th1 cells are characterised by expression of T box transcription factor (Tbet) and production of IL-2 and IFN- γ and are important for responses to intracellular pathogens(217). IFN- γ (type II IFN), which can accelerate disease in mice, is predominantly produced by Th1 and NK cells(218, 219). IFN- γ serum concentrations are increased in SLE patients as compared to healthy controls, and correlate with disease activity, with some studies suggesting a predominance of peripheral Th1 cells in LN(220, 221).

Th2

Th2 cells play an important role in humoral mediated immunity and are characterised by expression of transcription factor GATA binding protein 3 (GATA3) and production of cytokines such as IL-4, IL-5, and IL-13(217, 222). Although blockade of IL-4 ameliorates autoantibody titres and LN in mice, human studies do not clearly support a pathogenic role for IL-4, since levels are decreased or unchanged in comparison to healthy controls(223-226). Instead, decreased IL-4 receptor (IL-4R) engagement may lead to differentiation of human age associated B cells (ABCs), at least in vitro, suggesting a disease inhibitory role for this cytokine(227). IL-5 is increased in SLE serum, and skin keratinocytes in comparison to healthy controls, suggesting a potential role for Th2 cells and IL-5 in cutaneous manifestations(228). IL-13 is a Th2 pro-fibrotic cytokine, which can also upregulate expression of MHC-II on B cells, and elevated mRNA levels in human SLE serum have been correlated with disease activity, additionally IL-13 has been observed to be increased in the urine and kidneys of people with LN(229-232). In lupus prone mice, IL-13 expression can be localised to T cells infiltrating the glomeruli, suggesting a role for Th2/IL-13 axis in SLE mediated tissue damage(233, 234).

Th17

Th17 cells are a subset of CD4 T cells which produce pro-inflammatory IL-17 family cytokines and express transcription factor RAR-related orphan receptor gamma t (ROR γ t)(117). SLE patients with active disease have an expanded IL-17A producing circulating Th17 population(235). This may be explained by increased responsiveness to cytokine IL-23, elevated in the serum in SLE, and associated with non-response to therapy(236, 237). Metabolic defects in SLE T cells, with reliance on glycolysis pathways, may also favour the development of Th17 cells(238).

The pathogenicity of Th17 may be due to the inverse relationship between Th17 and Tregs, with increased Th17 resulting in reduced Treg cell numbers(239). IL-17 can also promote neutrophil and monocyte recruitment, and has been suggested to activate B cells via

BAFF(240). Within the kidneys of people with LN, areas of T cell infiltration have prominent IL-17 expression, linked to disease activity and poor treatment response (236).

T follicular helper cells (Tfh)

Tfh cells are central to T cell dependent immune responses and express the transcription factor B cell lymphoma 6 (Bcl6)(241). Tfh derived IL-21 and signalling through costimulatory molecules such as inducible T cell costimulatory (ICOS) and CD40 promotes B cell differentiation in GCs(242). Increased frequency high affinity antibody producing B cells and plasmablasts with somatic mutations in the blood of people with SLE are likely GC derived and therefore dependent on Tfh interactions(243). Tfh cells are expanded in lupus prone murine models, notably in Sanroque mice which have a mutation in Roquin-1, resulting in overexpression of ICOS(244, 245). Blocking co-stimulatory molecules ICOS and CD40 in lupus prone mice can reduce autoantibody production, delay renal disease, and prolong survival(246, 247). In the blood of patients with SLE, increased numbers of circulating IL-21 producing Tfh like cells have been observed and PD-1 expression on these cells correlated with higher circulating plasmablasts and autoantibody titres, suggestive of aberrant GC functions(248). A renal Tfh like population has been described using kidney scRNA-Seq in from LN human biopsy samples (202). Furthermore, Tfh co-localise with and interact with cognate B cells in the LN kidney, which could serve as a source for high affinity autoreactive B cells targeting renal antigens(190, 249). In summary, Tfh appear to play a major pathogenic role in SLE.

Tregs

Aberrant activity of regulatory CD4 T cells is associated with the development of autoimmune disease. These cells are characterised by expression of transcription factor Forkhead box P3 (FoxP3) and IL-2 receptor alpha (CD25), plus other markers associated with auto-reactive lymphocyte regulation such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4)(220) . The main function of Tregs is in the maintenance of peripheral

tolerance via modulation of aberrant T cell responses to prevent autoimmunity(117). Whether Treg numbers change in SLE is unclear, but they do appear to have functional impairments(250). An increased population of CD4⁺FoxP3⁺CD25⁻ cells has been described in human SLE, with impaired ability to suppress IFN- γ production, however the origin of these CD4⁺FoxP3⁺CD25⁻ cells is unclear, they may represent previously activated T cells expressing Treg markers but lacking their functional capacity, or dysfunctional or exhausted Treg cells(251, 252).

In humans Tregs are subset by their expression of marker CD45RA into three groups, with the first and second (resting Tregs and activated Tregs), retaining their suppressive effects and the third (non-suppressive Tregs), having a more inflammatory phenotype(253). In active SLE resting and non-suppressive Tregs were elevated peripherally, with resting Tregs demonstrating suppressive defects, and numbers correlating with disease activity(254).

1.3.5.5.3 CD8 T cells

CD8 T cells have the capacity to differentiate into cytotoxic T lymphocytes (CTLs), which recognise antigens in the context of MHC I, and induce apoptosis in target cells via release of cytotoxic granules such as granzyme and perforins, and cytokines such as IFN- γ (117). CTLs thus play a role in infection response and maintenance of peripheral tolerance via removal of autoreactive cells(255). Circulating CD8 T cells appear hyporesponsive in human SLE, with a reduced frequency of effector memory (EM) CD8 T cells, the primary cytotoxic subset, and decreased numbers of central memory (CM) CD8 T cells, which serve as a reservoir for EM CD8 T cells and generate more EM upon antigenic engagement(256). These cells also show decreased expression of granzyme B and perforins, and impaired IFN- γ on stimulation in vitro in comparison to healthy controls, suggesting that dysfunctional cytotoxic activity might be perpetuating systemic inflammation(256, 257). In vivo accelerated autoimmunity is observed in lupus prone mice deficient for perforin (297).

Dysfunctional CD8 cytotoxicity has been linked to changes in the expression of surface markers signalling lymphocytic activation molecule family member 4 (SLAMF4) and SLAMF7(256, 258).

Chromatin landscape studies utilising assay for transposase-accessible chromatin sequencing (ATAC-seq) in tumour infiltrating CD8 T cells have identified enhanced expression of NAD⁺ regulator CD38, with this CD38⁺CD8 T cell population demonstrating impaired cytotoxic capacity(259). In SLE, higher numbers of CD38⁺ CD8 T cells are associated with increased susceptibility to infections, and reduced cytotoxic activity is attributed to CD38 dependent reduced expression of effector genes including EOMES and TBX21(Tbet)(260). A case report of successful treatment of refractory SLE with anti-CD38 antibody daratumumab requires validation in larger controlled studies(261). Impaired metabolism in SLE CD8 T cells may also contribute to their hyporesponsive phenotype; increased type I IFN stimulation coupled with TCR activation results in increased NAD⁺ consumption, mitochondrial dysfunction, exhaustion, and enhanced stress related cell death(262). However, the contribution of activation or exhausted cytotoxic T cells to tissue mediated damage is unclear(263-265).

1.3.5.5.4 Double negative (DN) T cells

DN T cells express the $\alpha\beta$ chains of the TCR, but lack expression of CD8, CD4 or NK cell markers(266). These cells are present in the kidneys in LN, and correlate with kidney function in paediatric SLE(267, 268). In lupus prone murine model MRL/lpr, DN T cells are expanded and transfer of DN T cells from aged mice with disease to younger mice pre-disease accelerated autoimmunity. This phenotype may reflect failure of Fas mediated deletion of self-reactive T cells, akin to autoimmune lymphoproliferative disorder, although blocking CD8 T cells abolished the production of DN T cells, suggesting that these cells originate from CD8 T cells which have downregulated CD8 expression, reproducible by repeated TCR stimulation by autoantigens and apoptotic debris (268-270). DN T cells in

SLE have enhanced proliferation and tissue migration capacity, and their pathogenicity may be due to production of pro-inflammatory cytokines IL-17 and IFN- γ , which can promote Th17 cell differentiation(266, 271).

1.3.5.6 B cell dysfunction in SLE

Aberrant B cell activation results in the production of high affinity autoantibodies in SLE(272). Pathogenicity of B cells in SLE is evidenced by the efficacy of B cell depletion therapies such as Rituximab (anti-CD20), licensed for the treatment of refractory SLE(273). Rapid repopulation of memory and plasma B cells after cessation of Rituximab is associated with early relapse(274, 275).

Increased polyreactive / autoreactive immature and naïve B cells in SLE patients suggest defects in early tolerance checkpoints during B cell development with these autoreactive circulating naïve B cells serving as potential precursors to production of pathogenic autoantibodies (276, 277).

Peripheral B cells from SLE patients have enhanced somatic hypermutation and abnormalities in receptor editing, which contribute to peripheral autoreactivity and development of high affinity B cell receptors(278, 279). Circulating B cells subsets are altered, with increased proportions of antigen experienced CD27⁺ memory B cells and plasma cells(280). Increased pools of memory B cells in SLE have reduced reactivation threshold via reduced expression of Fc γ RII β and can also be stimulated via TLR stimulation and mediators such as BAFF, with increased BAFF levels correlating with anti-dsDNA antibodies as well as disease activity(281-283).

Within the naïve B cell pool in SLE is an expanded population characterised by absent CD27 expression, however unlike naïve B cells this population is IgD⁻ and its expansion correlates with disease activity, renal manifestations, and autoantibody titres(284). A CD95⁺ subset within this IgD⁻CD27⁻ (DN) B cell population is specific to SLE patients and not found

in infection driven inflammation(285). This DN CD95⁺ B cell population expresses higher levels of MHC II and activation markers such as CD86, consistent with a GC derived activated B cell memory population(272, 285). Recent studies identified two DN subsets, DN1 (CXCR5⁺CD19⁺CD21⁺) and DN2 (CXCR5⁻CD19⁺CD21⁻CD11c⁺), with DN2 accounting for the expansion of DN B cell population in SLE(286, 287). DN2 are also expanded with aging, autoimmunity, infection, and transplant rejection and are known as ABCs(288, 289). The expansion of ABCs has been reported in other autoimmune diseases such as RA, Crohn's disease, and multiple sclerosis, suggestive of a role for these cells in generalised immune dysregulation(289). Human and murine SLE peripheral ABCs require Tbet and TLR7 signalling for their generation and are Tbet⁺(286, 289, 290). In a female patient with a TLR7 gain of function mutation as the cause of monogenic SLE, ABCs were expanded in blood, with this study recapitulating the genetic defect in Bcl6^{flox/flox}; Cd23^{cre} mice to suggest that accumulation of ABCs contributes to GC independent autoantibody production(115).

Studies of ABCs in lupus prone mice and in vitro co-culture, suggest these cells can differentiate into plasmablasts, and depletion of this population ameliorates disease and reduces autoantibody titres, suggesting a pathological role(291) (287). In human SLE CD11c⁺ Tbet⁺DN B cells/ABCs correlate with development of skin and renal disease. As IL-21 is increased in SLE, this could hypothetically promote plasma cell differentiation from the expanded CD11c⁺Tbet⁺DN B cell/ABC population, enhancing numbers of autoantibody secreting cells(286, 287). Th17 and IL-17 may contribute to sustaining plasma cells, since transfer of Th17 depleted human SLE PBMCs into immunodeficient mice results in attenuated plasma cell numbers in comparison to mice receiving nondepleted PBMCs, while IL-17 enhanced plasma cell survival(292).

Both plasma cells and ABCs are present in the kidneys in LN patients, however a trajectory analysis did not show a path between ABCs and plasma cells(190, 287). While broad spectrum targeting by inhibiting the general B cell marker CD20 or B cell survival marker

BAFF are currently used for SLE treatment, better understanding of pathways involved in the expansion of specific B cell populations in disease is needed to enable the development of therapeutics specifically targeting pathogenic B cell subsets(293).

1.4 Lupus Nephritis

LN is a glomerulonephritis characterised by IC deposition and immune cell infiltration into the kidney and a serious complication of SLE(64). 20-65% of people with SLE will develop renal involvement, with progression to ESRD in 10-22% of those affected, requiring RRT in the form of dialysis or a kidney transplant(294, 295). Blacks, Hispanics, and Asians with SLE have a higher risk of developing LN in comparison to Caucasians(74). Aside from ethnicity other risk factors likely include younger age(78). Paradoxically, the gender bias is switched in LN, with males more like to develop renal disease (male: female,1.1-1.7:1), characterised by more aggressive presentation and increased risk of progression to ESRD(296, 297). This illustrates the heterogeneous presentation of SLE, and thus the limitation in using any single animal model to mimic the disease. Within SLE cohorts, renal disease confers a high risk of mortality, with an estimated patient mortality rate of 46.7% within 5 years of being diagnosed with ESRD(298). Strikingly there has been no reduction in the rate of development of ESRD, and no reduction in the mortality rates for LN in the last 30 years(298-300).

1.4.1 Clinical picture of LN

Initial presentation of renal lupus can vary from microscopic haematuria (blood in the urine) or modest proteinuria (40-49%), nephritic syndrome (renal impairment with haematuria) (12-29%), nephrotic syndrome (severe protein leak with oedema and hypoalbuminuria) (30-36%) or rapidly progressive glomerulonephritis (GN) (2-3%)(301, 302). A recent decline in patients presenting with renal insufficiency and an increase in those presenting with isolated urine abnormalities suggests improvements in early diagnosis, but this has not led to a

change in the distribution of biopsy based histological severity, highlighting the limited concordance of clinical and histological parameters(301, 302).

1.4.2 Uses and limitations of histological classification of LN

The ISN/RPS classification of LN standardises the reporting and interpretation of LN biopsies using histopathological features to group LN into 6 different and non-consecutive classes(59). The first two classes are primarily characterised by changes in the mesangium. Class I (minimal mesangial LN) is defined by the presence of mesangial IC deposits with histologically normal glomeruli, whereas class II (mesangial proliferative LN) additionally includes mesangial hypercellularity and mesangial matrix expansion(303). Class III (focal LN) features endocapillary and/or extracapillary GN involving <50% of the glomeruli and can be subclassified as active or inactive, focal (only in some glomeruli), segmental (only in part of the glomerulus) or global (the entire glomerulus), with subendothelial deposits(303). Class IV (diffuse LN) describes endocapillary and/or extracapillary GN involving >50% of the glomeruli. Class V (membranous LN) is characterised by subepithelial deposits, this class can be found in conjunction with class III or IV, and severe sclerosis is also a feature of this class(303). Class VI (advanced sclerotic LN) is defined by global sclerosis, without any residual activity in $\geq 90\%$ of glomeruli(303). IC deposition is seen across all classes, with typical positive immunofluorescence staining for immunoglobulins (IgG, IgM, IgA) and complement proteins (C3, C1q) highly specific to LN, though not all these are present in all biopsy samples, therefore IC positivity in conjunction with other histological features are used for diagnosis and classification(303).

Class III and IV and mixed class (class V+III/IV) are associated with worse outcomes, however disease progression can occur in people initially presenting with class I and II disease, with such risk difficult to predict(304, 305). The prognostic value of subcategories global and segmental in class III/IV disease is controversial, with poor interobserver consensus between pathologists in defining lesions as segmental or global, and no clear

differences in clinical outcomes between the class IV-S and IV-G subcategories despite serological and morphological differences(60, 306).

A retrospective analysis demonstrated a poor association between the ISN/RPS classification and clinically relevant outcomes such as overall renal survival and renal flare, limiting ability to stratify patient treatment or predict disease progression(307). The assumption that in a heterogenous disease such as LN, all patients within the same class of disease will have the same prognosis, independent of the type of lesions, might negatively impact treatment(307). There is also discordance between clinical and histological remission, in one study, 62% of patients with histological remission had clinically active disease and one third of patients with complete clinical remission did not achieve histological remission, including cases with active histological disease characterised by high chronicity indices and predictive of poorer renal prognosis (61, 308). This discordance may reflect the limitations of current histopathology in distinguishing active disease from chronic 'old' damage, and the relevance of chronic damage and nephron loss for future renal progression even in the absence of active inflammation. The main emphasis of ISN/RPS is glomerular injury with little importance given to tubular injury, however there is positive correlation between tubulointerstitial damage and poor renal prognosis, therefore minimal inclusion of tubular injury may further limit the prognostic value of the ISN/RPS classification(309-311).

Increased renal disease activity, or flares, are associated with decline in renal function, poorer response to therapy and a greater likelihood of progression to ESRD or mortality in LN, but biopsy carries significant bleeding risk, limiting its use as a tool to track disease activity(312, 313). Hence molecular disease markers that can translate to blood or urine biomarkers are needed to improve disease monitoring. The ISN/RPS classification does consider molecular markers of disease, however glomerular lesion transcriptomic signatures have been described with possible relevance for treatment response,

highlighting an opportunity to harness such signatures to advance diagnostic and prognostic pathological classification for SLE(314).

Transcriptomic analysis on bulk renal biopsy tissue samples from LN patients detected higher levels of adaptive immune infiltrate characterised by increased expression of markers associated with antigen presentation and activation, and this correlated with reduced eGFR and increased renal tubular damage, highlighting syndecan 1 (SDC1) as inversely correlated with LN infiltrate and damage(315). Immune transcriptomic profiling on bulk LN renal tissue before and after induction of treatment suggested that responders had increased expression of type I interferon genes, while non-responders had elevated T cell activation transcripts(316), whereas blood mRNA sequencing identified signatures for active lupus nephritis attributed to granulocytes and plasmablasts(317). The above studies pooled RNA from all cells, obscuring the cellular origin of these RNA signatures. ScRNA-seq overcomes this limitation by uniquely labelling the RNA from each cell(318). ScRNA-seq in 21 LN kidneys suggested that a high IFN score (derived from combining expression of interferon stimulated genes) in tubular populations correlated with poor treatment response and identified tubular immune related pathways such as TNF signalling which distinguished between proliferative (class III/IV) and membranous disease (class V)(319). However, this study only discriminated 3 tubular populations, and did not explore glomerular signatures, with glomerular cells poorly represented in the scRNA-seq dataset. Nevertheless, this work suggests that overlaying molecular signatures to histopathology can identify pathways relevant to disease, allowing for better stratification of LN.

1.4.3 Mechanisms of LN

1.4.3.1 IC mediated damage in disease

The presence of autoantibodies is required for the development of LN, with circulating autoantibodies detectable on average 3 years prior to the onset of symptoms and

deposition of ICs a hallmark of LN. (320). Several hypotheses have been proposed to explain IC mediated kidney damage, the first is that preformed circulating ICs are passively deposited, resulting in subsequent inflammation and damage to the glomeruli(65). However, it has been difficult to detect or isolate preformed ICs from the blood of SLE patients, and in murine studies transfer of ICs into non-autoimmune mice did not result in kidney deposition, suggesting a low GBM affinity or increased hepatic clearance(321, 322). Indeed, ICs transferred into lupus prone MRL/lpr mice attenuated disease in both pre-disease mice and nephritic mice, suggesting that these ICs had the potential to reduce rather than promote autoantibody production(323).

A second theory is that circulating autoantibodies bind to “planted antigens” in the kidney forming ICs *in situ*, these autoantigens could arise from accumulation of apoptotic debris, with cationic parts of nucleosomes able to bind to anionic GBM components serving as a bridge to facilitate autoantibody binding(324-326). Human LN serum antibodies, in conjunction with protein eluted from the kidneys of nephritic autoimmune mice NZB/W, bind to histone H1 and DNA, and colocalise to electron dense deposits associated with the GBM, suggesting binding to trapped chromatin material(327, 328). Supporting this hypothesis, circulating concentrations of anti-nucleosome antibodies in people with SLE have been observed to predict likelihood of renal specific relapse(329, 330).

The third hypothesis suggests cross-reactive antigens on the surface of renal resident cells drive autoantibody mediated injury(65). While anti-dsDNA or anti-nuclear antibodies are classically described, other nephritogenic antibodies could be important, with antibodies to vimentin, α -enolase and annexin A1 also reported in human LN glomeruli and serum(331, 332). Injection of anti- α -enolase antibodies into immune competent BALB/c mice resulted in a quarter of mice developing proteinuria and renal lesions in line with class IV LN, whereas injection into immune deficient SCID mice resulted in 50% developing proliferative GN, suggesting a distinct role for renal antigen specific autoantibodies in disease(331).

Isotypes differences may be relevant for disease with IgG2 and IgG3 most common in the kidney, and anti-C1q IgG4 seen mainly in conjunction with class V LN(331, 333).

Not all people with SLE and IC deposition develop symptomatic renal disease, indicating that other mechanisms are required for the development of LN. Complement component C1q is found in ICs in the kidney, and high anti-C1q antibody titres correlate with renal disease and activity(334, 335). Murine experiments suggest that anti-C1q antibodies can be damaging, anti-C1q administration into mice with existing C1q deposition caused renal damage, proposed to be via anti-C1q binding to C1q and stimulating Fc γ R activation(64, 336). However, some people with LN have no circulating anti-C1q antibodies at diagnosis(64). Thus, while IC deposition plays a role in mediating renal immune infiltration and *in situ* activation, the extent of renal damage depends on mechanisms that remain to be fully elucidated.

1.4.3.2 Immune mechanisms in LN

Immune infiltrate into the kidneys is composed of both innate and adaptive cells, with a role for both arms of immunity in mediating renal tissue damage (Figure 1.5).

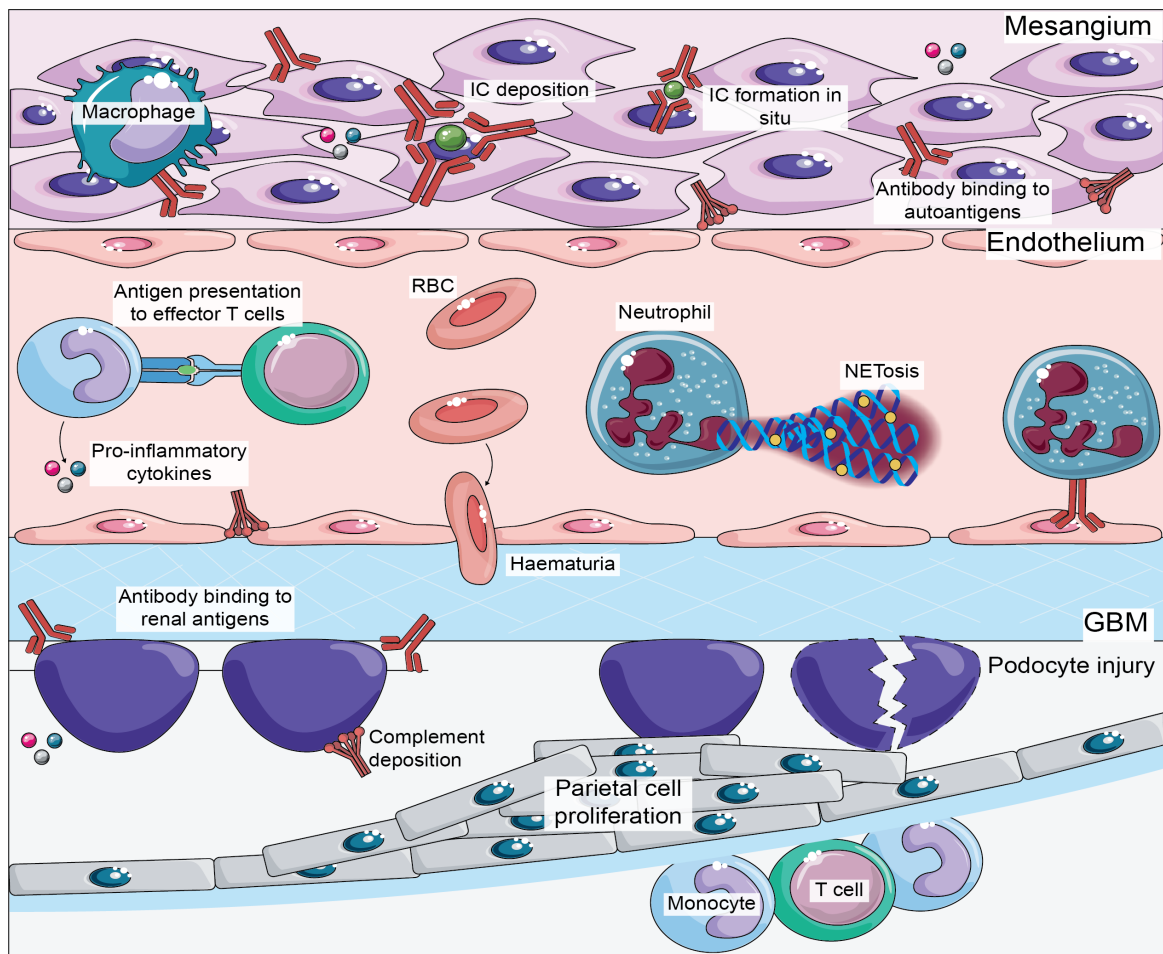


Figure 1.5: Immunological pathways implicated in LN.

Autoantibodies targeting “planted” glomerular antigens, IC and complement deposition in the kidney causes innate and adaptive immune cell infiltration. Innate cells bind via Fc receptors to immunocomplexes in the kidneys. IC deposition results in the production of proinflammatory mediators by resident cells which are chemotactic for innate immune cells. Innate immune cells release type I IFNs leading to the infiltration and activation of adaptive immune cells and further production of cytokines, chemokines. Neutrophils in glomerular capillaries undergo NETosis, increasing the burden of inflammatory mediators and autoantigens in the renal environment. Progressive damage results in mesangial matrix expansion, podocyte effacement, parietal cell proliferation and tubular damage. If left unchecked, glomerular, and tubular inflammation can lead to interstitial fibrosis and glomerulosclerosis, finally progressing to ESRD. Adapted from (5, 6)

1.4.3.2.1 Innate immune components in LN

Neutrophils are the most abundant leukocyte in circulation and are present in kidneys of people with active LN(166). Impaired NET degradation predisposes to renal disease in SLE suggesting a link between neutrophils and renal damage(166). Neutrophils are thought to access deposited ICs via migration through the glomerular endothelial fenestrae, here they can bind via their Fc γ R to the Fc portion of ICs, and once activated undergo NETosis

resulting in glomerular damage(337). In mice deficiency or blocking of Fc γ R prevents IC mediated activation and recruitment of neutrophils and protects from autoimmune diseases(338). Multiphoton imaging shows that in normal physiology these cells migrate through the glomerular vasculature, but in an in vivo model of GN neutrophils are shown to be retained in the glomerular capillaries during inflammation via Mac-1 (CD11b/CD18)(339). NET mediated damage and deposition in the kidney may increase the burden of “planted antigens”, for autoantibodies to bind(165). Decreased NET degrading capacity in SLE serum is associated with LN, low complement levels and increases titres of circulating anti-histone and anti-DNA antibodies(340). In lupus prone MRL/lpr mice neutrophil elastase from NETs degrades vascular endothelial cadherin expressed by glomerular endothelial cells, impairing endothelial cell contacts, and causing vascular leakage, which contributes to glomerular protein leak(341).

DC populations have been shown to accumulate in the kidneys with disease in murine models (e.g., MRL/lpr and NZB/W) and humans, however their role in the development of LN remains poorly understood (342, 343). In lupus prone *SLE1Tg7* mice overexpression of TLR7 in CD11c⁺ cells, which includes DCs, results in an expanded CD11b⁺ cDC population in the glomeruli, with enhanced ability to promote T cell proliferation(181). The relevance of this population in LN outside the context of TLR7 overexpression is unclear, but renal CD11c⁺DCs promote T helper cell responses in MRL/lpr and NZB/W lupus prone mice(343). In renal tissue from humans with lupus, immature DCs, or DCs expressing markers CD86, and DC-lysosome associated membrane glycoprotein (DC-LAMP), required for DC maturation, appear localised to the tubulointerstitial space, with higher TGF β expression in cDC populations in fibrotic samples as compared to non-fibrotic(342, 344). In autoimmune mice, and people with class III/IV disease, tubulointerstitial DCs produce C1q, which can opsonise and thus enhance phagocytosis of apoptotic and necrotic cells, but in the context of the LN, could result in enhanced uptake and presentation of renal antigens further perpetuating disease(345).

In lupus nephritis flare a unique inflammatory monocyte derived DC (infDC) population, is localised to the periglomerular and tubulointerstitial space, in proximity to T cells suggesting crosstalk between these two populations driving T cell activation(346). Despite the importance of DCs in systemic disease, IFN production and antigen presentation, the contribution of this population towards the development of renal disease remains unclear.

Serum NK cell numbers, cytotoxicity and proliferative capacity are reduced in LN, perhaps because these cells are migrating to inflamed tissue (347, 348). Killer cell lectin like receptor 1 (KLRK1/NKG2D) is one of the main activating receptors expressed by NK cells, with increased expression of its ligands Rae1 and MICA observed in the glomeruli of MRL/lpr mice and people with LN respectively(349). In MRL/lpr mice, NKG2D ligand expression correlates with infiltration of mature activated NK cells characterised by increased IFN- γ production(349).

NK cells localise to the periglomerular region and tubulointerstitium, within inflammatory infiltrates, in people with active LN(350). Transcriptomic analysis of human LN renal immune cells identifies two distinct NK populations with the larger population characterised by cytotoxic genes such as PRF1, and the smaller population described as tissue resident NK cells, suggesting a possible role for the renal disease microenvironment in modulating NK phenotype(190, 351).

Renal monocyte infiltrates in LN correlate with doubling of serum creatinine and worsening renal disease(352). Circulating monocytes can differentiate in situ into macrophages or DCs depending on cues from the renal microenvironment(353). CD16⁺ non-classical monocytes accumulate in the glomeruli in both murine lupus models (e.g., MRL/lpr, NZB/W and Yaa mice) and humans with LN, correlating with disease severity (202, 203). Non-classical patrolling monocytes promote GN independent of autoantibody production, as is demonstrated by the development of disease in Rag deficient mice in which there are no mature B or T cells, but not in NR4A1 deficient mice in which they are no patrolling

monocytes, even in the presence of IC deposition, autoantibody production and systemic disease(203). Patrolling monocytes can monitor the glomerular vasculature in both inflammatory and non-inflammatory settings via integrins $\beta 2$ and $\alpha 4$ (354). In GN they can additionally enhance neutrophil recruitment via TNF, increasing glomerular neutrophil retention and generation of reactive oxygen species (ROS)(354). Proinflammatory 6-sulfo-LacNAc⁺ (slan) monocytes (slanMo), recruited via IC engagement of CD16, may be important for neutrophil recruitment and retention in GN via production of TNF α and neutrophil chemoattractant CXCL2, and also drive a feedback loop in which these cells stimulate CX3CL1 production by endothelial cells perpetuating monocyte recruitment(355, 356). Consistent with this, scRNA-seq in LN kidney identified high expression of CX3CL1 on epithelial cells with its receptor CX3CR1 highly expressed on infiltrating monocyte and macrophage populations(190). Targeting non-classical monocytes in lupus prone TNFAIP3-interacting protein 1 mutant mice relieves renal disease even in the presence of continued systemic disease, suggesting a specific monocyte driven pathway to LN potentially amenable to drug targeting(203)

Macrophages are present in glomerular lesions in LN, and numbers of CD68⁺ macrophages at biopsy correlate with disease parameters such as anti-dsDNA titres and response to treatment(189, 357, 358). Adult renal macrophages can originate from two sources, firstly embryonic yolk sac progenitors which are prenatally seeded, able to self-renew and long lived, and secondly from bone marrow derived haematopoietic stem cells, which become monocytes that infiltrate tissue and differentiate into macrophages(359). The relative contribution from bone marrow progenitors varies across age and with disease(359-361). Distinct roles have been suggested for monocyte and yolk sac derived macrophages in the kidney during disease, with monocyte derived macrophages important for IC uptake and presentation while yolk sac derived macrophages may regulate leukocyte infiltration(362). Macrophages can be divided into M1 and M2 categories, with the M2 further subdivided

into M2a, M2b and M2c(353). However, evidence for the M1/M2 division in LN kidney is mixed, and macrophages may acquire an alternative profile characterised by expression of pro-inflammatory, reparative/degradation and regulatory genes, that does not fit with either the M1 or M2 phenotype(363-366).

The role of M1 vs. M2 like macrophages in LN may be context dependent. M2 like macrophages, particularly M2a and M2c, are associated with pro-fibrotic TGF β expression and the presence of these subtypes correlated with renal interstitial fibrosis score(189). In MRL/lpr, and in murine nephritis due to administration of anti-GBM antibodies, macrophage depletion through pharmacological inhibition of colony stimulating factor 1 receptor (CSF-1R) is protective, but as monocytes abundantly express CSF-1R simultaneous monocytic inhibition may account for this effect (367, 368).

Human LN renal macrophage populations have been described using scRNA-seq. A tissue resident macrophage cluster expressed higher levels of interferon stimulated genes and inhibitors of TLR signalling and lower levels of pro-inflammatory genes, in comparison to healthy controls(190). Clusters corresponding to activated, phagocytic and M2 like macrophages were expanded in the LN kidneys, with trajectory analysis suggesting a transition from activated monocyte/macrophages into a phagocytic phenotype and ending in alternatively activated M2 macrophage characterised by phagocytic markers such as MERTK and CD163, consistent with previous studies showing abundance of M2 macrophages in LN(189, 190). M2 differentiation is associated with a higher disease activity score and with proliferative and mixed classes as compared to pure membranous LN(369, 370). Comparable injury associated LN macrophages have been described between four lupus murine models and renal myeloid cells from LN patients, characterised by markers associated with lipid metabolism and phagocytosis, which could be relevant to renal remodelling during injury in LN(371). This latter study demonstrates scRNA-seq can identify shared immune profiles between human and murine LN, but did not examine non-immune

cell subsets, and so cannot explore the role of the LN renal environment in polarising macrophages.

1.4.3.2.2 Adaptive immune components in LN

T cells make up a significant portion of the kidney immune infiltrate in LN, with numbers correlating with the extent of chronic renal damage(263, 350, 372). A predominance of CD8 T cells in periglomerular immune infiltrate or the tubulointerstitium predicts poor renal outcomes, pointing to a role for these cells in mediating renal damage(263, 373).

LAMP1, a marker of cytotoxic degranulation, is expressed on CD8 T cells in human LN, localised to peritubular infiltrate, and positively correlated with proteinuria(374). A potentially exhausted renal CD8 T phenotype has been described in MRL/lpr mice; however transcriptomic analysis of human LN biopsy samples found cytotoxic CD8 T cells without markers of exhaustion(190, 264, 375). Whilst this may reflect heterogeneity in CD8 activity between murine models and LN patients, 16/24 participants in the Arazi *et al* study were on some form of immunosuppressive treatment which may have altered the renal transcriptomic phenotype(190). In MRL/lpr mice and in people with LN, the presence of CD4 T cells with a Th1 bias correlates with renal damage, while Th2 cells correlated with hypercellularity only(376-378). Tfh cells have also been identified in LN biopsy samples in association with B cells, though their clinical relevance is unknown(249). Evidence for Th17 cells in the development of nephritis mainly comes from correlations with circulating Th17 cells(379). DN T cells represent another major source of IL17 production and are found in the kidneys of people with LN(266). IL17 promotes epithelial to mesenchymal transition and production of further chemokines and cytokines by both renal and non-renal cells, promoting further immune cell infiltration(379). Genetic deletion of IL17, or proteins involved in IL17 signalling, ameliorates renal disease in animal models(380). In summary, T cells are present in the biopsy samples of LN patients, with CD8 cells particularly linked to pathology, but the overlap between murine and human LN T cell activity remains debated.

ScRNA-seq in human LN kidneys identified B cells subsets consisting of plasmablasts, interferon stimulated B cells, naïve B cells and ABCs(190). Plasma cell infiltration in the kidneys is associated with histological disease. These long-lived plasma cells are reliant on cytokines such as IL-6 and BAFF for their survival and are found in nephritic but not pre-nephritic mice, suggesting downstream disease amplifying effects rather than a disease initiation function(381). Whilst the role of B cells in autoantibody production has been established in SLE, the relative role of different subsets in the development of LN remains to be determined.

1.4.3.3 Role of resident renal cells in disease pathogenesis

Renal resident cells interact with and contribute to the renal inflammation seen in disease and their roles as active participants in lupus pathology are increasingly recognised.

MC dysfunction is a key element of the classification of LN(303). Anti-dsDNA antibodies deposit in the mesangium and can bind to cross reactive annexin II and α -actinin on the surface of MCs in vitro(382, 383). When MCs are cultured with LN serum, anti-dsDNA antibodies or cytokines relevant to SLE ($\text{TNF}\alpha$, $\text{IFN}\gamma$, $\text{IFN}\alpha$ and $\text{IL1}\beta$), they produce pro-inflammatory cytokines such as macrophage colony stimulating factor (M-CSF), and upregulate extracellular matrix proteins (e.g., COL4A1, COL1A1), matrix metalloproteinases (MMP9) and inhibitors of matrix metalloproteinases(384). These factors contribute to extracellular matrix remodelling, a feature of mesangial matrix expansion in LN(384).

In vitro evidence supports a role for MCs as antigen presenting cells (APCs). MCs stimulated with $\text{IFN}\gamma$ upregulate MHC II and co stimulatory molecules CD40 and CD80(385). To assess antigen presentation to CD4 T cells in the context of a specific antigen, $\text{IFN}\gamma$ stimulated murine MCs were isolated and pulsed with ovalbumin (OVA). Co-culture with OTII CD4 T cells, which have restricted TCR specificity to OVA, resulted in T

cell proliferation and activation(385). This suggests that in LN MCs could act as additional APCs to infiltrating T cells, perpetuating inflammation.

MCs can also influence macrophage polarisation in vitro, with skewing to an M1 phenotype observed when co-cultured with IFN γ stimulated MCs and M2 phenotype when co-cultured with platelet derived growth factor BB (PDGF-BB) stimulated MCs(386). The role of MCs in macrophage polarisation in vivo is not clear, however resting MCs produce CCL2 which drives M2 polarisation(386). It is possible that MC cytokine secretion contributes to the switches in macrophage polarisation states seen with disease progression(386).

MC localised expression of a proliferation inducing ligand (APRIL), which stimulates B cell survival, is reported in LN biopsy samples(387, 388). In vitro MC stimulation with BAFF, found in SLE serum, enhances MC proliferation, suggesting another possible driver for LN mesangial expansion(389). MCs also have ITGA8 dependent phagocytic capacity, with impaired phagocytosis in MCs from ITGA8 knockout mice (45, 390). Injured MCs may switch to a more fibroblast/pericyte like phenotype in models of nephrotoxic GN, diabetes and focal segmental glomerulosclerosis, which could impact MC phagocytic capacity, however this has not been shown in a LN model(52). Much of the evidence for MC function is based on in vitro models, and their contribution to LN in vivo remains to be elucidated.

Loss of podocytes, or podocyte foot process effacement, damages the glomerular filtration barrier and leads to proteinuria in LN. Expression of podocyte specific markers such as synaptopodin, WT1, and nephrin is diminished in biopsies from people with proliferative LN and urinary nephrin and synaptopodin levels correlate with disease, likely due to urinary podocyte shedding(391, 392). Podocyte antigen specific antibodies such α -enolase and annexin A1 suggest a role for these cells as disease targets(331). Like MCs, transgenic T cell experiments in vitro suggest podocytes have antigen presenting capacity(393). Anti-GBM disease is less severe in mice with podocyte specific MHC II deletion, in comparison to wildtype controls, with decreased immune cell infiltrate and activated effector memory

CD4 T cells, suggesting podocytes can drive T cell activation *in situ*, though this has not been directly shown in LN(393).

Podocytes may also clear IgG via neonate Fc receptor (FcRn), FcRn deficient mice have increased renal IC deposition and delayed IgG clearance, however this is not a podocyte specific deletion therefore the observed effect may be due to loss of expression in cell types such as proximal tubules(25).

The NLRP3 inflammasome is important for conversion of IL1 β and IL18 to their active forms and is activated in LN biopsy podocytes and proteinuric lupus prone mice, with pro-inflammatory cytokine IL1 β expression(394). Necroptosis and NLRP3 activator receptor protein kinase 3 (RIP3) is also expressed in the LN human podocytes, and pharmacological inhibition of RIP3 inhibits NLRP3 and necroptosis in MRL/lpr mice and reduces anti-dsDNA titres, and renal immune infiltrate while preserving renal function(395). Given that aberrant cell death is a feature of SLE, inhibition of necroptosis and IL1 β production could be therapeutically useful, but the cell types involved and a causal effect for necroptosis have not been fully established in SLE(395).

The role of GECs in LN is poorly understood, although breaches in contact between endothelial cells are associated with increased vascular leakage in LN(337). Cytokine stimulation of GECs, to model the LN inflammatory environment *in vitro* causes upregulation of pro-inflammatory cytokines, chemokines, adhesion molecules (VCAM1) and co-stimulatory molecules (ICOS-L), suggesting a possible role for GECs in leukocyte recruitment, adhesion, and activation(396). Hyaluronic acid (HA), a component of the endothelial glycocalyx, may contribute to T cell homing and tissue extravasation via CD44 expression on T cells, enzymatic removal of HA with hyaluronidase reduces T cell homing and improves survival in lupus prone mice(397). Likewise, expression of TRIM27, involved in endothelial glycocalyx preservation, is increased in GECs in people with LN and

suppression of TRIM27 in GECs in vitro or in MRL/lpr mice reduced endothelial injury and VCAM1 expression(398).

In vitro IC stimulation of endothelial cells promotes secretion of endothelin-1 causing disruptions in endothelial-endothelial cell contacts and endothelial injury, which may promote leukocyte infiltration(399). Clinical trials to assess the therapeutic potential of endothelin antagonists (e.g., sparsentan) are currently underway for IgA nephropathy which is another IC mediated disease(400)

Renal tubular epithelial cells (RTECs) can produce cytokines, chemokines, and mediators which interact with immune cells(370). By in situ hybridisation, LN RTECs have been identified as major producers of IFN α in severe disease, this may have an autocrine effect with immunoproteasome activity (LMP7), and increased RTEC MHC I expression with the potential to activate CD8 T cells(401). In MRL/lpr mice CD8 T cells are found close to the proximal tubules, and activated CD8 T cells can cause RTEC apoptosis, suggesting that antigen cross presentation by RTECs may activate CD8 T cells, which then induce RTEC apoptosis and thus tubulointerstitial damage in LN(402). In LN glomerular cell pathology predominates, however these data demonstrate the need for a better understanding of tubular damage for disease stratification and treatment.

Many of the studies contributing to the understanding of renal cells in LN have not been independently validated or were performed in vitro, limiting extrapolation to physiological setting. Mechanistic studies are largely from in vivo animal models with limited evidence from human disease, in part due to difficulties in obtain renal tissue. Increasingly the role for renal cells in LN is becoming recognised and a better understanding of crosstalk with infiltrating cells is needed for better prediction and classification of disease.

1.4.4 Treatment and management of LN in the clinical setting

There is no cure for SLE or LN, the aim of current treatments for LN is to slow down kidney damage and progression to ESRD. Choice of treatment is based on clinical variables and histological classification and is centred around broad-spectrum suppression of the immune system(16). Complete response to therapy is defined by proteinuria <0.5-0.7g/24hours, with partial response characterised by 50% reduction in proteinuria(293).

Class I and II LN are generally treated with a background of hydroxychloroquine, with the addition of renin angiotensin aldosterone system (RAAS) blocking agents if proteinuria is >0.5g daily, further addition of low dose glucocorticoids (GCs) alone or in conjunction with azathioprine may be used if protein excretion is >1g daily(16, 403). Evidence for immunosuppression in Class V LN is not strong and the mainstay of treatment is similar to non-proliferative disease with the addition of immunosuppression only in the context of nephrotic syndrome(403).

Proliferative disease (Class III and IV) carries poorer prognosis, and treatment is divided into two phases, an induction phase using an intensive immunosuppressive agent lasting between 3-6 months and a less intense but prolonged maintenance phase to prevent flares (404). MMF and CYC are the most commonly used immunosuppressants(405, 406). MMF inhibits inosine-5'-monophosphate dehydrogenase which is required for the *de novo* production of purines such as guanosine, since B and T cells rely on *de novo* purine synthesis during replication, MMF preferentially inhibits B and T cell DNA replication(407). CYC is an alkylating agent which forms crosslinks between adjacent DNA strands at guanine position 7 leading to replication failure and cell death, with a dominant effect in T cells resulting in reduced IFN γ and selective inhibition of Tregs(408). Rituximab, a monoclonal antibody targeting CD20, is an alternative induction agent, commonly used for refractory or relapsing LN(403). Voclosporin, a novel calcineurin inhibitor, showed superior efficacy in achieving remission versus placebo when combined with MMF and low dose

GCs(370, 409, 410). During the maintenance phase immunosuppression is continued with the lowest dose possible that can control renal and extra renal symptoms(16).

The major limitation in the use of these drugs is the unfavourable side effect profile. All these drugs increase infection risk due to immune suppression. Side effects associated with CYC include leukopenia, infertility, and increased risk of malignancy(411). MMF has lower ovarian and gonadal toxicity, making it a viable treatment for women who are of childbearing age, but it is teratogenic in pregnancy. Side effects include leukopenia, nausea, and diarrhoea(412). Adherence to medication represents an additional challenge, with non-adherence a likely factor contributing to LN progression(413).

Newer biologic therapies based on immune mechanisms are starting to reach clinical practice. The monoclonal antibody belimumab blocks soluble BAFF, thereby inhibiting most classes of circulating B cells. The addition of Belimumab to standard therapy reduced risk of a composite end point including proteinuria, in comparison to placebo, however more long-term studies are needed to validate these observations(414).

Obinutuzumab, a new CD20 targeting monoclonal antibody, achieves better B cell depletion than rituximab(415). In phase II clinical trials in proliferative LN, participants receiving Obinutuzumab in conjunction with MMF and low dose steroids had improvements in renal function at week 52, and B cell depletion was maintained in 94% of patients more than 4 months post the last infusion, phase III clinical trials are underway(416). Belimumab and obinutuzumab broadly suppress B cells, but do not adequately deplete plasma and memory B cells, therefore newer T cell-based therapy approaches in the form of chimeric antigen receptor (CAR) T cells may offer a targeted option(417). A single study utilising CD19 CART cells in patients with refractory SLE resulted in complete remission of disease, with no relapse observed even after reconstitution of B cells. B cell profile composed primarily of naïve B cells with few plasma and memory B cells detected, therefore demonstrating the feasibility of such an approach in the future(417)

Other new therapeutic strategies not yet approved in the UK include type I interferon receptor antagonism for SLE, with trials currently underway to assess its effect on LN(176, 418).

Since complement activation is prominent in LN, complement inhibitors might be expected to be efficacious. Eculizumab inhibits of complement C5, preventing the formation of the membrane attack complex, and has been used for lupus nephritis complicated by thrombotic microangiopathy(419). Ravulizumab like eculizumab binds to C5 and is being currently evaluated in phase II clinical trials for use in proliferative LN(420). Avacopan is a complement 5a receptor antagonist licensed for the treatment of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis, a systemic disease characterised by auto antibodies to neutrophil cytoplasmic granules(421). This drug is blocks C5a binding to C5aR on neutrophils, inhibiting neutrophil activation and migration, with clinical trials for its use in LN currently underway(421). Daratumumab inhibits CD38, which is highly expressed on plasma cells, and has been shown to be effective in a small study in active refractory LN(261, 422). Other notable drugs in trials for treatment of LN include itolizumab (CD6 monoclonal) and CFZ533 (anti-CD40 preventing T cell co-stimulation)(423). The development of drugs based off mechanistic information will be crucial to improve clinical outcomes.

1.5 Modelling molecular mechanisms of SLE and LN

Murine models are important tools for studying cellular mechanisms underlying disease and identifying therapeutically targetable pathways, because access to human kidney and other tissue is limited and animal models permit manipulation to explore function and test candidate therapies. Murine models of SLE can be divided according to spontaneous and inducible disease, with variations in onset, renal involvement, and systemic phenotype(424).

1.5.1 Murine Models

1.5.1.1 *Spontaneous models of SLE*

Spontaneous disease arises due to underlying genetic predisposition to autoimmunity. Crossing of NZBxNZW results in F1 offspring (NZB/W) which develop autoantibodies including anti-dsDNA, splenomegaly, hypergammaglobulinemia and IC mediated GN, onset is after 6 months with a bias towards females like human disease, an additional 10 strains are derived from NZBxNZW F1 mice including NZM2410 which develop IC mediated GN faster than NZB/W mice(425, 426). Mice with the NZM2410 susceptibility loci (sle1,2,3) bred on C57BL6 background permit more accessible study of transgenes and genetic knockouts(425).

MRL/lpr mice have a spontaneous retrotransposon insertion in the apoptotic regulator CD95 (Fas) leading to premature termination of transcription and reduced CD95 expression(427). These mice develop a full panel of lupus antibodies seen in humans, nephritis, and additional manifestations such as skin lesions and vasculitis, splenomegaly and lymphadenopathy develop with accumulation of DN T cells due to impaired apoptosis. Onset is earlier in female mice, with 50% mortality at 22 weeks(123). BXSB mice have a translocation of the *Yaa* locus, consisting of an estimated 16 genes, from the X to the Y chromosome, resulting in twofold increase in *Tlr7* expression and an SLE-like phenotype with more severe in males, like human LN(428)

1.5.1.2 *Inducible models of SLE*

Inducible models include injecting wild type mice with a terpenoid alkane, tetramethylpentadecane (pristane). In this model onset is up to 6 months, not all mice develop nephritis, and this compound causes complications unrelated to SLE(429, 430). An alternative approach uses topical application of the TLR7 agonist imiquimod on the ears of wild type lab strains (BALB/c, FVB/n and C57BL/6) for 8 weeks, resulting in an activated

immune phenotype, antinuclear autoantibodies (ANAs) and glomerulonephritis, but this model has been reported to be associated with high mortality and differences in onset, manifestations, and severity of disease dependent on the murine strain utilised, with BALB/c mice observed to have a more rapid onset of disease(431). Injection of mice with antibodies targeting GBM components is useful for the study of pathways downstream of IC deposition but does not fully model SLE(432).

1.6 Current knowledge of LN from single cell transcriptomics

The advent of newer transcriptomic technologies has made it possible to study heterogeneity within LN in greater depth and detail, by profiling the transcriptome of individual cells. Bulk RNA-seq analysing the global profile of the kidney has provided some insights but bulk RNA studies are hampered by the abundance of tubular over glomerular renal tissue, which can mask glomerular signals. To address this Peterson et al, used laser capture microdissection (LCM), to collect glomeruli specifically, and demonstrated upregulation of pathways associated with myeloid lineage cells(433). However, bulk approaches are unable to delineate the precise identities of these samples, or distinguish infiltrating immune from resident tissue cells, which is essential in a complex disease such as LN.

ScRNA-seq technologies provide a way to study the transcriptome of individual cells at a high resolution and have been key in advancing understanding of cellular heterogeneity within seemingly homogenous populations and the relative contributions of cell types in disease and health. The input for single cell experiments can be cells or nuclei dissociated from tissue or extracted from cell lines, followed by a method for single cell isolation prior to mRNA profiling(434). Droplet based isolation methods are the most commonly used and encapsulate a single cell in a microfluidic droplet, within which cDNA is produced by reverse transcription(435). Each cDNA fragment consists of a unique barcode which is specific to the cell and a unique molecular identifier (UMI) specific to the cDNA fragment. After

sequencing and mapping of reads to a reference genome, the combination of the unique cell barcode and UMI are used to generate a cell by gene count matrix for downstream analysis(434)

The feasibility of scRNA-seq has been demonstrated in both healthy human kidney tissue and renal disease samples, identifying a range of cell types, with diseases studied including diabetic nephropathy, IgA nephropathy, LN, and renal carcinoma, with similar findings observed in mouse kidney tissue(436-439). The only officially published human single cell dataset in LN comes from the Accelerated Medicines Partnership (AMP), a large multi-centre project, studying rheumatoid arthritis and SLE utilising 'omic' approaches. In this study kidney biopsy samples from 24 LN patients and 10 controls were analysed using two approaches, kidney cells were analysed as a whole, or immune cells were purified by sorting on immune markers such as CD45(190, 319). Analysis of kidney cells (mostly tubular and some glomerular) from SLE patients revealed markers of fibrosis and interferon signature genes (ISGs) in patients with poor response to treatment(319). The immune cell analysis revealed 21 distinct immune cell clusters, corresponding to subtypes of B, T, and myeloid cells(190, 319). This were further segregated based on phenotype, for example subsetting of T cells revealed effector, Treg and Tfh populations and suggested a role for resident epithelial cells in recruitment of immune cells(190). An additional dataset from the same multi-centre project, yet to be published, compared renal myeloid populations from people with LN with those found in the kidneys of immune 4 murine models of LN, pre and post the development of nephritis(440). This analysis identified expanded classical and non-classical monocyte and resident macrophage signatures which were shared between people with LN and nephritic mice and suggested that these populations correlated with chronicity and activity indices of disease(440).

Single cell sequencing datasets in the kidneys have limited representation of glomerular populations and to address this several studies have utilised single-nuclei RNA sequencing

(snRNA-seq)(441). Comparative studies between the two modalities in the kidney have demonstrated increased signatures of stress due to enzymatic dissociation in the scRNA-seq dataset, compared to snRNA-seq, with all glomerular populations represented in the snRNA-seq data(441). SnRNA-seq can be performed on frozen tissue, allowing for the use of stored samples in biobanks, without overt loss of cell populations, however immune populations have been observed to be less well represented with this method, with fewer total transcripts detected when compared with scRNA-seq due to discarding of cytoplasmic RNA(442). SnRNA-seq is therefore an attractive approach for glomerular based diseases like LN, whereby both infiltrating and renal populations can be captured.

1.7 Summary and aims of thesis

Published human LN data is predominantly immune focused, and therefore not suited to the study renal tissue-immune crosstalk. The advent of new technologies has made it possible to dissect underlying mechanism more effectively. However, a better understanding of the crosstalk between intrarenal and immune cellular responses in LN injury is still needed to better predict prognosis and develop more targeted therapies. Use of human tissue to study LN is attractive but has several limitations. Diagnosis, and therefore biopsy, can be late in the disease and dominated by signals of fibrosis and chronic renal damage, rather than active pathways driving disease and damage to already limited tissue might make isolation of certain vulnerable cell populations more difficult(318). Furthermore, findings are confounded by inherent human heterogeneity, environmental, drug related and genetic factors, which can only be circumvented by large samples sizes and are associated with high financial costs. Murine models represent a relatively inexpensive and rapid method for the study of such a heterogenous disease on a homogenous background, allowing for the dissection of potentially causative pathways whilst limiting the role of potential confounders such as environment. Additionally, disease onset can be monitored in such models allowing for the study of changes associated with early disease processes, minimising the effect of non-specific fibrotic signals associated with chronic renal damage.

Hypothesis: Discovery based approaches using renal tissue from models of early LN can elucidate molecular and cellular changes in non-immune and immune resident and infiltrating cells associated with disease progression and identify potential therapeutic targets.

To study early disease two murine models of SLE with renal disease were chosen, the first a TLR7 inducible model using agonist imiquimod on BALB/c mice and the second MRL/lpr

mice in which disease develops spontaneously because of a deficiency in apoptotic mediator *Fas*.

Aim 1: Establish a platform to characterise LN in vivo.

Chapter 3 of this thesis characterises and compares both the systemic immune and renal phenotype of two murine LN models using flow cytometry and histological techniques. The aim of these experiments was first to develop a platform for studying LN in vivo and to assess suitability to model of early disease, and second to generate a comprehensive phenotyping dataset to support in-depth transcriptomic analysis in Aim 2.

Aim 2: Identify both overlapping and model specific immune and tissue single cellular transcriptomic signatures in early LN.

Chapter 4 describes the analysis from snRNA-seq experiments carried out on the kidneys of both the LN models. SLE is a heterogeneous disease and therefore changes in one of the two models studied here may provide insight into the disease heterogeneity in the kidneys. Whereas overlapping features shared between models may have broad clinical relevance and lead to the identification of potentially therapeutic pathways. This chapter goes on to explore immune cell localisation within the kidney by piloting sub cellular resolution spatial transcriptomics and provides protein level validation of key candidate targets for downstream evaluation.

Aim 3. Functionally validate potential novel LN genes and pathways as candidate therapeutic targets

Chapter 5 begins to explore targets identified from snRNA-seq analysis, with the aim of functionally validating these as candidate therapeutic targets. This chapter first describes an interventional in vivo experiment carried out in MRL/lpr mice, targeting leukocyte homing protein *Itga4* with a blocking antibody (PS/2) which prevents binding of *Itga4* to its ligands. The second part of this chapter describes in vitro experiments carried out in a

CRISPR/Cas9 generated *ITGA8* knockdown human mesangial cell line to determine whether this mediates the acquisition of a more fibrotic phenotype as suggested in chapter 4.

In summary, chapter 3 establishes a platform for in vivo exploration of early LN in two murine models of disease, generating an in-depth phenotyping dataset. Having established the suitability of these models as models of early disease, chapter 4 generates an extensive renal and immune transcriptomic dataset, identifying shared and altered cellular signatures associated with early disease and chapter 5 describes results from experiments functionally exploring transcriptomically identified disease signatures. Chapter 4 and 5 additionally demonstrate that this integrated renal snRNA-seq approach can be interrogated for potential therapeutic targets with a similar approach applicable to human pathology.

Chapter 2 : Materials and Methods

2.1 Animal work

All experiments were carried out in compliance with UK Home Office Guidelines and the Animals Scientific Procedure Act 1986 (amended 2013). Procedures were reviewed by the clinical medicine animal care and ethical review body (AWERB) and conducted under Home Office Project Licenses P84582234 and P79A4C5BA. All experiments were carried out on female age matched mice. I would like to acknowledge Tanya Cheetham, and all the staff at the Functional Genetics Facility for their help with colony maintenance, in particular I would like to thank Peter Hinton for his help with procedures.

2.1.1 Breeding and maintenance

Female BALB/cOlaHsd mice were purchased from Envigo at 4-5 weeks of age. MRL/MpJ (#000486) and MRL/lpr (#000485) were purchased from The Jackson Laboratory. For all in vivo experiments with MRL/MpJ or MRL/lpr mice, female 15-week-old mice were used. Animals were housed in specific pathogen free individually ventilated cages and kept on a 12- hour light/dark cycle from 8:00-20:00, with food and water available freely.

2.1.2 In-vivo imiquimod treatment and monitoring

BALB/c female mice aged 6 weeks were treated topically with either 5% Aldara (Imiquimod) cream (Meda Pharmaceuticals) or Vaseline (Unilever) control on both ears, 3 times weekly for 5 and 8 weeks. MRL/lpr and MRL/MpJ were weighed 3 times weekly from the age of 12 weeks. Mice urinary protein levels were monitored using One Step Protein Urine test strips (AcuPrime) at the same time as treatment/weighing. Urine collection was done at week 8 for imiquimod treated mice and controls and at week 14 for MRL/lpr and MRL/MpJ mice. Briefly, mice were individually housed in cages containing hydrophobic Kit4Kat cat urine sample collection sand (KIT4CAT, Hyperdrug), for a maximum of 3 hours. Collected urine

was centrifuged at 10,000g for 10 minutes at 4°C, supernatant was removed and sent to the MRC Harwell Mary Lyon Centre, for analysis of total protein and creatinine.

2.1.3 In vivo Itga4 blockade

Mice were administered InVivoMAb anti-Itga4 (PS/2) (#BE0071, Bioxcell) or InVivoMAb isotype control IgG2b (#BE0090, Bioxcell) intraperitoneally at a dose of 250µg per mouse once a week for 8 weeks.

2.1.4 Sample processing

All mice were euthanised using an appropriate Schedule 1 method. Whole blood collected for serum isolation was spun at 17,000g for 10 minutes at 4°C, supernatant removed and subsequently stored at -20°C. Whole blood for plasma isolation was extracted into Microvette 200 Lithium heparin tubes (201292, Sarstedt), spun at 2000g for 10 minutes at 4°C, supernatant was removed and sent to the MRC Harwell Mary Lyon Centre, for analysis of serum creatinine and albumin. Spleen, thymus, bone marrow (BM) and mesenteric lymph nodes (MLN) were harvested and placed in RPMI 1640 media (Sigma) containing 2% foetal calf serum (FCS) (Sigma) and 20mM HEPES solution (Sigma). Tissues were processed to single cell suspensions by mashing through 70µm cell strainers (Fisher Scientific, Loughborough, UK), and the total number of white blood cells were counted with a haemocytometer using white blood cell counting fluid (1.5% acetic acid, 0.5% methyl violet in water). Harvested kidneys were either snap frozen in liquid nitrogen and stored at -80°C or placed in 10% neutral buffered formalin (NBF) (HT501128, Sigma) for 24 hours and thereafter shifted into 70% ethanol prior to being processed for paraffin embedding.

2.1.5 Flow cytometry

2.1.5.1 Surface staining

Cells were plated at a density of $0.5-1 \times 10^6$ into 96-well round bottom plates (Greiner) and washed twice with FACs buffer (Hanks Buffered solution (Sigma) containing 2% FBS,

20mM HEPES and 0.05% Sodium Azide (Sigma). For surface staining, samples were resuspended in antibody staining cocktail containing 10% Fc Block, single stain controls were resuspended with single antibody stains and incubated on ice for 30 minutes. Cells were washed twice, resuspended in FACs buffer, and run on the BD FACS CANTO10c. Antibodies used for surface and intracellular staining are detailed in Appendix A. Data was analysed using FlowJo software (BD).

2.1.5.2 Intracellular staining

Intracellular staining was performed using True Nuclear Transcription Factor Buffer Set (Biolegend) according to the manufacturers protocol. Cells were resuspended in FACs buffer prior to being run on the BD FACS CANTO10c. Data was analysed using FlowJo software (BD).

2.1.6 Calculation of absolute numbers of cells from flow cytometry

Each population was quantified as a percent of the lymphocyte gate in FlowJo. The calculated percentage values were multiplied by the total number of white blood cells (section 2.1.4), to obtain the absolute number of lymphocytes for each individual population.

2.2 Molecular Biology

2.2.1 Western blot

Cell lysate was prepared from cultured cells using supplemented RIPA buffer (sc-24948, Santa Cruz Biotechnology). After lysis the suspension was centrifuged for at 12,000g at 4°C for 15 minutes, and supernatant used for quantification. Protein concentration of each sample was determined by Pierce™ BCA assay (23225, Thermofisher). 20-25ug of lysate was made up in a solution of NuPAGE™ LDS Sample Buffer (4X) (NP0007, Invitrogen) and NuPAGE™ Sample Reducing Agent (10X) (NP0009, Invitrogen) and denatured by boiling for 5 minutes at 95C, prior to being loaded onto a 4-20% Mini-PROTEAN® TGX™ Tris-HCl gel (Bio-Rad) and run at 120V for 60 minutes. Proteins were transferred onto a

nitrocellulose membrane (10600012, Cytiva) and blocked in 5% non-fat milk diluted in TBS-T (150mM NaCl (7647145, Sigma), 50mM Trizma[®] Hydrochloride pH 7.4 (T2194, Sigma) and 0.05% TWEEN[®]-20 (9005645, Sigma)) for 60 minutes at room temperature. Membranes were incubated with anti-ITGA8 (PA71555, Thermofisher; 1:1000) overnight and then incubated with HRP linked anti-Rabbit IgG (111035003, Jackson ImmunoResearch; 1:5000). Monoclonal anti- β -actin peroxidase (A3854, Sigma; 1:10000) was used as a loading control. Protein bands were detected using Amersham ECL prime (RPN2232, Cytiva).

2.2.2 ELISA

Sera IgG concentration was measured by sandwich ELISA using Bethyl laboratories mouse IgG quantification kits (E90-131, Cambridge Biosciences) according to the manufacture's protocols. Serum was diluted 1:12000 and developed using TMB substrate (00-4201-46, Lifetechnologies). Absorbance was measured at 450nm on a BioTek ELx808 plate reader.

2.2.3 Anti-nuclear and anti-dsDNA staining

For ANA titres the positive control was a positive sample from the 3i's cohort(443). Serum samples were diluted 1:100 and with Hep-2 slides (A Menarini Diagnostics) for 30 minutes at room temperature in a humidifying chamber, washed with PBS (14190144, Gibco) and MilliQ water, thereafter bound antibody was detected by incubation with FITC conjugated anti mouse IgG (H+L) (1:500; 626511, Thermofisher) for 20 minutes at room temperature in a humidifying chamber. Slides were washed and mounted (38015, A Menarini Diagnostics) prior to imaging on Evos M5000 Imaging system. Images were analysed using multiparametric image analysis in ImageJ and intensity was scored from 0-4 based on control samples and FITC QC slide (A Menarini Diagnostics). Scores were manually checked by eye by an independent blinded researcher.

For the detection of anti-dsDNA titres serum positive control was serum from mouse model of graft versus host disease(444). Experimental samples were diluted 1:50 and incubated with Crithidia lucillae slides (26110, Bio-Rad), all subsequent steps were the same as above. Images were scored by eye by an independent blinded researcher for positivity.

2.3 In vitro assays

2.3.1 Cell culture

The mesangial cell line, conditionally immortalised using temperature sensitive simian virus 40 large T antigen (tsSV40) and human telomerase (hTERT), was a kind gift from Professor Gavin Walsh and Professor Moin Saleem at the University of Bristol(445). Mesangial cells were cultured in R10 complete media (RPMI 1640 media with 10% FCS, 20mM HEPES solution, 2mM L-glutamine (G7513, Sigma), 100units/mL penicillin, 100ug/ml streptomycin (P0781, Sigma) 1X minimum non-essential amino acids (11140050, Gibco), 1mM sodium pyruvate (11360070, Gibco), 55µm 2-Mercaptoethanol (21985023, Gibco) and 1X insulin-transferrin-selenium (41400045,Gibco)) and incubated at 33°C, 5% (v/v) CO₂ for routine passage. For antibiotic selection after electroporation mesangial cells were incubated in R10 complete media further supplemented with 0.4µg/ml puromycin (A1113803, Gibco). Mesangial cells were differentiated at 37°C, 5% (v/v) CO₂ for 10 days, prior to being used for further experimentation(445).

2.3.2 Cytokine stimulation

Differentiated mesangial cells were seeded on 6 well plates (Greiner) at a density of 500,000 cells/well in antibiotic free R10 complete media and left overnight. The next day media was removed and replaced with either fresh antibiotic free media or antibiotic free media containing 10ng/ml of cytokines TNF α (570102, Biolegend), IFN α (592702, Biolegend), IFN γ (570202, Biolegend) and IL1 β (PHC0814, Thermofisher). Cells were incubated in cytokine containing media for 24 hours.

2.3.3 Electroporation

Electroporation of mesangial cells for genetic manipulation was with the help of Dr Phalguni Rath and Katie Holden. Cells were prepared for electroporation using the Neon™ NxT Electroporation System 10-μL Kit (Thermofisher) as per the manufacturer's instructions and electroporated using the Neon™ NxT Electroporation System (Thermofisher) with the following electroporation parameters; 1400V; 20ms; 2 pulses. Each electroporation reaction consisted of 100,000 cells resuspended in Buffer R. For CRISPR/Cas9 experiments, Alt-R s.p. HiFi Cas9 nuclease V3 (1081060, IDT), was incubated with sgRNAs (Synthego) targeting *ITGA8* exon 7 (Cr1-5'acctctgccatcaagactaaagg) and exon 27 (Cr2-5' tgtgcagtgggacgactcgaagg) at room temperature for 15 minutes for formation of ribonucleoprotein complexes. The ribonucleoprotein complexes were subsequently added to cell suspension in Buffer R and electroporated. For overexpression plasmid transfection, 500ng of plasmid (pRP[Exp]-EGFP/Puro-CAG>hITGA8[NM_003638.3], VB900007-7973bfv, VectorBuilder) was added directly to the cell suspension in Buffer R and electroporated. After electroporation cells were seeded in a 12 well plate containing R10 complete media for further culture.

2.3.4 Single clone sorting and screening

Sorting of electroporated mesangial cells was done with the help of Ghada Youssef in the WTCHG flow cytometry facility. Electroporated mesangial cells were prepared at a concentration of 1×10^6 /ml and sorted into 96 well plates (1 cell/well) using the FACS Aria Fusion Cell Sorter. DNA was isolated from clones using QuickExtract™ DNA Extraction Solution (QE09050, Cambio) as per the manufacturers protocol and screened for deletion using the following PCR primers after which the products were visualised via gel electrophoresis:

CR1:

Forward- 5' tccagctcatttatcatgcttgg 3'

Reverse- 5' aagaggggaagacgcgatgag 3'

Product size-214bp

CR2:

Forward- 5' aagactctgacgtgttccac 3'

Reverse – 5' cccagtgagcagtgtgtatg 3'

Product size-189bp

CR1-CR2:

Forward- 5' tccagctcatttatcatgcttgg 3'

Reverse – 5' cccagtgagcagtgtgtatg 3'

Product size-239bp

2.3.5 RNA extraction and quantitative real time PCR (qPCR)

RNA was extracted from cultured cells using PureLink™ RNA Mini Kit (12183018A) as per the manufacturers protocol and quantified using a NanoDrop 1000 spectrophotometer (Thermofisher). Reverse transcription was carried out on 1000ng of RNA per sample using LunaScript® RT SuperMix Kit (E3010, NEB) as per protocol. Expression levels of genes of interest were quantified by qPCR using PowerUp™ SYBR™ Green Master Mix (A25776, Thermofisher). The primers are listed in Table 2.1. Fold changes for all genes were calculated relative to endogenous control *TUBB* using the $2^{-\Delta\Delta CT}$ method for quantification.

Table 2.1: qPCR primer sequences

Gene	Orientation	Sequence (5'-3')
FN1	Forward	acaacaccgaggtgactgagac
	Reverse	ggacacaacgatgcttctgag
COL4A1	Forward	gccagcaaggtgttacaggatt
	Reverse	agaaggacactgtgggtcatctatt
PDGFRB	Forward	agacacgggagaatacttttc
	Reverse	agttcctcggcatcattaggg
CXCL12	Forward	ctcaacactccaaactgtgccc
	Reverse	ctccaggtactcctgaatccac
ACTA2	Forward	ctatgcctctggacgcacaact
	Reverse	cagatccagacgcatgatggca
CCN2	Forward	cttgccaagctgacctggaaga
	Reverse	ccgtcggtagatactccacaga
C7	Forward	gtggtttggctactgttgaggg
	Reverse	tccaagaggaccagcaactcca
TUBB	Forward	ctggaccgcatctctgtgtact
	Reverse	gccaaaaggacctgagcgaaca

2.4 Histology

2.4.1 Tissue processing and embedding

Formalin fixed tissue was transferred to 70% ethanol and further processed by an automated tissue processor; Here tissue was dehydrated via sequential incubation in two baths of 70% ethanol, two baths of 90% ethanol, three baths of 100% ethanol and three baths of Neo-Clear™ (Sigma), thereafter tissue was incubated in three baths of melted wax and subsequently embedded into paraffin blocks. Sections of 3µm were cut using a HistoCore MULTICUT R microtome (Leica).

Frozen tissue was directly embedded in OCT (KMA-0100-00A) and then cut into 10µm sections using a Leica CM1950 Cryostat (Leica).

2.4.2 Histological Stains

Stained sections for all protocols were mounted with DPX mountant (44581, Sigma) prior to being scanned using the Leica Aperio CS2 slide scanner at 40x magnification. Sections

were kindly reviewed by renal pathologist's Prof. Ian Roberts and Dr Maria Soares for classification of disease.

2.4.2.1 H&E

Slides were deparaffinised in 3 changes of Neo-Clear™ for 5 minutes each and rehydrated in a series of graded alcohol (100%,90%,70% and 50%) for 5 minutes each. Slides were incubated in distilled water for 5 minutes and then placed in neat haematoxylin (105174, Merck) for 10sec, then run in distilled water for 5 minutes and then dipped in acidic alcohol (5ml concentrated HCl (34.5-38%) in 0.5L 70% ethanol). Subsequently slides were washed in distilled water for 5 minutes and incubated in 3 changes of alcohol (50%, 70% and 90%) and then placed in Eosin Y solution (102439, Merck) for 1 minute. Slides were then rinsed twice with 100% alcohol and placed in 2 changes of Neo-Clear™ for 5 minutes each and then mounted.

2.4.2.2 Periodic Acid Schiff Staining

Slides were deparaffinised in 3 changes of Neo-Clear™ for 5 minutes each and rehydrated in different concentrations of alcohol (100%,100%,95% and 95%) for 5 minutes each. Slides were incubated in 2 changes of distilled water for 5 minutes and then placed 0.5% periodic acid solution (104821, Merck) for 5 minutes. Slides were rinsed in 3 changes of distilled water and stained in Schiff's reagent (109033, Merck) for 10 minutes at room temperature and then rinsed in lukewarm tap water for 10 minutes. Slides were then placed in haematoxylin (105175, Merck) for 30sec, and rinsed in distilled water for 1 minute prior to being placed in 1X Scotts tap water substitute (S5134, Sigma) for 1 minute. Slides were rinsed in distilled water for 30sec and then dehydrated in two changes of 100% alcohol and 3 changes of Neo-Clear™ for 1 minute each and then mounted.

2.4.3 Immunohistochemistry

2.4.3.1 *Paraffin embedded tissue*

Slides were deparaffinised with Neo-Clear™ rehydrated in a series of graded alcohols, and placed in Tris buffered Saline (TBS) (150mM NaCl (Promega), 50mM Trizma® Hydrochloride pH 7.6 (Sigma)). Enzymatic antigen retrieval using 0.75mg/mL PRONASE protease, *Streptomyces griseus* (Sigma) was done at 37°C for 45 minutes and stopped by incubating with TBS at 4°C for 1 hour. Slides were then blocked with blocking buffer (TBS containing 1% bovine serum albumin (BSA) (Sigma), 10% goat serum (MP Biomedicals) and 0.01% TWEEN®-20) for 1 hour at room temperature. Slides were incubated with antibodies against C3 (MP Biomedicals) and C1q (JL-1) (Cambridge Biosciences) diluted 1:100 overnight at 4°C. For detection of IgG, slides were incubated with anti-mouse IgG (H+L) (Invitrogen) for 30 minutes at room temperature. Slides stained with an unconjugated primary antibody were incubated with Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen) secondary antibody 1 hour at room temperature. All slides were counterstained with DAPI (Cambridge Biosciences) at a concentration of 1µg/mL and incubated for 5 minutes. Sections were mounted with VECTASHIELD® Antifade Mounting Media (Vector Laboratories) and visualised with a Leica SP8 confocal microscope.

2.4.3.2 *Frozen tissue*

Frozen sections were fixed in two changes of ice-cold acetone (24201, Sigma) for 10 minutes each with air drying in between. Sections were then washed three times with TBS and incubated in TBS for 5 minutes prior to being blocked with blocking buffer for 1 hour at room temperature. Thereafter slides were incubated with primary antibodies at 1:100 dilution against Itga4 clone R1-2 (103629, Biolegend) and Vcam1 clone 429 (141061082, Thermofisher) overnight at 4°C. Slides were then stained with Alexa Fluor 594 conjugated goat-anti-rat IgG (H+L) (A11007, Thermofisher) for 1 hour at room temperature, counterstained with DAPI, mounted and visualised as above.

2.4.3.3 Quantification of fluorescence

Cumulative intensity was measured across equivalent numbers of glomerular and non-glomerular regions defined by nuclei and brightfield. Glomerular intensity was then background corrected for each glomerulus using the following formula:

Corrected total glomerular fluorescence (CTGF) = Cumulative intensity - (Area of glomerulus × Mean fluorescence of background readings)

2.4.4 Electron Microscopy

Harvested kidney tissue was placed in 3% glutaraldehyde 0.1M cacodylate buffer for fixation. Samples were sent to the University of Southampton Biomedical Imaging Unit where they were processed by Catherine Griffiths and Anton Page for electron microscopy. Briefly samples were washed and incubated for post fixation in 2% osmium tetroxide for 2 hours, washed and then incubated in 4% neodymium acetate solution for 30 minutes. Samples were dehydrated by incubation in increasing concentrations of ethanol for 20 minutes each (70%, 90% and 100%). Samples were incubated in acetonitrile for 30 minutes, placed in liquid epoxy resin for 6 hours and then polymerised by being heated at 60°C for 16 hours. Resin blocks were sectioned at a thickness of 70nm and stained with lead citrate prior to imaging. Images were acquired using a FEI Tecnai T12 Transmission Electron Microscope.

2.5 Transcriptomic profiling

2.5.1 scRNA-seq tissue processing

Kidneys were harvested and decapsulated on ice. All steps took place on ice unless stated otherwise. For glomeruli enrichment the whole kidney was used whereas, for cortex enrichment equal portions of renal cortex were cut out from each mouse and used in the subsequent dissociation steps. The tissue was finely minced and passed through 100µm strainer (Fisher Scientific). Cortex cells were then resuspended in enzyme mix made up in

DPBS containing cold active protease from *Bacillus licheniformis* (10mg/mL) (Sigma), 0.5M EDTA (Santa Cruz Biotechnology) and 125U/mL Dnase-1 (VWR). Cells from the whole kidney were passed by gravity flow through a 70µm filter, flow through was discarded and the filter was turned upside down and rinsed with enzyme mix, and liquid collected. Cells in enzyme mix were triturated for 15s every 2 minutes for 10 minutes. Samples were transferred into Miltenyi C-tubes (Miltenyi Biotec) and run on twice Miltenyi gentleMACS brain program_03 twice. Single cell dissociation was confirmed by microscope visualisation and the enzymatic reaction was stopped by addition of DPBS containing 10%FCS (10%FCS/DPBS). Samples were pelleted, resuspended in DPBS containing 0.01% BSA (0.01%BSA/DPBS) and passed through a 30µm filter. Red blood cells were lysed by ammonium-chloride-potassium (ACK) buffer. Cells were washed and finally resuspended in 0.01%BSA/DPBS. Concentration of live cells was determined using trypan blue (Sigma) and resuspended at a concentration of 1000cells/µl and loaded on to the Chromium controller for generation of gel in emulsion bead (GEMs) (10X-Genomics).

2.5.2 SnRNA-seq tissue processing

Imiquimod experiment: All steps took place on ice unless stated otherwise, whole kidneys were minced and placed into a Dounce tissue grinder (HOM3584, SLS) containing Nuclei lysis buffer 1 (Nuclei EZ lysis buffer (NUC101, Sigma) supplemented with cOmplete™ EDTA-free protease inhibitor (5892791001, Roche), 0.5% RNasin® plus RNase inhibitor (N2615, Promega) and 0.5% SUPERase•In™ RNase inhibitor (AM2696, Thermofisher)). Tissue was homogenised 20-30 times then passed through a 100µm strainer and transferred back into the Dounce tissue grinder. Tissue was homogenised 10-15 times and incubated for 5 minutes on ice. Homogenate was passed through a 30µm strainer (4350040, pluriSelect) and centrifuged for 5 minutes at 500g. Supernatant was discarded, and the pellet was resuspended in Nuclei lysis buffer 2 (Nuclei EZ lysis buffer supplemented 0.1% RNasin® plus and 0.1% SUPERase•In™) and incubated on ice for 5 minutes prior to

centrifugation for 5 minutes at 500g. Supernatant was discarded and nuclei were resuspended in nuclei wash buffer (DPBS with 1%BSA and 0.2U/μl of Protector RNase inhibitor (03335402001, Roche), counted using trypan blue and resuspended at a concentration of 1000-12000 nuclei/μl and loaded on to the Chromium controller (10X-Genomics).

MRL experiment: Nuclei was isolate from 50mg of kidney cortex using Chromium Nuclei Isolation Kit (1000494, 10X Genomics) as per manufacturers protocol. Nuclei were stained with SYTOX™ AADvanced™ Dead Cell stain (S10349, Thermofisher) at a dilution of 1:500, prior to sorting 500,000 nuclei using the FACS Aria Fusion Cell Sorter with the help of Ghada Youssef. Nuclei were counted and resuspended as above.

2.5.3 Library prep and sequencing

After gel bead in emulsion (GEM) generation all subsequent library prep and sequencing steps were carried out by the Oxford Genomics Centre for sc/snRNA-seq. Sequencing was performed on a NovaSeq6000 system (Illumina).

2.5.4 Bioinformatics analysis

Raw sequencing data was mapped against mm10-2020-A and quantified with 10X-Genomics Cellranger count v4.0.0 (scRNA-seq), v6.1.1 (imiquimod snRNA-seq) and v6.7.0 (MRL snRNAseq) for the generation of gene expression matrices, subsequently analysed using R package Seurat(446). Data was filtered to remove doublets and low-quality cells, filtering criteria for individual datasets is reported in Chapter 4. For clustering and dimensionality reduction log-normalised and scaled gene expression counts were used. Clusters were identified using “FindClusters” feature on Seurat and the “FindAllMarkers” function was used to identify genes unique to a cluster based on differential expression between clusters for population annotation.

Slingshot v210.0 was used for trajectory analysis, log-normalised populations of interest were subset from the rest of the dataset and provided as input for the trajectory inference step(447). Pseudobulk differential state analysis was performed using muscat v1.17.0(448). Gene counts were aggregated per sample for each cluster generating a pseudo-bulk RNA count matrix, and differential expression was carried out between groups per cluster. For cluster vs cluster differential expression, the “FindMarkers” function in Seurat was used. Gene ontology-based gene set enrichment analysis was performed using clusterProfiler v4.10.0 function “gseGO” using a ranked gene list as input(449). Ligand receptor analysis was performed using CellChat v1.5.0, only pathways with an adjusted pvalue <0.05 for communication probability were used for subsequent analysis(450). Data was visualised using ggplot2 R package v3.4.4 and thresholds for significance are defined within each figure.

Raw data from the Arazi et al study was taken from ImmPort repository (accession code SDY997) and analysed in line with the published paper(190).

2.5.5 Calculation of gene-set scores

Genes used for calculation of interferon and exhaustion scores are listed in Appendix B-C. The interferon response gene set was taken from overlapping genes between 3 IFN modules (M1.2, M3.4 and M5.12) identified in the blood of people with SLE(451). For T cell exhaustion associated genes, overlapping genes between the following sets were taken from MSigDB; GSE9650_EFFECTOR_VS_EXHAUSTED_CD8_TCELL_DN, GSE9650_NAIVE_VS_EXHAUSTED_CD8_TCELL_DN, and GSE9650_GP33_VS_GP276_LCMV_SPECIFIC_EXHAUSTED_CD8_TCELL_UP(452, 453). Genes were converted from human to mouse orthologs using biomaRt v2.58.0, and all genes were found to have mouse orthologs.

For calculating the gene set score, expression of genes in a set were averaged across all cells and then ranked from highest to lowest. Based on expression genes were divided into

25 bins containing roughly equal numbers of genes. 100 controls genes were sampled randomly from each bin and mean expression was calculated for the set of control genes. The mean expression of control genes was subtracted from mean expression of genes in the gene set, giving an overall score per cell, this score was averaged across clusters by group to give a mean cluster/group score(454)

2.5.6 Converting murine to human annotations

Mouse MGI gene symbols were first converted to murine Ensembl ids using biomaRt v2.58.0. For conversion to human Ensembl ids, mouse Ensembl ids from the mmusculus_gene_ensembl database were mapped to human Ensembl orthologs from the hsapiens_gene_ensembl database using the dec2021.archive version of Ensembl. Genes with no human ortholog were removed, whereas expression for genes with one-to-many mappings was averaged per gene.

2.5.7 Dataset integration

Integration for datasets carried out in this thesis were done using Harmony batch correction, with 20 clustering rounds set for each round of integration and 20 rounds of integration in total. Tolerance for both parameters was set to “Inf” to ensure that the algorithm did not stop before the chosen number of rounds was completed(455)

2.6 Statistical Analysis

Data are expressed as mean $\pm$ 95% confidence interval unless stated otherwise. Results were analysed using unpaired students t-test, multiple t-tests using the Holm-Sidak method or one way ANOVA using the Holm-Sidak method to determine statistical significance and to correct for multiple comparisons, using GraphPad Prism 10 software (Prism, San Diego, USA). $P \leq 0.05$ was taken to be statistically significant.

Chapter 3 : Immune and renal phenotyping of mouse models of LN

3.1 Introduction

Murine models provide crucial biological information which has furthered our understanding of disease processes and translated into the development of clinically relevant therapeutics(1). More particularly, understanding of renal disease initiating events necessitates the study of early disease and in vivo this requires the use of models with a predictable time course of disease involving the kidneys and phenotypes translatable to human aetiology.

Several established models of murine SLE exist (section 1.5.1), with variability in disease features, therefore model choice was determined by development of renal disease, time and predictability of disease onset, histological features, and severity of renal disease. These criteria are largely determined by the mechanism underlying disease development, hybrid spontaneous models such as NZB/W have slow disease onset, whereas polycongenic models derived from the NZM harbouring combinations of susceptibility loci (Sle1-3) have demonstrated variable disease, with both sets of models requiring complex breeding for maintenance of disease phenotype(425).

The genetics of the background strain has a modifying effect in combination with specific mutations. Notably, models on the popular C57BL/6 background displaying significantly delayed renal disease, as exemplified by the change in phenotype observed between different strains harbouring the *lpr* mutation(456). Inducible models generally can be applied to any background strain and therefore represent an attractive option; however, there is variability in disease penetrance and induction period ranging from 8 weeks in the case of TLR7 agonist imiquimod to 6 months in the case of mineral oil pristane(430, 431).

Based on the aforementioned criteria, two murine models with differing aetiological drivers were chosen. The first, induced by topical application of TLR7 agonist imiquimod (IMQ) (henceforth referred to as the IMQ model) has been observed to cause disease after 4 weeks of drug application and mortality after 8 weeks of administration, the flexibility of this model in being applied to any murine strain, and comparatively rapid onset of systemic and renal disease made this a suitable choice(431). The second model chosen was *Fas* deficient MRL/lpr mice. Female MRL/lpr mice present with palpable lymphadenopathy by 12 weeks of age and urine abnormalities are detectable by 15 weeks leading to a 50% mortality observed in female mice at 20 weeks. The rapid onset and high penetrance of spontaneous disease, as well as the underlying pathology, made the MRL/lpr model suitable for the studies described in this thesis(457).

Other models with SLE-like features were tested for suitability, including the Roquin1 mutant Sanroque mice and the Bm12 inducible model. However, these models were found to have a significantly delayed and inconsistent pattern of renal disease on the C57BL6 background. Mortality due to systemic disease frequently occurred prior to development of renal phenotype and therefore they were deemed unsuitable for the purpose of this thesis.

The aims of the experiments described in this chapter were first to characterise and confirm the presence of systemic disease by immune phenotyping and assess autoantibody production and renal disease using a combination of histological and immunofluorescence methods to define disease features and relevance to human lupus nephritis. Additionally, I was looking for models that could be representative of early disease. The second aim was to generate a complementary phenotyping dataset which could be compared with extensive cellular transcriptomic profiling carried out in the next chapter. Therefore, this chapter describes phenotyping experiments that confirm the presence of an autoimmune phenotype in both models. The findings describe a series of novel findings relevant to human disease with implications for future discovery.

3.2 Imiquimod model of LN

3.2.1 Imiquimod treatment for 5 weeks results in only mild immune activation without detectable autoantibodies.

Imiquimod is a TLR7 agonist previously shown to induce a lupus like phenotype with renal involvement(431). Increased mortality (20%) has been described with 8 weeks of treatment with imiquimod, and 100% mortality observed after 15 weeks(431). Considerable variability in mortality rates has been reported in studies using this model, with some studies observing mortality as early as 4 weeks and others observing no mortality even at time periods later than 8 weeks(458, 459). Whilst subtle methodological differences in treatment regimen may be one explanation, differing disease severity and mortality may in part be explained by complex interactions between differing environmental variables and the immune system. Additionally, reported disease phenotype varied by murine strain, with systemic disease severity and histopathological renal scores greatest in BALB/c mice when compared with other strains (FVB/n and C57BL/6) for all time points assessed, suggesting enhanced susceptibility to IMQ induced systemic and renal disease in BALB/c mice(431).

Based on the published findings, BALB/c mice were chosen for IMQ experiments and pilot experiments were initially limited to 5 weeks of treatment during which mice were treated with either topical imiquimod or Vaseline (as a control), on both ears, three times weekly(431). Treatment for 5 weeks was well tolerated, characterised by mild immune activation, but with absence of anti-nuclear antibodies (ANAs) (Figure 3.1A-B). Serum and urinary analysis identified no significant alterations in serum creatinine and urinary protein with treatment, indicating normal renal function (Figure 3.1C-D). Periodic acid Schiff's staining identified no differences between treated and untreated mice by light microscopy, however treatment did result in glomerular positivity for IC deposition (C1q, C3 and IgG) (Figure 3.1E-F). Taken together, treatment for 5 weeks resulted in a mild systemic and renal autoimmune phenotype without the presence of ANAs, which are a hallmark of SLE.

Treatment for a longer duration of 8 weeks was therefore assessed and resulting in a more pronounced immune phenotype, positivity for ANAs and early renal disease detectable by light microscopy, described below. All further experiments represent data from mice treated for 8 weeks unless stated otherwise.

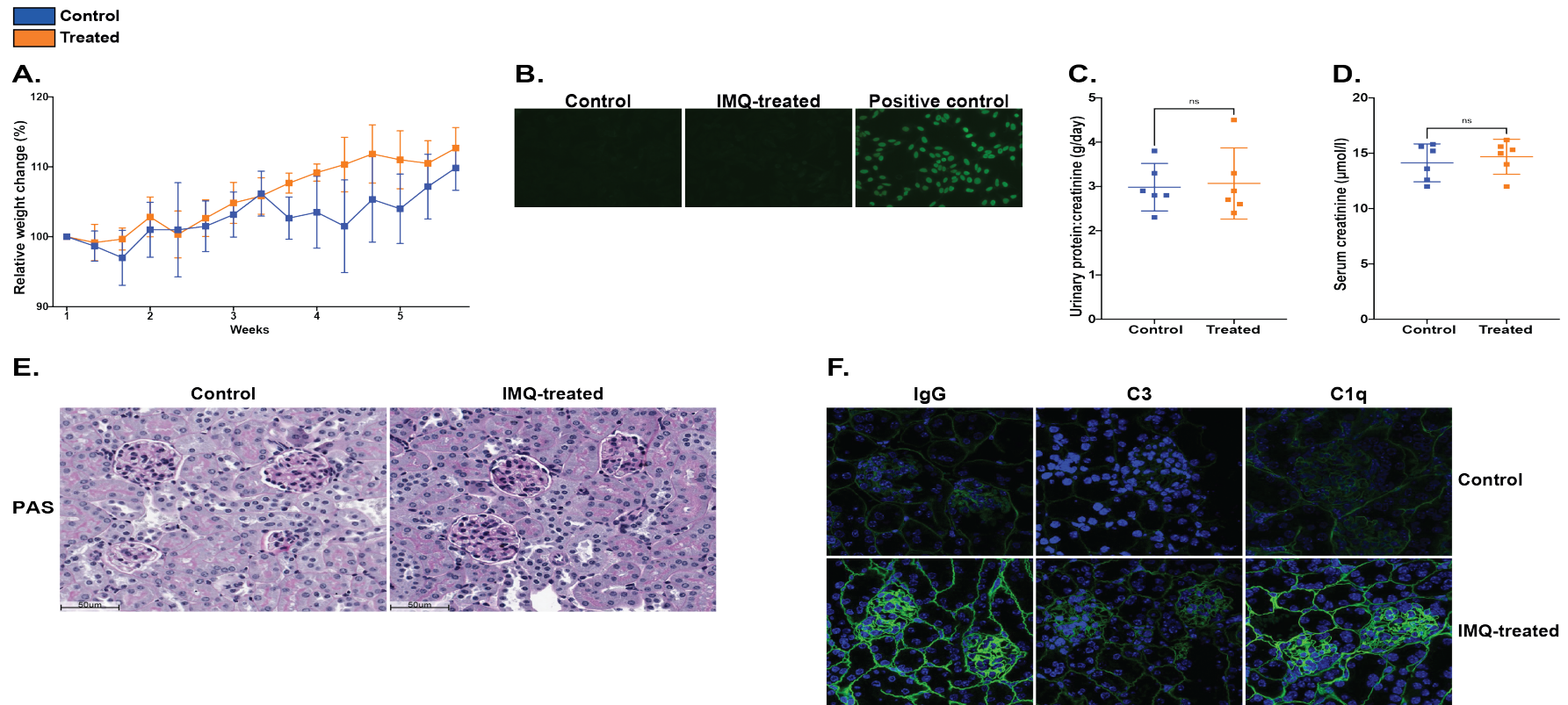


Figure 3.1 IMQ treatment for 5 weeks results in renal immune complex deposition but no detectable ANAs.

Mice were treated for 5 weeks with IMQ or Vaseline control. **(A)** Percentage change from starting weight of IMQ treated and Vaseline control mice treated for 5 weeks. **(B)** Representative images of ANAs in mouse sera detected by indirect immunofluorescence. **(C)** Urine protein creatinine ratio at 5 weeks for Vaseline control and IMQ treated mice. **(D)** Serum creatinine levels in Vaseline control and IMQ treated mice at 5 weeks. **(E-F)** Mice were treated for 5 weeks with IMQ or Vaseline control, culled and kidneys fixed in 10%NBF for 24 hours, processed and embedded into paraffin, prior to being cut into 3 μm sections. **(E)** Periodic-acid Schiff (PAS) stain of kidneys from Control (left panel) and IMQ treated (right panel) mice. Bar = 50 μm . **(F)** Immunofluorescence staining of kidney sections of control and IMQ treated mice for detection of IgG, C3 and C1q (green) with DAPI (blue) (63X magnification). Sera for ANAs was diluted 1:100. Data are mean $\pm$ 95% CI, analysed by a student t-test **(C-D)**. Data and images are representative of three independent experiments, each with 5-6 mice per group.

3.2.2 Imiquimod treatment for 8 weeks is well tolerated

Treatment for 8 weeks was not associated with differences in weight or mortality but was associated with splenomegaly (Figure 3.2A and B). A trend to an increase in urinary protein was observed in mice treated for 8 weeks however this was not significant, and there was no significant difference observed in serum creatinine between treated and untreated mice (Figure 3.2C and D). In mice tubular secretion accounts for 35-50% of creatinine clearance in the steady state and increases with disease (up to 70%), resulting in an overestimation of glomerular filtration, therefore renal damage resulting in a loss of creatinine clearance by >50% would be needed to see any impact on the glomerular filtration rate, casting doubt on the sensitivity of creatinine as a marker for measuring mild to moderate changes in glomerular filtration rate in mice(460). Glomerular damage sufficient to cause an increase in serum creatinine is unlikely to be observed in the context of early disease.

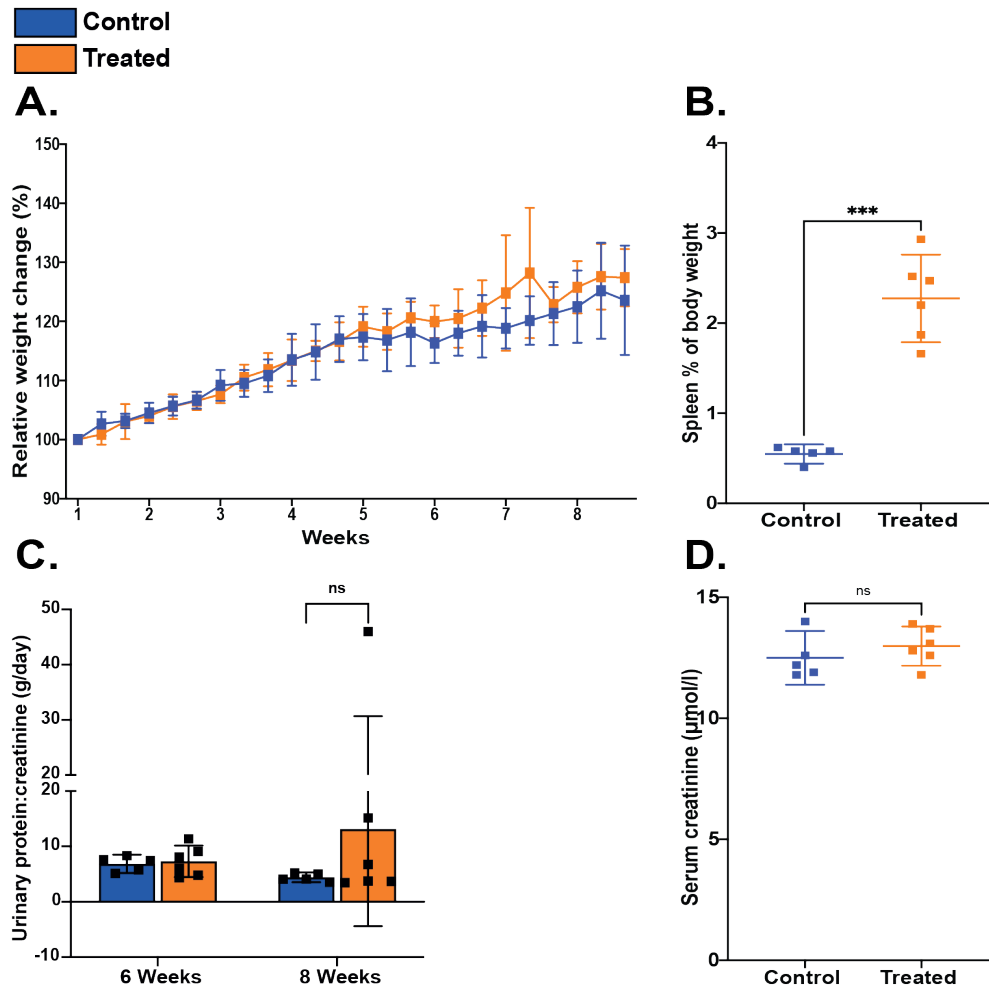


Figure 3.2: IMQ treatment is well tolerated for 8 weeks.

Mice were treated for 8 weeks with IMQ or Vaseline control. **(A)** Percentage change from starting weight of IMQ treated and Vaseline control mice treated for 8 weeks. **(B)** Spleen as a percentage of body weight in IMQ treated and Vaseline control mice for 8 weeks. **(C)** Urine protein creatinine ratio at 6 weeks and 8 weeks for Vaseline control and IMQ treated mice. **(D)** Serum creatinine levels in Vaseline control and IMQ treated mice at 8 weeks. Data are mean $\pm$ 95% CI, analysed by a student *t*-test *** $P \leq 0.001$ and representative of six independent experiments, each with 5-6 mice per group.

3.2.3 Imiquimod treatment causes T cell activation

No effect of treatment was observed on T cell development in the thymus (Figure 3.3A-E).

Within the splenic T cell CD4 compartment, flow cytometric analysis demonstrated increased numbers and proportions of activated effector memory (EM) CD4 T cells (CD44^{hi}CD62L^{low}) and increased numbers CD69⁺CD4 T cells (Figure 3.3F-H), with CD69 expression being a marker of early T cell activation. This finding is consistent with the

activation profile reported in peripheral circulating T cells from SLE patients and with findings from BXSB mice in which disease is also TLR7 driven(118, 461, 462). T-follicular helper (TFH) (PD1⁺ICOS⁺CXCR5⁺) and Tbet⁺CD4 T cells were significantly increased with treatment (Figure 3.3F-G). Although Tbet expression is associated with commitment to the Th1 lineage, further phenotyping of this increased Tbet⁺CD4 T cell population would be needed to confirm this, however this finding is consistent with previously published data from this model(463). Absolute numbers and proportions of Tregs (CD25⁺FoxP3⁺) were significantly increased with treatment, which is in contrast to a previous study in the same model which observed a decrease in splenic Tregs(463). However, the findings are in line with observations in TLR7 driven B6.Yaa.Sle1, which presents with increased splenic Tregs, and with MRL/lpr and NZB/NZW mice, in which biphasic changes occur with increased Tregs in early disease and decreased Tregs in later(118, 250) .

A trend toward increased activation within the CD8 T cell compartment (Figure 3.3I-J), with increased numbers and proportions of CD8⁺CD69⁺ and CD8 effector memory cells did not reach statistical significance but mirrors similar findings observed in Yaa mice(118). Additionally, significantly increased numbers of CD8⁺Tbet⁺ and numbers and proportions of CD8⁺PD1⁺ cells were induced with IMQ treatment, markers which are typically associated with an “exhausted” phenotype in CD8 T cells and are expressed on CD8 T cells in blood of patients with SLE. Whilst PD1 expression (based on MFI) is increased on CD8 T cells in TLR7 overexpressing B6.Sle1.Tg7 mice, an increase in the absolute numbers and proportions of this population in TLR7 SLE models has to the best of our knowledge not been shown before(222, 361). Taken together, these data demonstrate that aberrant T cell activation is a feature strongly associated with this model of systemic autoimmunity.

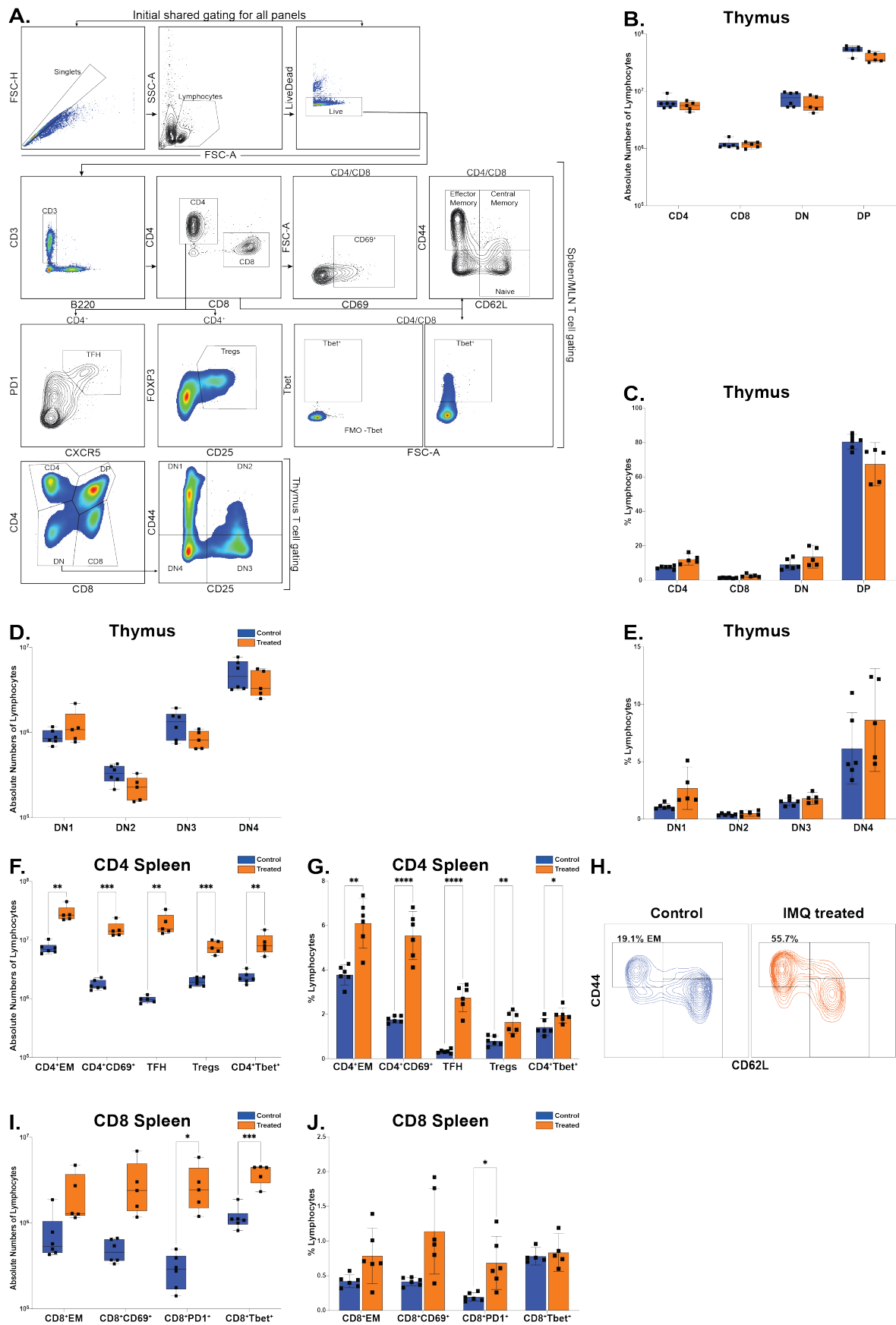


Figure 3.3: Splenic and thymic T cell populations.

Mice were treated for 8 weeks with IMQ or Vaseline control, culled and immune organs taken for analysis by flow cytometry. **(A)** Initial gating on singlets, lymphocytes and live cells was used in all panels. For T cells in the spleen and mesenteric lymph node (MLN), T cells were gated on CD3 expression and further gated on CD4 and CD8 expression. Effector Memory cells are defined as CD4⁺/CD8⁺CD44^{hi}CD62L^{low}; TFH are defined as CD4⁺PD1⁺ICOS⁺CXCR5⁺; Tregs are defined as CD4⁺CD25⁺FoxP3⁺. Thymic T cells were gated on CD4 and CD8 expression. Thereafter double negative T cells were further divided into developmental stages based on CD44 and CD25 expression. **(B-C)** Absolute numbers and proportion of lymphocytes of thymic T cell populations. **(D-E)** Absolute numbers and proportion of lymphocytes of thymic double negative T cell populations. **(F-G)** Absolute numbers and proportion of lymphocytes for splenic CD4⁺ T cell populations. **(H)** Representative contour plots of CD44 and CD62L expression on splenic CD4⁺ T cells from Vaseline controls (left) and IMQ treated (right), the percentage of the EM population is shown. **(I-J)** Absolute numbers and proportion of lymphocytes for splenic CD8⁺ T cell populations. Boxplot whiskers in **(B/D/F/I)** show min and max values and line represents the median. Bar plots in **(C/E/G/J)** show means $\pm$ 95% CI. Significance was determined by multiple t-tests using the Holm-Sidak method to correct for multiple comparisons * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and data are representative of six independent experiments, each with 5-6 mice per group.

3.2.4 Imiquimod treatment causes B cell activation and autoantibody production

No differences in bone marrow B cell development were observed with treatment (Figure 3.4A-C). Treated mice had significantly increased numbers and proportions of activated splenic B cells as denoted by expression of CD69 or CD86. Additionally, increased numbers of germinal centre (GC) like B cells (CD95⁺PNA⁺GL7⁺IgD^{low}), age associated B cells (ABCs) (CD19⁺B220⁺CD21⁺CD11c⁺CXCR5⁺), and plasma cells (CD138⁺), were observed in treated mice, with the first two populations found to also be increased as a proportion of lymphocytes (Figure 3.4D-F). Numbers and proportion of marginal zone (MZ) B cells were lower in treated mice, in line with the data published in this model (Figure 3.4B-C). Decreased MZ B cells have been observed in mice with the Yaa mutation, independent of disease, however this decrease is not observed in non TLR7 derived murine models of SLE, suggesting this to be a unique effect of TLR7. The mechanism underlying this decrease is not clear, however previous studies have demonstrated positive selection of low affinity autoreactive B cells in the MZ and therefore it is possible that in disease these activated autoreactive cells exit from the MZ compartment resulting in the observed decrease(464).

Increased IgG titres were observed in the serum of treated mice and incubation of serum on human epithelial cell 2 (Hep-2) slides followed by detection with labelled IgG antibody showed positivity for anti-nuclear antibodies (ANAs), with a homogenous pattern of staining (Figure 3.4G-H). Although ANAs are frequently seen in SLE patients, they are not specific to SLE, but rather a generic marker of dysregulated immunity. To test for anti-dsDNA antibodies, which are pathognomonic in SLE, serum was incubated with *Crithidia luciliae* slides. In this assay positivity for anti-dsDNA antibodies is denoted by staining of the kinetoplast which is detected via indirect immunofluorescence. No positive kinetoplast staining was observed in treated mice (Figure 3.4H). Since other studies published in this model have shown anti-dsDNA antibody positivity using ELISA based assays, we cannot exclude a discrepancy due to methodological differences. Whilst ELISAs have been observed to have higher sensitivity when compared with the *Crithidia luciliae* indirect immunofluorescence test, the latter has been shown to have a higher specificity with ELISAs producing more false positive results, hence the *Crithidia luciliae* indirect immunofluorescence test is often used for confirmation of an ELISA result(465). Taken together our data demonstrate treatment with topical Imiquimod for 8 weeks causes increased B cell activation and autoantibody production although no detectable production of high levels of anti-dsDNA antibodies.

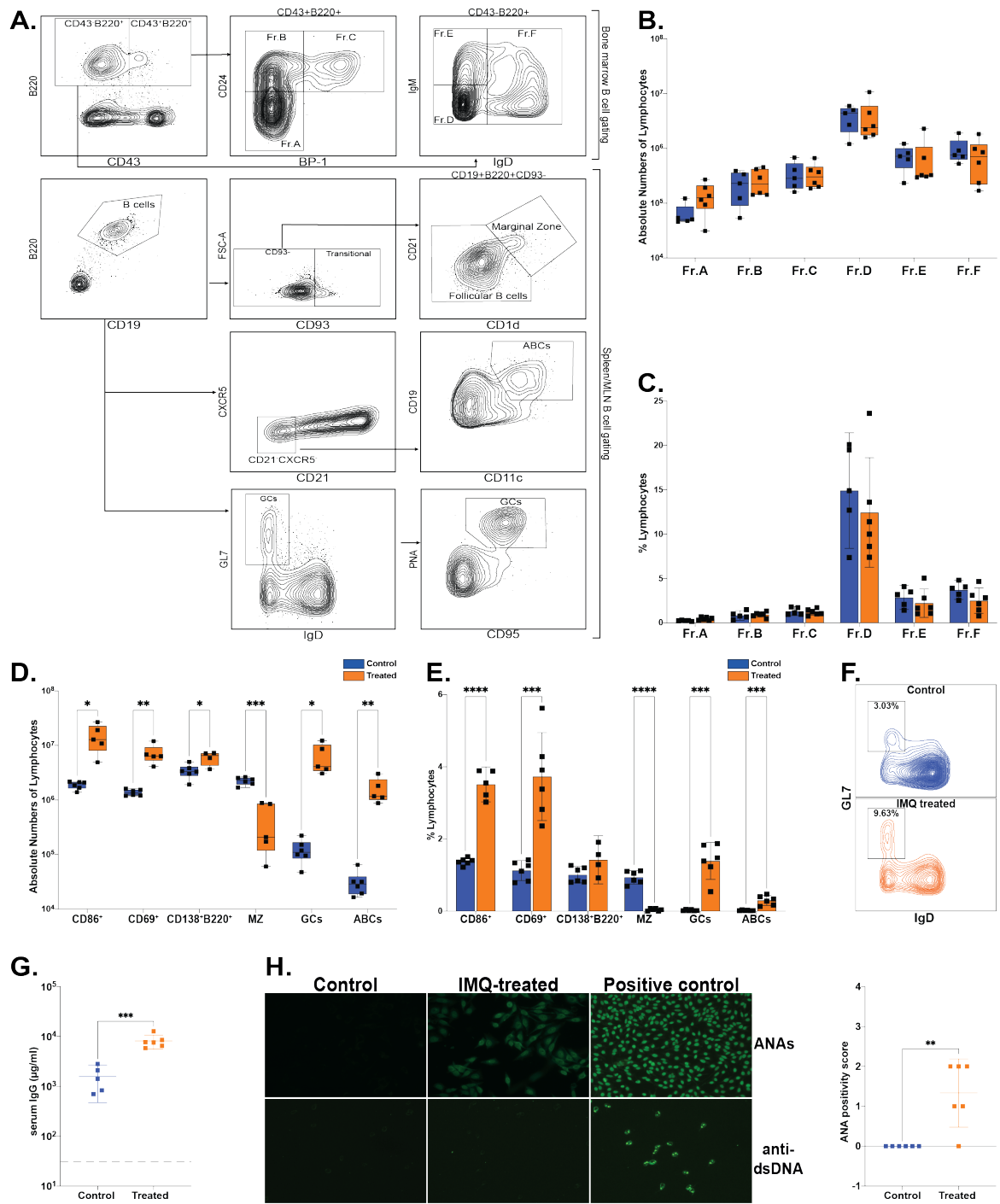


Figure 3.4: Splenic and bone marrow B cell populations.

Mice were treated for 8 weeks with IMQ or Vaseline control, culled and immune organs taken for analysis by flow cytometry. After initial gating as shown in **Figure 3.3A (A)** Gating strategy: Bone marrow B cells were gated into Hardy Fractions **(A-F)**. Splenic CD3⁺ cells were gated out; B cells were identified as B220⁺CD19⁺ and used for further gating of subpopulations. Marginal zone B cells are defined as CD1d^{hi}IgM^{hi}; germinal centre B cells are defined as CD95⁺GL7⁺PNA⁺IgD^{low}; ABCs are defined as CD19⁺ B220⁺CD21⁺CD11c⁺CXCR5⁻. **(B-C)** Absolute numbers and proportion of lymphocytes of bone marrow B cell populations. **(D-E)** Absolute numbers and proportion of lymphocytes of splenic B cell populations. **(F)** Representative contour plots of IgD and GL7 expression on B cells from Vaseline controls (top) and IMQ treated (bottom), the percentage of the GC population is shown. **(G)** Serum IgG was assessed by sandwich ELISA using sera from treated and untreated mice. **(H)** Representative images of ANAs (top panels) and anti-dsDNA (bottom panels) in mouse sera detected by indirect immunofluorescence. ANAs score based on staining positivity, by an independent blinded researcher. Sera for ELISA was diluted 1:12000. Dotted line in **(G)** corresponds to 2x background. Sera for ANAs was diluted 1:100 and for anti-dsDNA was diluted 1:50. Boxplot whiskers in **(B/D)** show min and max values and line represents the median. Bar plots in **(C/E)** show means $\pm$ 95% CI. Significance was determined by multiple t-tests using the Holm-Sidak method to correct for multiple comparisons * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and data are representative of six independent experiments, each with 5-6 mice per group.

3.2.5 Imiquimod induced LN with glomerular IC deposition.

Haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) stained renal formalin fixed paraffin embedded histology sections showed mesangial hypercellularity, diffuse endocapillary hypercellularity, glomerulomegaly, karyorrhexis (cells with fragmented nuclei irregularly spaced within the cytoplasm) and myeloid cell infiltration in the glomerulus of IMQ treated mice, not observed in controls (Figure 3.5A-B). Mild podocyte effacement, myeloid cell infiltration and the presence of electron dense deposits in the subendothelial and mesangial space were visualised with transmission electron microscopy (TEM). By immunofluorescence these deposits were confirmed to be IC deposition, with strong glomerular positivity for C1q, C3 and IgG, and a linear pattern of staining in the kidneys of treated mice (Figure 3.5E and F). Clinical interpretation of renal sections and TEM images was done by renal histopathologists Professor Ian Roberts and Dr Maria Soares within the Oxford University Hospitals Trust, who graded the histopathological features as being consistent with class II human LN.

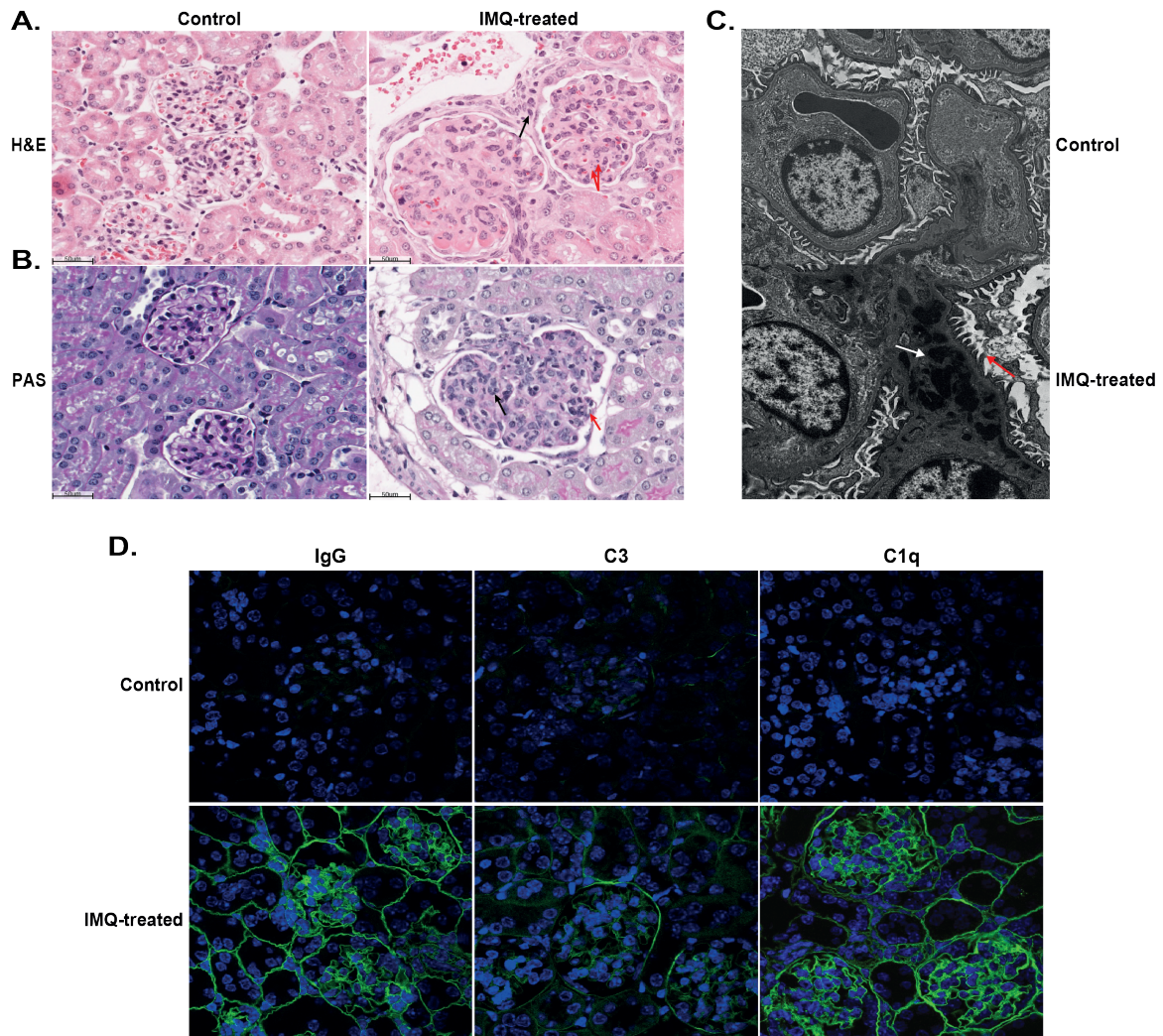


Figure 3.5: IMQ induced glomerulonephritis and immune complex deposition.

Mice were treated for 8 weeks with IMQ or Vaseline control, culled and kidneys fixed in 10%NBF for 24 hours, processed and embedded into paraffin, prior to being cut into 3 μ m sections. **(A-B)** Stained kidney sections from Vaseline control (left top and bottom panels) and IMQ treated mice (right top and bottom panels) (40X magnification). **(A)** Haematoxylin and Eosin (H&E) showing immune infiltrate (black arrow) and karyorrhectic debris (red arrow). **(B)** Periodic-acid Schiff (PAS) showing mesangial hypercellularity (black arrow) and endocapillary hypercellularity (red arrow). Bar = 50 μ m. **(E)** TEM of glomeruli (Top: Control; Bottom: IMQ treated), showing the presence of electron dense mesangial deposits (white arrow), podocyte foot process effacement (red arrow) (1770X magnification). **(F)** Immunofluorescence staining of kidney sections of control and IMQ treated mice for detection of IgG, C3 and C1q (green) with DAPI (blue) (63X magnification). Images are representative of six independent experiments, each with 5-6 mice per group.

3.3 MRL/lpr spontaneous model of Lupus nephritis

3.3.1 Fas mutation causes spontaneous T cell activation

MRL/lpr mice develop accelerated systemic autoimmunity because of a spontaneous mutation in apoptotic regulator Fas (CD95)(457). The congenic background MRL/MpJ strain is also predisposed to autoimmunity but developing disease over a much longer period of time with symptoms in females typically presenting at 12 months(457). Disease in MRL/lpr mice is characterised by the presence of autoantibodies starting at ~6 weeks of age, palpable lymphadenopathy by 12 weeks with proteinuria beginning at 15 weeks(466). Mortality associated with renal disease is reported in female MRL/lpr mice starting at 16-17 weeks of age with 50% mortality by ~20 weeks, therefore 15 weeks was chosen as a suitable time point for assessment of phenotype to have consistency without unacceptable adverse events(456). For all experiments MRL/lpr mice were compared with congenic background MRL/MpJ strain.

MRL/lpr mice at 15 weeks had lymphadenopathy and splenomegaly (Figure 3.6A). Flow cytometric analysis of T cell populations demonstrated increased numbers and proportions of double negative (DN) T cells in mesenteric lymph nodes (MLN), spleen and thymus in MRL/lpr mice, in line with previous studies demonstrating accumulation of DNT cells in secondary lymphoid organs as being the main driver of the observed lymphadenopathy in these mice(Figure 3.6B-D)(3,4). In line with enhanced DN T cells systemically, alterations were observed in thymic development, characterised by a decrease in the absolute numbers and proportions of double positive thymocytes and increased proportions of all double negative factions (DN1-4), with an increase in the absolute numbers of DN2 and DN3 cells (Figure 3.6E-F). These findings are consistent with previous studies demonstrating an important role for Fas mediated apoptosis in thymic selection for the maintenance of central tolerance, allowing the escape of autoreactive T cells into the periphery(467).

Within the splenic CD4 T cell subsets in MRL/lpr mice, increased numbers, and proportions of activated CD4 T cells (CD69⁺), EM (CD44^{hi}CD62L^{low}), TFH (PD1⁺ICOS⁺CXCR5⁺), Tregs (CD25⁺FoxP3⁺) and CD4⁺Tbet⁺ were observed (Figure 3.6G-H). Within the splenic CD8 T cell compartment, increased numbers, and proportions of activated CD8 T cells (CD69⁺), EM, CD8⁺Tbet⁺ and CD8⁺PD1⁺ cells were observed (Figure 3.6I-J). These data demonstrate aberrant T cell activation in MRL/lpr mice at 15 weeks compared to MRL/MpJ controls.

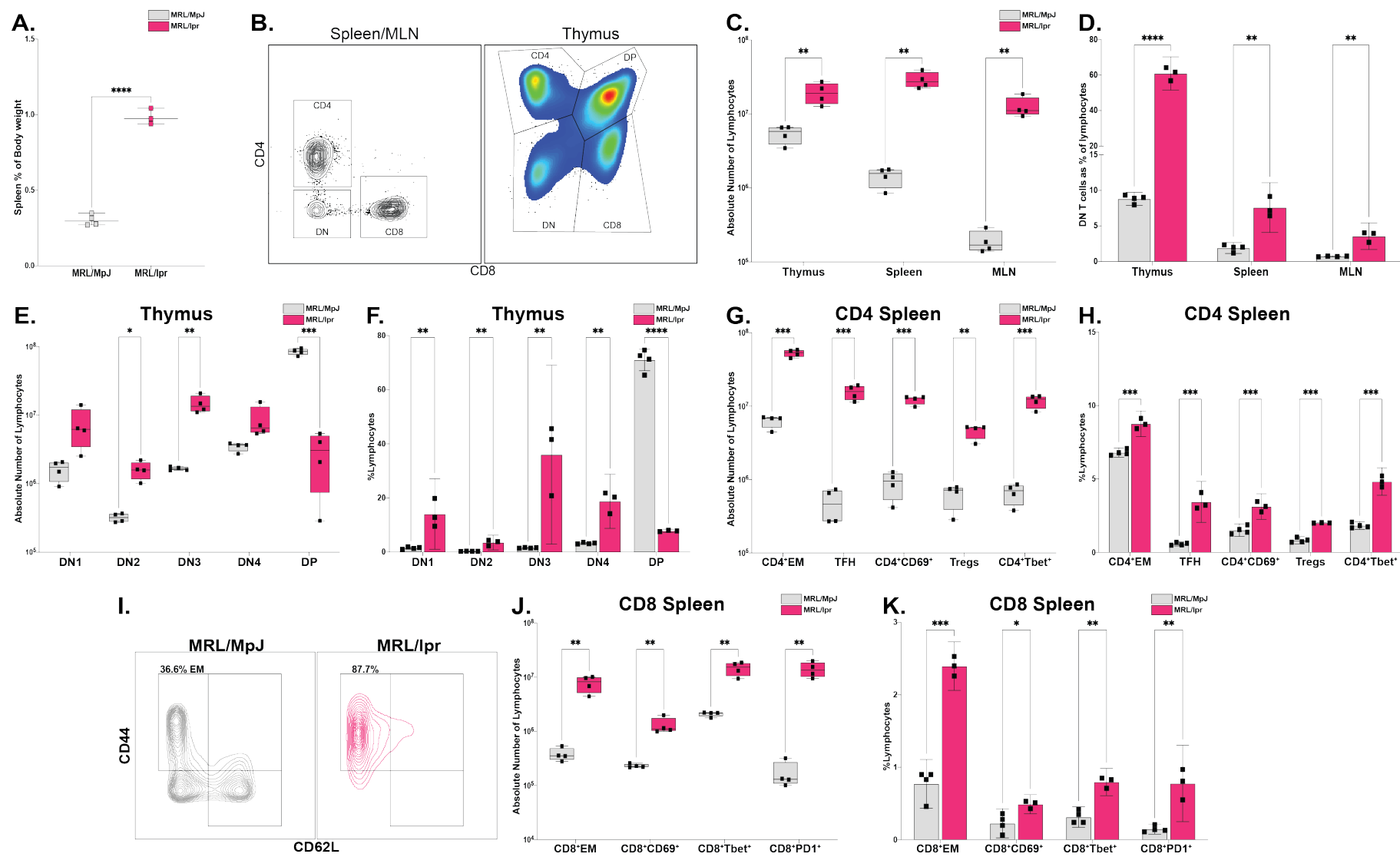


Figure 3.6: Fas mutation causes spontaneous T cell activation.

MRL/lpr and background strain MRL/MpJ were aged for 15 weeks, culled and immune organs taken for analysis by flow cytometry. **(A)** Spleen as a percentage of body weight in 15-week-old MRL/MpJ and MRL/lpr mice. After initial gating as shown in **Figure 3.3A (B)** Gating strategy: T cells were gated on CD3 expression and further gated as CD4⁺, CD8⁺ or CD4⁺CD8⁺ (CD3⁺CD4⁺CD8⁺DN T cells). Effector Memory cells are defined as CD4⁺/CD8⁺CD44^{hi}CD62L^{low}; TFH are defined as CD4⁺PD1⁺ICOS⁺CXCR5⁺; Tregs are defined as CD4⁺CD25⁺FoxP3⁺. Thymic cells (right panel) were gated on CD4 and CD8 expression as CD4⁺, CD8⁺, CD4⁺CD8⁺ (DN T cells) and CD4⁺CD8⁺ (double positive (DP)). **(C-D)** Absolute numbers and proportion of lymphocyte of double negative (DN) T cells in thymus, spleen, and MLN. **(E-F)** Absolute numbers and proportion of lymphocytes of thymic double negative T cell subpopulations and thymic double positive T cells. **(G-H)** Absolute numbers and proportion of lymphocytes for splenic CD4⁺ T cell subpopulations. **(I)** Representative contour plots of CD44 and CD62L expression on splenic CD4⁺ T cells from MRL/MpJ (left) and MRL/lpr (right), the percentage of the EM population is shown. **(J-K)** Absolute numbers and proportion of lymphocytes of splenic CD8⁺ T cell subpopulations. Boxplot whiskers in **(C/E/G/J)** show min and max values and line represents the median. Bar plots in **(D/F/H/K)** show means $\pm$ 95% CI. Significance was determined by multiple t-tests using the Holm-Sidak method to correct for multiple * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ and data are representative of four independent experiments, each with 3-5 mice per group.

3.3.2 Fas mutation causes spontaneous B cell activation and SLE specific anti-dsDNA autoantibody production

At 15 weeks no difference in B cell bone marrow development was observed in MRL/lpr mice (Figure 3.7A and B). However, increased numbers and proportions of activated B cells (CD69⁺ and CD86⁺), age related B cells (ABCs) and germinal centre (GC) like B cells were observed in the spleen and MLN (Figure 3.7A-C) of MRL/lpr mice. Increased numbers and proportions of plasma cells (CD138⁺) were observed in the spleen and MLN of MRL/lpr mice (Figure 3.7C-F). Incubation of serum with Hep-2 slides showed strong positivity for ANAs, with a homogenous pattern of staining, and indirect immunofluorescence of serum incubated with *Crithidia luciliae* slides showed positivity for anti-dsDNA antibodies, with staining of the kinetoplast, in the MRL/lpr mice, pathognomonic of SLE (Figure 3.7H)(5). Thus, female MRL/lpr mice at 15 weeks have an activated B cell phenotype in conjunction with circulating ANAs and SLE specific anti-dsDNA antibodies, indicating development of a lupus like systemic autoimmune disease.

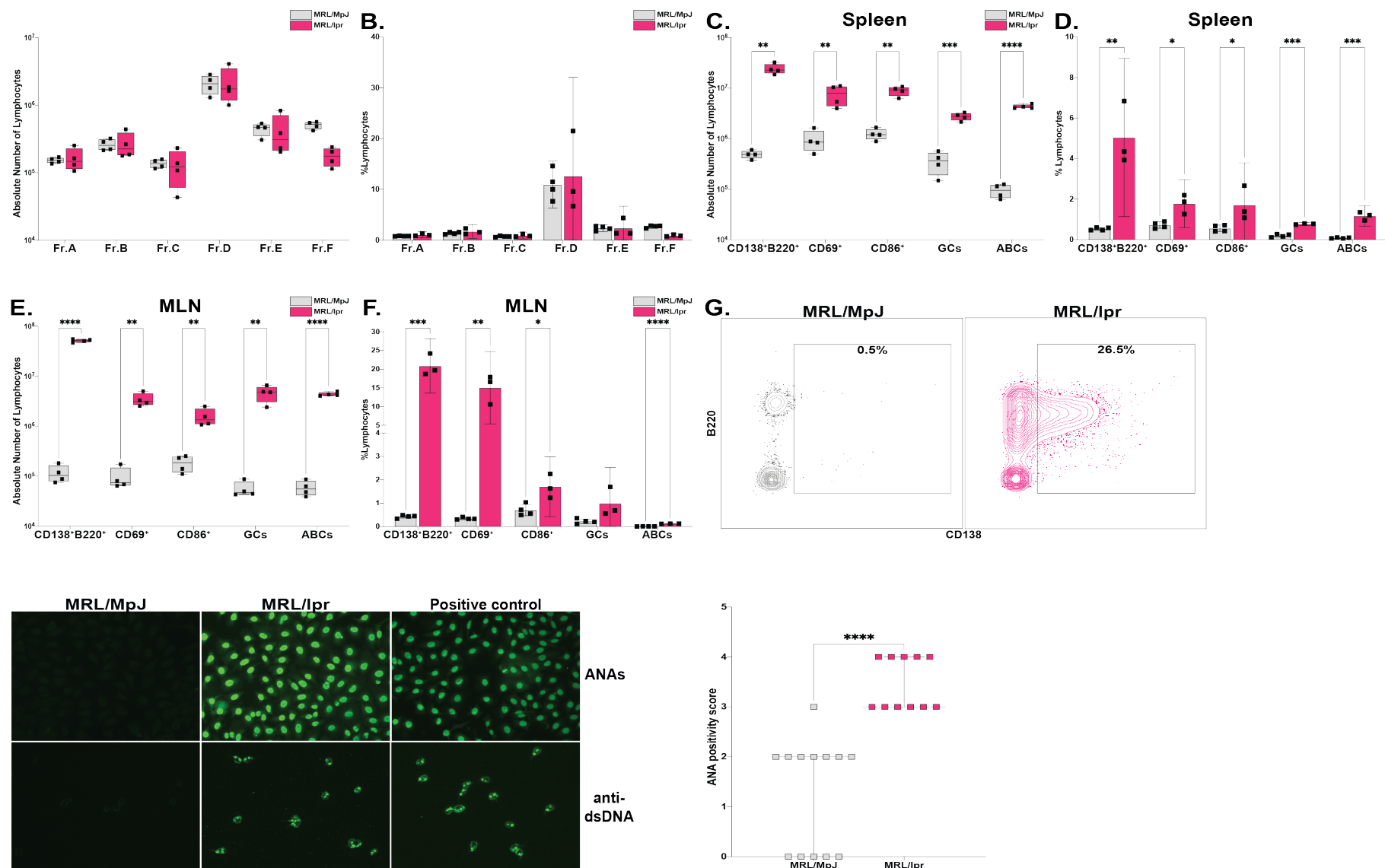


Figure 3.7: Fas mutation causes spontaneous B cell activation and autoantibody production.

MRL/lpr and background strain MRL/MpJ were aged for 15 weeks, culled and immune organs taken for analysis by flow cytometry. After initial gating as shown in **Figure 3.3A**. Bone marrow B cells were gated into Hardy Fractions **(A-F)**. B cells were identified as B220⁺CD19⁺ and used for further gating of subpopulations. Marginal zone B cells are defined as CD1d^{hi}IgM^{hi}; germinal centre B cells are defined as GL7⁺PNA⁺IgD^{low}; ABCs are defined as CD19⁺B220⁺CD21⁺CD11c⁺CXCR5⁺. **(A-B)** Absolute numbers and proportion of lymphocytes for bone marrow B cell populations. **(C-D)** Absolute numbers and proportion of lymphocytes of splenic B cell populations. **(E-F)** Absolute numbers and proportion of lymphocytes of MLN B cell populations. **(G)** Representative contour plots of B220 and CD138 expression on MLN B cells from MRL/MpJ (left) and MRL/lpr (right), the percentage of the CD138 population is shown. **(H)** Representative images of ANAs (top) and anti-dsDNA antibodies (bottom) in mouse sera detected by indirect immunofluorescence. ANAs score based on staining positivity, by an independent blinded researcher. Sera for ANAs was diluted 1:100 and sera for anti-dsDNA was diluted 1:50. Boxplot whiskers in **(A/C/E)** show min and max values and line represents the median. Bar plots in **(B/D/F)** show means $\pm$ 95% CI. Significance was determined by multiple t-tests using the Holm-Sidak method to correct for multiple * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ and data are representative of four independent experiments, each with 3-5 mice per group.

3.3.3 Fas mutation induces LN characterised by glomerular IC deposition and crescent formation

MRL/lpr mice had more pronounced renal involvement compared to the IMQ treated mice (Figure 3.2B and Figure 3.5A-B). However, they still represent early renal injury. MRL/lpr had no detectable difference in renal clearance, with no difference in serum creatinine or albumin observed when compared with the MRL/MpJ background strain (Figure 3.8A-B). However, in contrast to IMQ treatment, MRL/lpr mice secrete more protein in the urine with an increase in the urinary protein: creatinine ratio (uPCR) (Figure 3.8C). This indicates early glomerular damage but given the limitations associated with the use of creatinine as a marker of renal clearance in mice (section 3.3.2), impaired clearance cannot be precluded and can be checked by a gold standard method such as inulin infusion(460)

H&E and PAS-stained renal sections identified diffuse mesangial hypercellularity, endocapillary proliferation and karyorrhexis in MRL/lpr but not control kidney sections. Active focal crescents, characterised by parietal epithelial cell expansion, immune cell infiltration and disruption of part of the Bowmans capsule were present in the kidneys of MRL/lpr mice (Figure 3.8D-E). Tubular morphology was preserved, but scattered protein casts were observed, suggestive of early proteinuria and in line with the increased uPCR. Immunofluorescence confirmed glomerular positivity for IC and complement deposition (IgG, C3 and C1q), with a linear pattern of staining in the kidneys of MRL/lpr mice (Figure 3.8F). Clinical interpretation of renal sections for classification of disease was done with support from renal histopathologist Dr Maria Soares and disease features were graded as being consistent with class III focal LN.

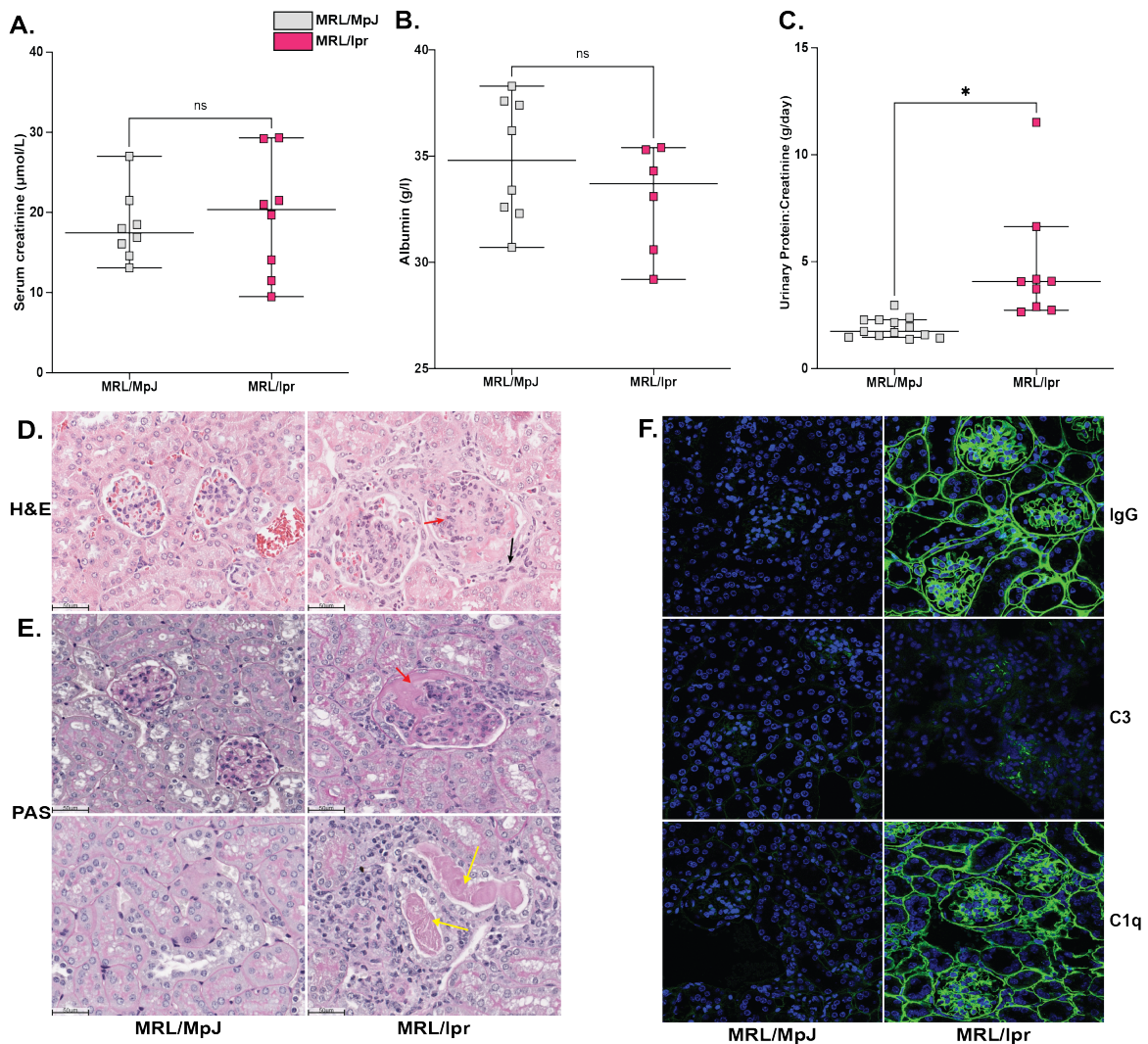


Figure 3.8: Fas induced IC deposition and crescent formation in the kidney.

Female MRL/lpr and background strain MRL/MpJ were aged for 15 weeks (A) Serum creatinine levels in MRL/MpJ and MRL/lpr mice aged 15 weeks. (B) Serum albumin levels in MRL/MpJ and MRL/lpr mice aged 15 weeks. (C) Urinary protein: creatinine ratio for MRL/MpJ and MRL/lpr mice aged 15 weeks. (D-E) Stained kidney sections from MRL/MpJ (left panels) and MRL/lpr (right panels) 15-week-old mice (40X magnification). (D) Haematoxylin and Eosin (H&E) showing immune infiltrate and segmental crescent formation (black arrow) and karyorrhectic debris (red arrow). (E) Periodic-acid Schiff (PAS) showing an active necrotic crescent (red arrow) and tubular protein casts (red arrow) Bar = $50\mu\text{m}$. (F) Immunofluorescence staining of kidney sections of MRL/MpJ and MRL/lpr mice aged 15 weeks for detection of IgG, C3 and C1q (63X magnification). Images are representative of four independent experiments, each with 3-5 mice per group.

3.4 Summary

Studies of human LN are frequently confounded by factors such as disease stage (typically late), treatment status and inherent human heterogeneity. In this context, murine models provide us with an opportunity to gain biological insight into disease initiating events;

however, the choice of model is dependent on several important factors, including the predictable time course of disease, development of renal disease and translatability to human disease.

Here we describe results from a combined immune and renal phenotyping approach to two selected murine models. Treatment with TLR7 agonist imiquimod for 8 weeks was shown to be well tolerated and without adverse events. The treated mice developed a systemic phenotype consistent with autoimmunity, associated with an activated B and T cell phenotype, with the latter characterised by increased activated and effector T cells populations in line with previous observations(431). Notably an increased population of CD8 T cells with an “exhausted” signature were observed representing a previously undescribed finding in the context of TLR7 driven models of SLE. Treated mice were shown to have positivity for serum ANAs but not anti-dsDNA antibodies, although the lower sensitivity of the assay used for anti-dsDNA antibodies may preclude detection of lower titres of antibodies. Similarly, the systemic phenotype in MRL/lpr mice was characterised by activated B and T cells, in addition to positivity for ANAs and anti-dsDNA antibodies, again in line with published findings of this model(457). Renal phenotyping of both models demonstrated histological features consistent with early renal disease. Renal creatinine clearance was preserved in both murine models, with urinary abnormalities (proteinuria) specific to MRL/lpr mice. Therefore, these two models represent a gradation of early renal disease from class II to class III LN, providing a basis for studying the cellular pathology of early active LN in the context of two different aetiologies, with the aim of identifying overlapping mechanistic pathways relevant to disease.

Chapter 4 : Renal cellular expression in lupus nephritis / renal transcriptomic signatures in lupus nephritis

4.1 Introduction

Molecular information can aid the stratification and treatment of disease, exemplified by the field of cancer biology, in which the use of whole genome and exome sequencing has identified cancer subtype genetic alterations, which can be screened for in patients and targeted specifically through the use biologics and small molecule inhibitors reducing the need for cytotoxic therapies(468). The advent of newer technologies has enabled in depth characterisation of disease processes, for example study of the tumour microenvironment with single cell technologies has begun to shed light on cell type heterogeneity and shared features across cancers(468). However, dissection of disease causative pathways from passive damage pathways can be difficult, especially later in disease.

In chapter 3 I characterised two murine models with systemic features of SLE and early active renal disease, providing tools for studying the cellular pathology of early SLE kidney disease. Molecular information in early LN is lacking and a better understanding of pathways modulating early disease may have important prognostic and therapeutic implications.

To understand the renal transcriptomic signatures and pathways associated with early LN at the resolution of individual cells, single-cell/single-nuclei RNA sequencing (sc/snRNA-seq), was performed on kidneys from the MRL/lpr and Imiquimod treated mice and their respective controls. SLE is a heterogenous disease, and I hypothesised that model specific pathway alterations, present in only one of the two models studied here, may provide insight into LN heterogeneity in the kidneys. Ultimately such insights could enable better stratification of patients and more targeted therapies. On the other hand, pathways shared by both the models could represent more general or unified LN pathways, relevant for the

development of broadly applicable but biologically targeted therapeutics. Therefore, the aim of the transcriptomic studies was to identify both overlapping and model specific cellular signatures with therapeutic potential.

4.2 Single-cell RNA sequencing (scRNA-seq) vs. single-nuclei RNA sequencing (snRNA-seq)

Conventional single cell RNA sequencing (scRNA-seq) generates cell specific quantification of both nuclear and cytoplasmic transcript abundance. A pilot scRNA-seq study was carried out utilising kidneys from IMQ treated and Vaseline controls treated for 8 weeks, with three mice per group. Prior to enzymatic dissociation, to enrich for glomerular cell types, I compared two different isolation methods. The first relied on a sieving method using the whole kidney to enrich for glomeruli, whereas the second method used the kidney cortex for subsequent dissociation. Cells with a gene count less than 200 or greater than 5000, plus cells with greater than 50% mitochondrial genes were removed, resulting in 7016 cells for downstream analysis.

Unsupervised clustering of cells generated 16 clusters, corresponding to tubular populations across the length of the nephron, including proximal tubules, distal tubules, thick ascending limb (TAL), connecting tubule (CNT) and cortical collecting duct (CCD) plus B, T and myeloid lineage cells and a single endothelial cell cluster (Figure 4.1A-B). Cell type identities were manually defined using canonical renal and immune markers derived from the published literature. Glomerular populations were not adequately represented with few cells detected expressing markers corresponding to podocytes, mesangial cells or parietal epithelial cells and the numbers too small to form discrete individual clusters.

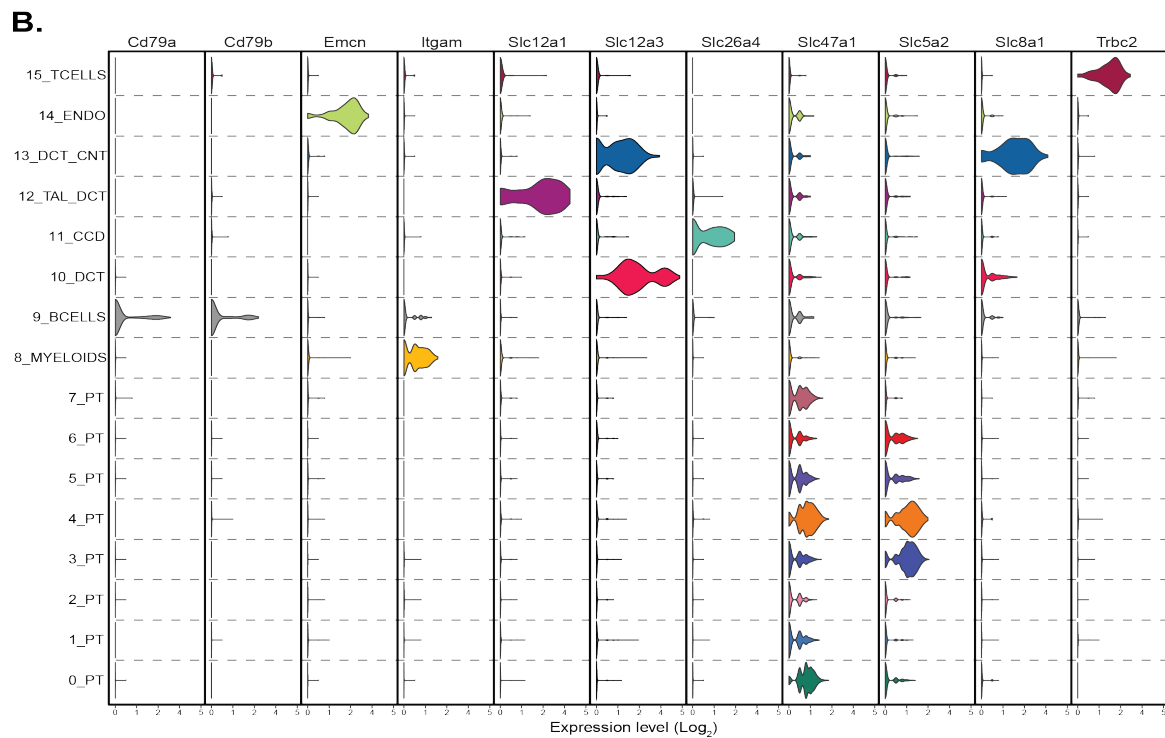
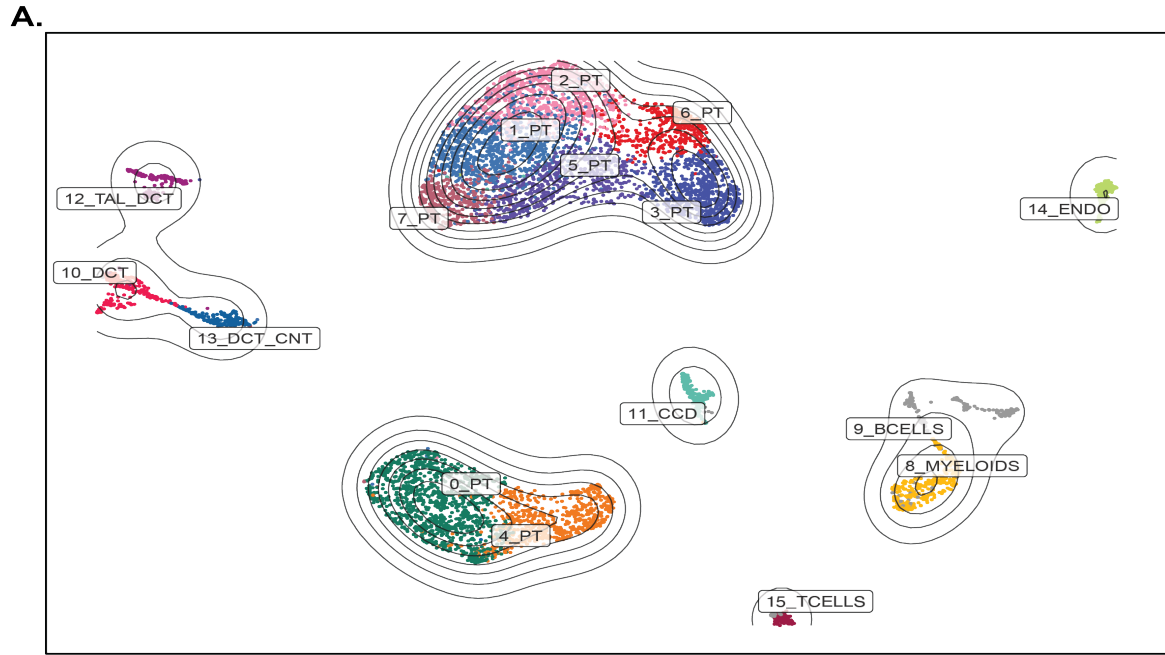


Figure 4.1: Pilot scRNA-seq study of IMQ treated and Vaseline control mice kidneys.

Mice were treated for 8 weeks with IMQ or Vaseline control, culled and kidneys taken processed for scRNA-seq ($n=3/\text{group}$). **(A)** Unsupervised clustering of 7016 cells identifies 16 clusters visualised using dimensionality reduction method UMAP and annotated by identity **(B)** Log2 transformed normalised expression of key markers genes used for annotation of cluster identity. **PT**: Proximal tubule; **TAL**: Thick ascending limb; **DCT**: Distal convoluted tubule; **CNT**: Connecting tubule; **CCD**: Cortical collecting duct; **ENDO**: Endothelial cells.

Much of the kidney cortex is made up of renal tubules (>90%), with glomerular cells contributing only around 3% of total epithelial cells(469). Similar to our data, published renal scRNA-seq have been dominated by tubular populations, with typically 80% of the captured cells being of tubular origin, and glomerular populations underrepresented or absent in some datasets(439, 470, 471). Glomeruli are relatively robust and adherent structures, resistant to dissociation, a feature that has been exploited for sieving based glomerular isolation methods, but likely contributes to depletion in scRNA-seq workflows(472).

Since glomeruli are the primary site of IC deposition and early histopathological changes (such as mesangial hypercellularity) in LN, adequate capture of glomerular populations is needed in this project. An alternative transcriptomic approach for tissue that does not readily dissociate into a single cell suspension is snRNA-seq(473). SnRNA-seq achieves comparatively superior capture of glomerular populations, without compromising capture of other renal populations, and minimises the transcriptomic changes (e.g., expression of stress response genes) associated with scRNA-seq dissociation(441, 473, 474).

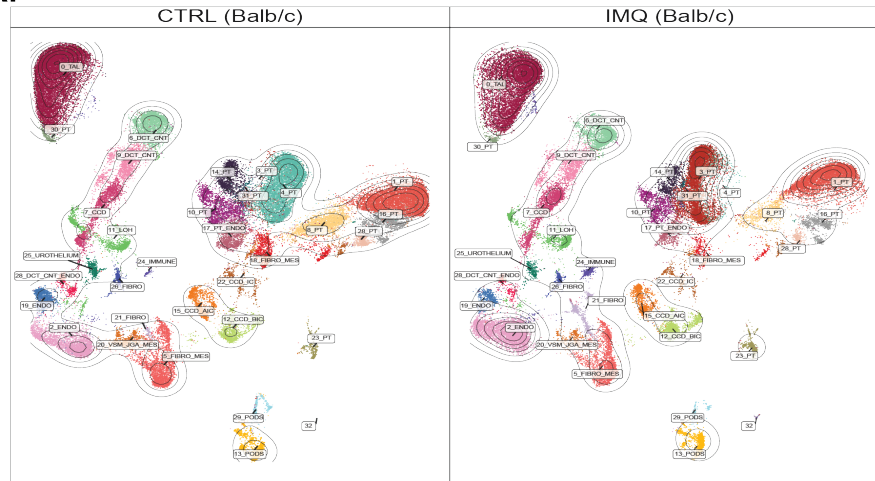
There are biases in cellular capture with both modalities. SnRNA-seq is biased towards the capture of epithelial and stromal populations and scRNA-seq towards leukocytes, with the frequency of immune populations found to be underestimated by snRNA-seq in the kidneys(473). However, studies reporting few immune cells in snRNA-seq kidney datasets were carried out in healthy tissue samples and subsequent snRNA-seq studies carried out in disease models have identified clusters representing B, T and myeloid cells, additionally, improvements in downstream analysis such as inclusion of intronic regions when mapping nuclear transcripts to the genome have resulted in better detection of gross immune populations(438, 473, 475). Balancing the above observations with the importance of capturing glomerular populations for the study of LN we adopted snRNA-seq for subsequent transcriptomic single cell experiments in this thesis.

4.3 Per model analysis of renal cellular expression profiles in lupus nephritis

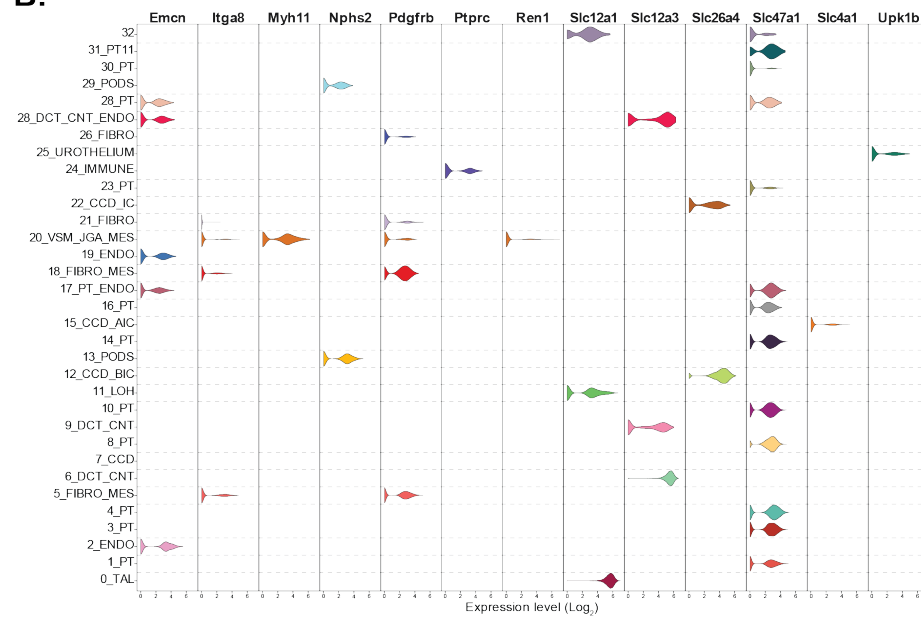
4.3.1 Analysis of stromal populations identifies mesangial alteration in IMQ treated mice

SnRNA-seq was carried out on nuclei extracted from the kidneys of mice treated with IMQ or Vaseline for 8 weeks, with three mice per group. The whole kidney was used for nuclei extraction. 20k nuclei per mouse were used for single nuclei library preparation and sequencing. After quality control steps to remove nuclei with less than 500 or greater than 4000 genes, and nuclei with greater than 5% mitochondrial genes, 101k nuclei were obtained for downstream analysis. 33 clusters were identified, corresponding to tubular, glomerular, stromal and a single immune population (Figure 4.2A-B). In contrast to the scRNA-seq analysis, podocytes (3.1%) and endothelial cells (11.2%) were represented. Visualisation of clustering in two-dimensional space via uniform manifold approximation and projection (UMAP) identified trend differences in non-immune glomerular populations between IMQ treated and Vaseline control mice particularly within the endothelial (2 and 19) and stromal clusters (5,18,20,21, and 26), with a significant increase in the proportions of cluster 2, endothelial cells, (Figure 4.2C).

A.



B.



C.

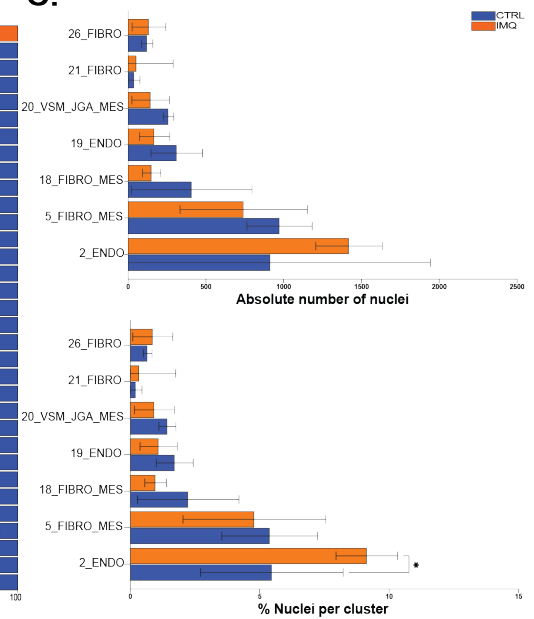


Figure 4.2: SnRNA-seq analysis identifies alterations in stromal populations in the kidneys of IMQ treated mice.

Mice were treated for 8 weeks with IMQ or Vaseline control, culled and kidneys processed for snRNA-seq (n=3/group). **(A)** Unsupervised clustering of 101k nuclei identified 33 clusters, visualised using dimensionality reduction method UMAP and annotated by cluster identity and split by treatment group; nuclei from Vaseline control mice (left panel) and nuclei from IMQ treated mice (right panel). **(B)** Log2 transformed normalised expression of key markers genes used for annotation of cluster identity. Bar plots represent percentage contribution to each cluster by group. **(C)** Absolute numbers (top panel) and proportions (bottom panel) of IMQ and Control nuclei within endothelial (2 and 19) and stromal clusters (5,18,20,21 and 26). Error bars represent standard deviation and significance was calculated using multiple t-tests comparing absolute numbers or proportions of IMQ and Control nuclei per cluster with Bonferroni correction to correct for multiple comparisons where $*P < 0.05$. **PT**: Proximal tubule; **TAL**: Thick ascending limb; **DCT**: Distal convoluted tubule; **CNT**: Connecting tubule; **ENDO**: Endothelial cells; **VSM**: Vascular smooth muscle; **JGA**: Juxtaglomerular apparatus cells; **FIBRO**: Fibroblast; **MES**: Mesangial cells; **PODS**: Podocytes; **PECS**: Parietal epithelial cells; **CCD-AIC**: Cortical collecting duct alpha intercalated cell; **CCD-BIC**: Cortical collecting duct beta intercalated cell; **CCD-IC**: Cortical collecting duct intercalated cell; **MYO**: Myofibroblast; **LOH**: Loop of Henle.

Sub-clustering these populations into 21 clusters allowed classification into distinct stromal and endothelial cell subtypes (Figure 4.3A). A decrease in mesangial cells (cluster 2), in conjunction with an increase in UMAP neighbour cluster 7, classified as fibroblasts, was observed in IMQ treated mice (Figure 4.3A and B). Differential expression analysis of the mesangial cluster identified increased expression of genes associated with fibrosis, including *Ccn2* (connective tissue growth factor), *F2r* (coagulation factor II receptor) and immune modulation *Tnfsf13b* (BAFF) and *Cxcl12* in the IMQ group (green dots in Figure 4.3C), suggesting a shift in mesangial cells towards a profibrotic state characterised by altered immune signalling. There was no signal for changes in genes related to cell death, and I hypothesised that the decreased numbers of mesangial cells may be explained by differentiation to a more fibroblast phenotype with IMQ treatment, contributing to the closely related and expanded fibroblast like cluster 7, which express *C7*, encoding complement factor 7.

Trajectory analysis is a method whereby individual cells are ordered in a lineage based on changes in transcriptional profile along an inferred “pseudotime”, enabling the modelling and visualisation of cellular differentiation in different conditions(447). Trajectory analysis was applied to test the hypothesis of mesangial to fibroblast differentiation. A pathway was constructed from 2_MES to the disease enhanced 7_C7_FIBRO cluster, but not to another *Cxcl12* expressing fibroblast cluster, suggesting that mesangial cell loss in disease may be a result of differentiation into a more matrix and complement producing cell type (Figure 4.3D). However, while trajectory inference has enabled biological inference of continuous cellular states, directionality can be skewed by population abundance forcing a particular lineage, and individually calculated trajectories might not be easily comparable due to differences in reduced dimensional space(476).

Differential expression analysis between mesangial cell (2) and fibroblast (7) cluster identified *Itga8* (integrin $\alpha 8$) (Figure 4.3E) amongst genes downregulated in the fibroblast

cluster. By pathway enrichment analysis, upregulated pathways in 2_MES vs. 7_C7_FIBRO included those involved in cell substrate adhesion, extracellular matrix organization and integrin signalling (Figure 4.3F) with *Itga8* the top differentially expressed gene to be shared amongst all these pathways.

Functionally, *Itga8* is expressed on mesangial and vascular smooth muscle cells and contributes to cell attachment and migration(477). Downregulation or loss of *Itga8* in mesangial cells has been associated with enhanced mesangial cell proliferation, release of fibrotic mediators and a more fibroblast like phenotype(477-479). Assessment of *Itga8* expression within the mesangial cluster showed a non-significant trend towards decreased expression in IMQ treated mice (Figure 4.3G), the lack of significance may be due to the lower number of nuclei in this cluster from IMQ treated mice leading to higher variability. Thus, the snRNA-seq data suggests that IMQ induced renal injury results in mesangial cell loss with transition towards a more profibrotic and inflammatory phenotype, associated with downregulation of *Itga8*. These mesangial changes could represent an early mechanism culminating in the irreversible fibrotic damage seen in severe LN. Further in vitro functional studies are required to validate this finding.

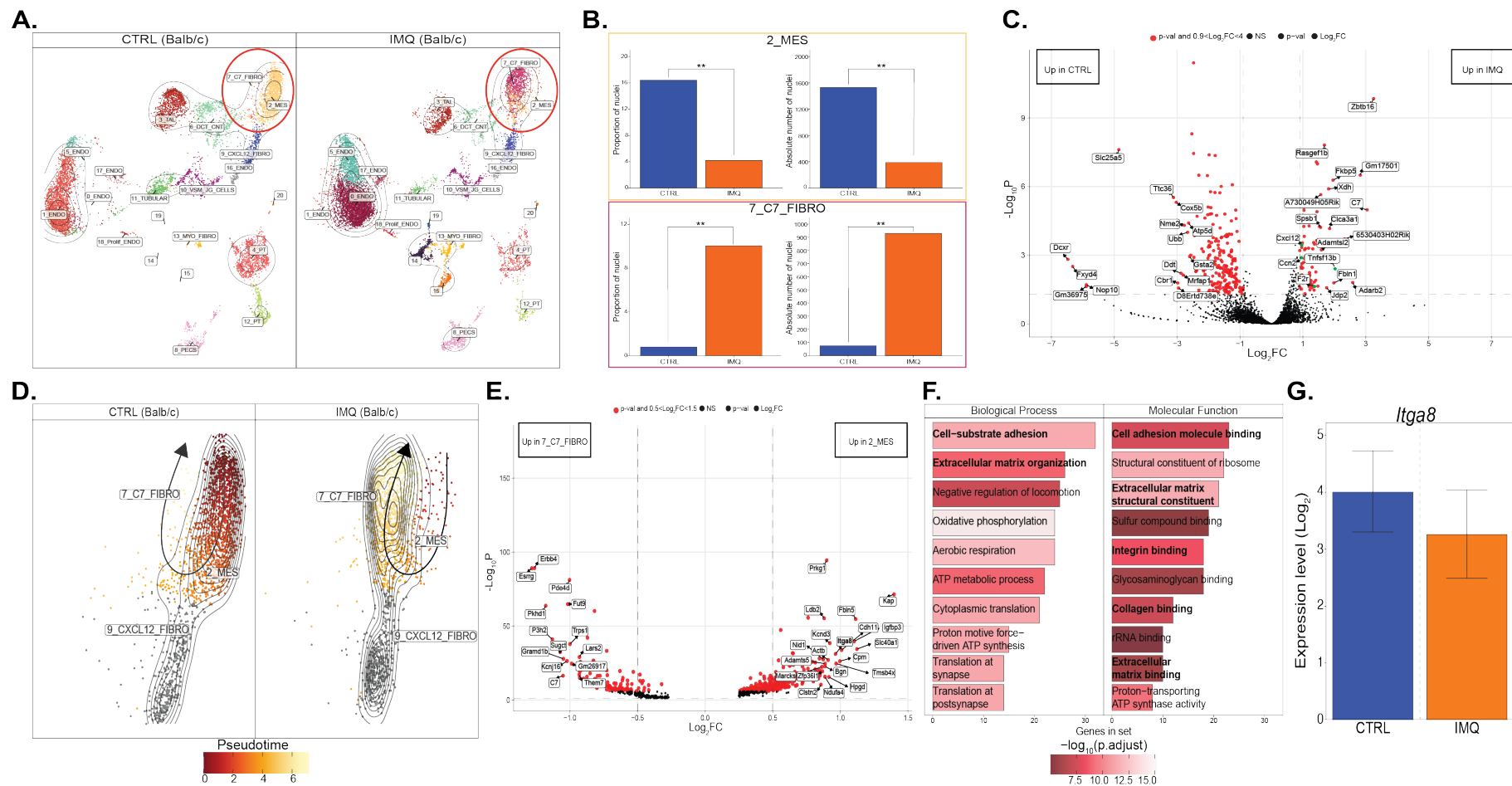


Figure 4.3: Focused analysis of stromal populations identifies mesangial cell alterations in the kidneys of IMQ treated mice.

Mice were treated for 8 weeks with IMQ or Vaseline control, culled and kidneys processed for snRNA-seq (n=3/group). **(A)** Focused analysis of stromal and endothelial populations identified 21 additional clusters, visualised using dimensionality reduction method UMAP, nuclei were annotated by cluster identity and split by treatment group; nuclei from Vaseline control mice (left panel) and nuclei from IMQ treated mice (right panel). **(B)** Proportion and absolute numbers of IMQ and Control nuclei within 2_MES (top panel) and 7_C7_FIBRO (bottom panel). Significance was calculated using multiple t-tests comparing absolute numbers or proportions of IMQ and Control nuclei per cluster with Bonferroni correction to correct for multiple comparisons where $^{**}P < 0.01$. **(C)** Visualisation of pseudobulk analysis derived differentially expressed genes between IMQ treated and Vaseline control mice within 2_MES cluster. Coloured dots (red or green) represent genes that met the threshold of $\log_2FC = 0.9$ and global p adjusted value < 0.05 , of these the top 15 genes based on absolute $\log_2FC$ are labelled. Pseudobulk analysis was used as it aggregates gene expression counts per sample within a cluster providing more power for statistical inference between groups per cluster, particularly important in clusters where there is disproportionate representation. **(D)** Inferred trajectory analysis of putative transition between 2_MES and 7_C7_FIBRO clusters, coloured by pseudotime overlayed onto a UMAP plot, representing the order of nuclei along this trajectory, with this plot split by treatment group; nuclei from Vaseline control mice (left panel) and nuclei from IMQ treated mice (right panel). **(E)** Visualisation of Seurat FindMarkers derived differentially expressed genes between 2_MES and 7_C7_FIBRO. Red dots represent genes that met the threshold of $\log_2FC = 0.5$ and p adjusted value < 0.05 , of these the top 20 genes based on absolute $\log_2FC$ are labelled. **(F)** Gene ontology analysis of genes upregulated in 2_MES when compared with 7_C7_FIBRO, upregulated genes were defined as in **(F)**, GO terms that met the threshold of p adjusted value < 0.05 were sorted by "Count" which represents the number of differentially expressed genes annotated with a particular GO term of which the top 10 are shown per GO ontology domain: GO terms associated with biological processes (left panel) and molecular function (right panel). Bars are labelled with descriptions of the relevant GO term; length of the bar indicates number of differentially expressed genes annotated with the given term and bar colour represents significance. **(H)** Bar plot of *Itga8* expression between Vaseline control and IMQ treated mice within 2_MES cluster, y axis shows the $\log_2$ transformed normalised expression and error bars represent standard deviation. **PT:** Proximal tubule; **TAL:** Thick ascending limb; **DCT:** Distal convoluted tubule; **CNT:** Connecting tubule; **ENDO:** Endothelial cells; **VSM:** Vascular smooth muscle; **JGA:** Juxtaglomerular apparatus cells; **FIBRO:** Fibroblast; **MES:** Mesangial cells; **PODS:** Podocytes; **PECS:** Parietal epithelial cells; **MYO:** Myofibroblast;

4.3.2 Interferon associated inflammatory signature highlights damage to renal proximal tubule populations in MRL/lpr mice

SnRNA-seq was carried out on nuclei extracted from the kidneys of 15-week-old MRL/lpr or MRL/MpJ mice, with three mice per group. Kidney cortex (50mg) was used for nuclei extraction and subsequent sorting, thereafter 20k sorted nuclei were used for single nuclei library preparation and sequencing. Nuclei with less than 500 and greater than 6000 genes and nuclei with greater than 5% mitochondrial genes were removed, resulting in a dataset of 46k nuclei for further analysis. Broad clustering identified 28 clusters (Figure 4.4A-B). Cluster 4 (4_Inflamed_PT) corresponds to a proximal tubule (PT) cluster which consisted predominantly of nuclei from MRL/lpr mice (Figure 4.4A red circle and Figure 4.4C). The mostly highly expressed genes in this inflamed PT cluster were interferon stimulated genes (*Gbp6*, *ligp1*, *Irf8*) and gene ontology-based gene set enrichment analysis of differentially expressed genes in 4_Inflamed_PT compared with remaining clusters, identified GO terms associated with response to interferon and interferon signalling (Figure 4.4D) indicating interferon mediated proximal tubule inflammation.

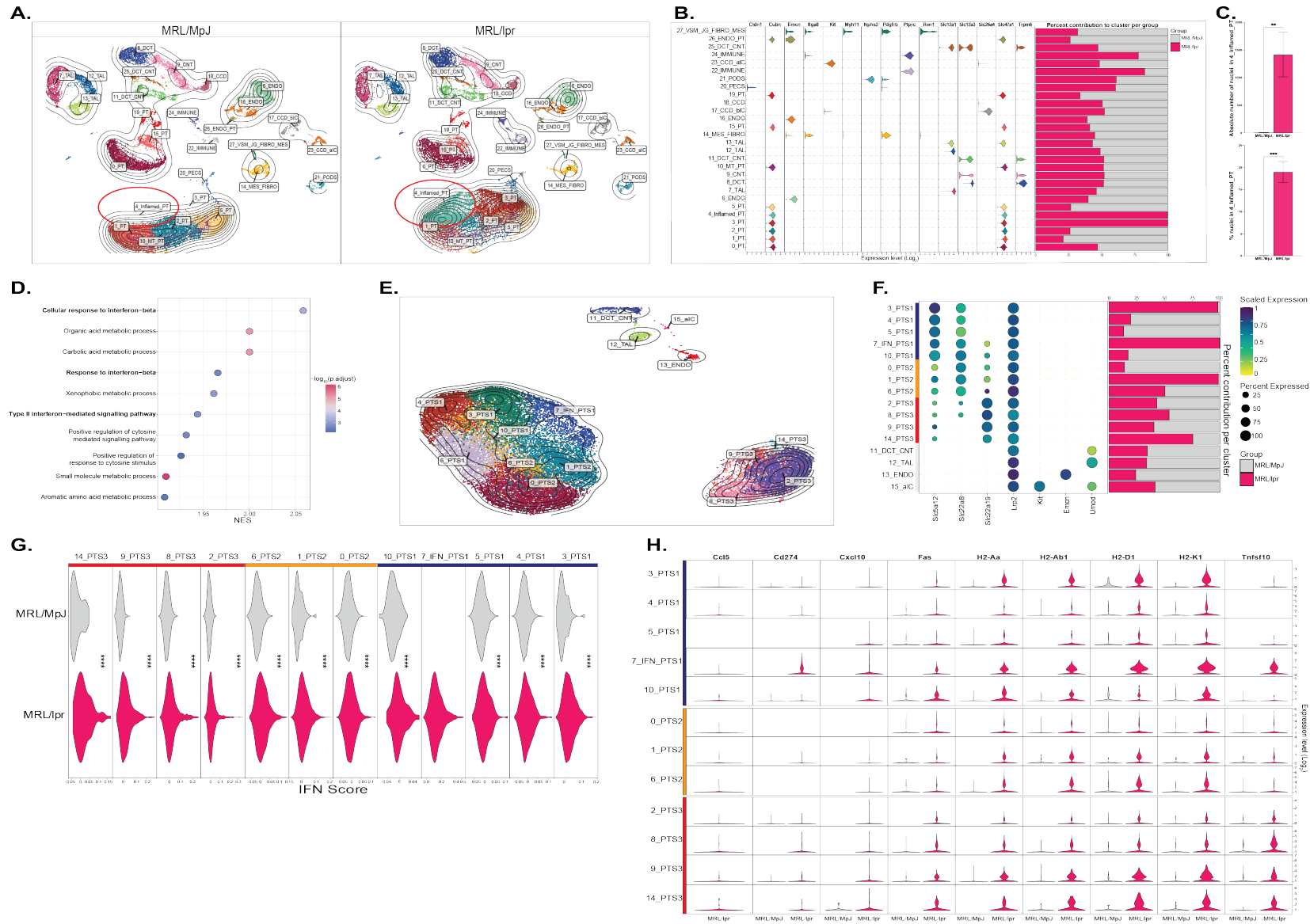


Figure 4.4: SnRNA-seq analysis identifies enhanced susceptibility of proximal tubules to interferon mediated damage.

MRL/lpr and background strain MRL/MpJ were aged for 15 weeks culled and kidneys processed for snRNA-seq ($n=3/\text{group}$). **(A)** Unsupervised clustering of 46k nuclei identified 28 clusters, visualised using dimensionality reduction method UMAP, nuclei were annotated by cluster identity and split by strain: nuclei from MRL/MpJ mice (left panel) and nuclei from MRL/lpr mice (right panel). **(B)** Log2 transformed normalised expression of key markers genes used for annotation of cluster identity. Bar plots represent percentage contribution to each cluster by group. **(C)** Absolute numbers (top panel) and proportions (bottom panel) of MRL/MpJ and MRL/lpr nuclei within 4_Inflamed_PT. Error bars represent standard deviation and significance was calculated using student t-tests where $**P<0.01$ and $***P<0.001$. **(D)** GO ontology-based gene set enrichment analysis of differentially expressed genes in 4_Inflamed_PT when compared with all other clusters, differentially expressed genes were defined as those with an absolute $\log_2\text{FC}>0.25$ and p adjusted value <0.05 . GO terms that met the threshold of p adjusted value <0.05 were sorted by the normalised enrichment score (NES) which represents the degree to which gene sets of GO terms are represented in the list of differentially expressed genes from 4_Inflamed_PT. Dot plot of top 10 enriched GO terms and dot colour represents significance. **(E)** Focused analysis of proximal tubule populations identified 16 additional clusters, visualised using dimensionality reduction method UMAP and annotated by cluster identity. **(F)** Mean expression dot plot of genes used for annotating clusters. Expression value is scaled from 0-1. Bar plots represent percentage contribution to each cluster by group. **(G)** Comparison of interferon response score between MRL/MpJ and MRL/lpr mice within proximal tubule clusters, visualised using a violin plot. Significance was calculated using Wilcoxon rank sum test with BH post-test where $****P<0.0001$. **(H)** Violin plot displaying expression of selected pseudobulk analysis derived differentially expressed genes between MRL/MpJ and MRL/lpr mice across proximal tubular clusters. **PT:** Proximal tubule; **TAL:** Thick ascending limb; **DCT:** Distal convoluted tubule; **CNT:** Connecting tubule; **ENDO:** Endothelial cells; **VSM:** Vascular smooth muscle; **JGA:** Juxtaglomerular apparatus cells; **FIBRO:** Fibroblast; **MES:** Mesangial cells; **PODS:** Podocytes; **PECS:** Parietal epithelial cells; **CCD-AIC:** Cortical collecting duct alpha intercalated cell; **CCD-BIC:** Cortical collecting duct beta intercalated cell; **CCD-IC:** Cortical collecting duct intercalated cell; **MYO:** Myofibroblast; **LOH:** Loop of Henle.

Focused sub clustering of all proximal tubule clusters resolved an additional 16 clusters, corresponding to different proximal tubule segments (Figure 4.4E-F). Nuclei from both mice were represented in 13 out of the 16 clusters, however clusters 1,3,5 and 7 were almost entirely composed of nuclei from MRL/lpr mice (Figure 4.4F). Interferon response scores, calculated from the average expression of a module of interferon stimulated genes were enhanced across proximal tubule clusters in MRL/lpr mice (Figure 4.4G). Additionally, increased expression of MHC class II (*H2-Ab*, *H2-Aa*) and MHC class I (*H2-K1*, *H2-D1*) genes were observed in nuclei from MRL/lpr mice across a subset of early (S1) and late (S3) PT clusters (Figure 4.4H), suggesting increased antigen presentation. Proximal tubule antigen presentation may be important in LN, since autoimmune T cell activity is required for the renal phenotype in MRL/lpr, and renal tubule specific ablation of MHCII genes reduced renal T cell infiltration and fibrosis in ureteric obstruction and folic acid induced models of renal fibrosis(480, 481).

The MRL/lpr specific S1 proximal tubule segment had the highest expression of apoptotic mediator *Tnfsf10* (TNF-related apoptosis inducing ligand /TRAIL), and the highest interferon score, alongside cluster specific enhanced expression of chemokines *Cxcl10* and *Ccl5*, both chemotactic for T cells (Figure 4.4H). In-vitro studies suggest that interaction of TRAIL with its ligand promotes survival and proliferation of interferon stimulated proximal tubular cells, in addition to production of proinflammatory stimuli(482). This could suggest that the LN specific PT cluster 7 may be expanded due to interferon stimulation supported by TRAIL. However, increased TRAIL expression did not correlate with the extent of PT expansion in MRL/lpr compared to MRL/MpJ controls (Figure 4.4F and H).

MRL/lpr mice have ~10 fold less wildtype *Fas* mRNA in the thymus, and instead have aberrantly sized non-functional chimeric transcripts containing sequences from the early retrotransposon (ETn) which are degraded during translation(483, 484). The expression of *Fas* in the kidney in the MRL/lpr model has not been examined. Paradoxically, expression

of apoptotic mediator *Fas* was increased across proximal tubule clusters in MRL/lpr mice compared to controls (Figure 4.4H, *Fas* logFC values; PT cluster 0 1.8886, PT cluster 2:4.8721, PT cluster 3 9.2789). The enhanced expression of *Fas* observed in this study could reflect non-functional chimeric transcripts in the kidney, which may be degraded by nonsense mediated decay during translation. The role of *Fas* in proximal tubules is unclear, with in vitro studies suggesting resistance to *Fas* mediated apoptosis, whereas others have demonstrated enhanced *Fas* mediated apoptosis in proximal tubules in the presence of inflammatory mediators including interferon(485, 486). In summary the snRNA analysis suggests that in the MRL/lpr model, populations of injured proximal tubules may serve as a source of inflammatory stimuli. Enhanced expression of immunomodulatory molecule *Cd274*, encoding programmed death ligand 1 (PD-L1), was observed on proximal tubules in MRL/lpr, particularly on cluster 7 (*Cd274* logFC value; 1.2049, Figure 4.4H). Data on PD-L1 expression in human LN PT is inconclusive, but in-vitro studies suggest a protective effect of this receptor against CD8 T cell mediated cytotoxicity(487-489).

PT is classically considered to be comprised of early, middle, and late segments with specific apical channels and functions, although snRNA-seq has shown that additional sub segments exist(490, 491). Interferon signalling has been described in MRL/lpr proximal tubule but the distribution between the PT segments has not been described. I identified the interferon score was highest in the S1 or early proximal tubule segment(218). Therefore, these data suggest a previously unreported specifically enhanced susceptibility of the S1 segment to interferon mediated damage as demonstrated by increased interferon score with higher expression of genes including *Cd274*, *Tnfrsf10*, *H2-Aa*, *H2-K1* within S1 clusters. This suggests an important role for S1 proximal tubule cells in antigen presentation and perpetuation of localised inflammation through secretion of chemotactic mediators.

4.4 Integrated snRNA-seq analysis of murine models of LN

Integrated analysis, combining the snRNA-seq data from IMQ and MRL/lpr and respective controls was carried out to determine overlapping disease signatures. To correct for numerical difference in nuclei between the two datasets, nuclei were downsampled prior to analysis, generating a dataset of 91K nuclei with equal numbers from MRL/lpr plus control compared to IMQ plus control, and integrated using Harmony to minimise batch and murine strain effects prior to downstream analysis(455)

4.4.1 Enhanced interferon response scores and infiltrating immune cells in murine LN kidneys

Clustering of the integrated dataset identified 30 clusters, across the 4 groups of mice (IMQ treated BALB/C, Vaseline treated BALB/C, MRL/lpr and MRL/MpJ), corresponding to renal tubular and glomerular populations and two immune clusters (Figure 4.5A). Differences between the murine control strains were observed, for example TAL clusters 3 and 29 were more abundant in BALB/c mice, but with no differences in the nuclei numbers due to disease, whilst tubular clusters 14_PT and 7_DCT_CNT predominated in MRL mice irrespective of LN (Figure 4.5A-C).

Cluster contribution also varied with disease as seen in clusters 23_IMMUNE and 25_IMMUNE, which were composed of nuclei primarily from diseased mice from both models, with some disease associated clusters unique to a specific model for example 17_Inflamed_PT in MRL/lpr mice (Figure 4.5B-C). An increased interferon response score was shared across the majority of clusters in nuclei from diseased mice (Figure 4.5D). Endothelial and immune clusters were observed to have higher interferon response scores when compared with the remaining clusters, with the highest interferon response score observed in 4_ENDO.

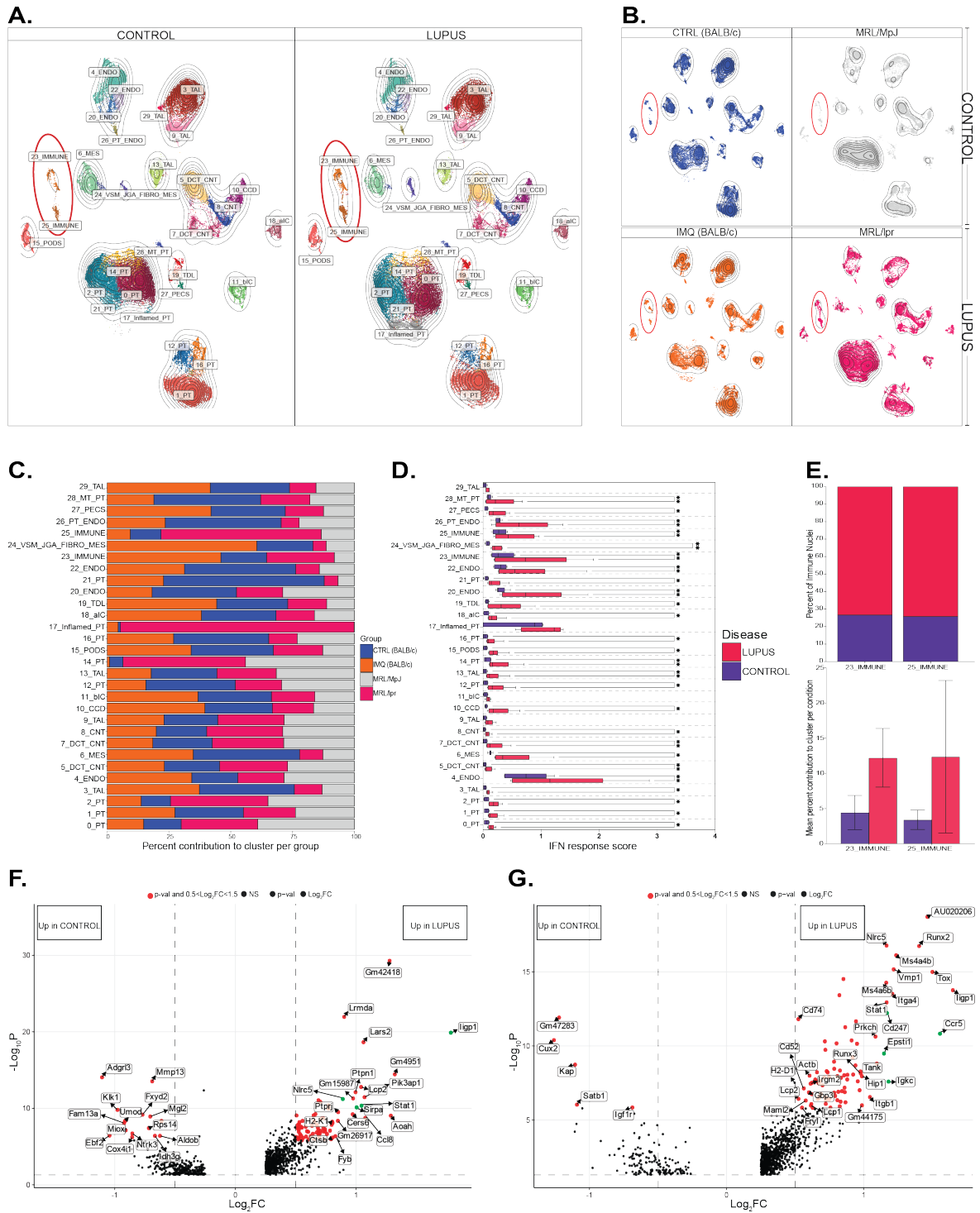


Figure 4.5: Integrated analysis of snRNA-seq datasets identifies enhanced interferon response score and infiltrating immune populations in the kidneys of diseased mice.

SnRNA-seq from IMQ treated mice and Vaseline controls and MRL/MpJ and MRL/lpr mice integrated using Harmony (A) Unsupervised clustering of 91k nuclei identified 30 clusters, visualised using dimensionality reduction method UMAP, nuclei were annotated by cluster identity and split by disease status; nuclei from control mice (Vaseline control and MRL/MpJ; left panel) and nuclei from diseased mice (IMQ treated and MRL/lpr mice; right panel). (B) Clusters as defined in (A) visualised using dimensionality reduction method UMAP, with nuclei coloured and split by group; nuclei from control mice (Vaseline control; top left panel, MRL/MpJ; top right panel) and nuclei from diseased mice (IMQ treated; bottom left panel, MRL/lpr; bottom right panel). (C) Percentage contribution to each cluster by group. (D) Comparison of interferon response score between Control and diseased mice across clusters, visualised using boxplots with whiskers representing the minimum and maximum values per cluster. Significance was calculated using Wilcoxon rank sum test with BH post-test where * $P < 0.05$ and ** $P < 0.01$. (E) Representation of Control and Lupus mice within immune clusters (23_IMMUNE and 25_IMMUNE) (top panel) and mean contribution of Control and Lupus mice within immune clusters (23_IMMUNE and 25_IMMUNE), error bars represent standard deviation (bottom panel). (F) Visualisation of Seurat FindMarkers derived differentially expressed genes between Lupus and Control mice within 23_IMMUNE cluster. Coloured dots (red or green) represent genes that met the threshold of $\log_2FC < 0.5$ and p adjusted value < 0.05 , of these the top 20 genes based on absolute $\log_2FC$ are labelled. (G) Visualisation of Seurat FindMarkers derived differentially expressed genes between Lupus and Control mice within 25_IMMUNE cluster. Coloured dots (red or green) represent genes that met the threshold of $\log_2FC < 0.5$ and p adjusted value < 0.05 , of these the top 20 genes based on absolute $\log_2FC$ are labelled. **PT**: Proximal tubule; **TAL**: Thick ascending limb; **DCT**: Distal convoluted tubule; **CNT**: Connecting tubule; **ENDO**: Endothelial cells; **VSM**: Vascular smooth muscle; **JGA**: Juxtaglomerular apparatus cells; **FIBRO**: Fibroblast; **MES**: Mesangial cells; **PODS**: Podocytes; **PECS**: Parietal epithelial cells; **CCD-AIC**: Cortical collecting duct alpha intercalated cell; **CCD-BIC**: Cortical collecting duct beta intercalated cell; **CCD-IC**: Cortical collecting duct intercalated cell; **MYO**: Myofibroblast; **LOH**: Loop of Henle

Immune cell populations were expanded in the LN kidneys, accounting for ~70% of total immune nuclei and corresponding to a mix of resident and infiltrating immune cells (marked in Figure 4.5A-B and Figure 4.5E). Differential expression between disease and control mice within the two immune clusters (23_IMMUNE and 25_IMMUNE) identified enhanced expression of interferon response genes (*Iigp1* and *Stat1*), pattern recognition receptors including *Nlrc5* (NOD like receptor family CARD domain containing 5) and phagocytosis related genes such as *Sirpa* (signal regulatory protein alpha) within 23_IMMUNE in diseased kidney (Figure 4.5F). Within cluster 25_IMMUNE (Figure 4.5G), LN groups had enhanced expression of genes associated with leukocyte homing such as *Ccr5* and the interferon stimulated gene *Epsti1* (epithelial stromal interaction 1). The latter is upregulated in patients with SLE and important in monocyte adhesion to vasculature(492). TCR receptor gene *Cd247* and immunoglobulin gene *Igkc* were also upregulated, suggesting this cluster included infiltrating immune cells.

4.4.2 LN associated renal B, T, and myeloid cells, with differences in immune composition between models of disease

Further clustering of the renal immune cells (23_IMMUNE and 25_IMMUNE) identified an additional 17 clusters corresponding to B, T, and myeloid cells (Figure 4.6A and B). Differences in immune cell numbers and composition were observed between models of disease, with the highest number of immune nuclei in MRL/lpr kidneys (Figure 4.6C), and T cells (both CD4 and CD8) contributing the largest proportion of immune cells (Figure 4.6D), in line with flow cytometry experiments in which T cells accounted for the highest proportion of CD45⁺ cells in MRL/lpr kidneys(264).

While T cell expansion was predominantly seen in MRL/lpr compared to control, both IMQ and MRL/lpr kidneys were characterised by expansion of innate populations (Figure 4.6C and D). These differences in immune populations may be explained by the different drivers of disease, with Fas expression important for adaptive immune cell homeostasis and tolerance, whereas TLR7 agonism is associated with activation of innate immune components(290, 493). Parallels can be drawn between the immune profile of this study and human LN transcriptomic studies, in which enhanced T cell and innate populations (e.g., macrophages) were observed in LN biopsies, with T cell and myeloid cells found to be the most abundant populations(190)

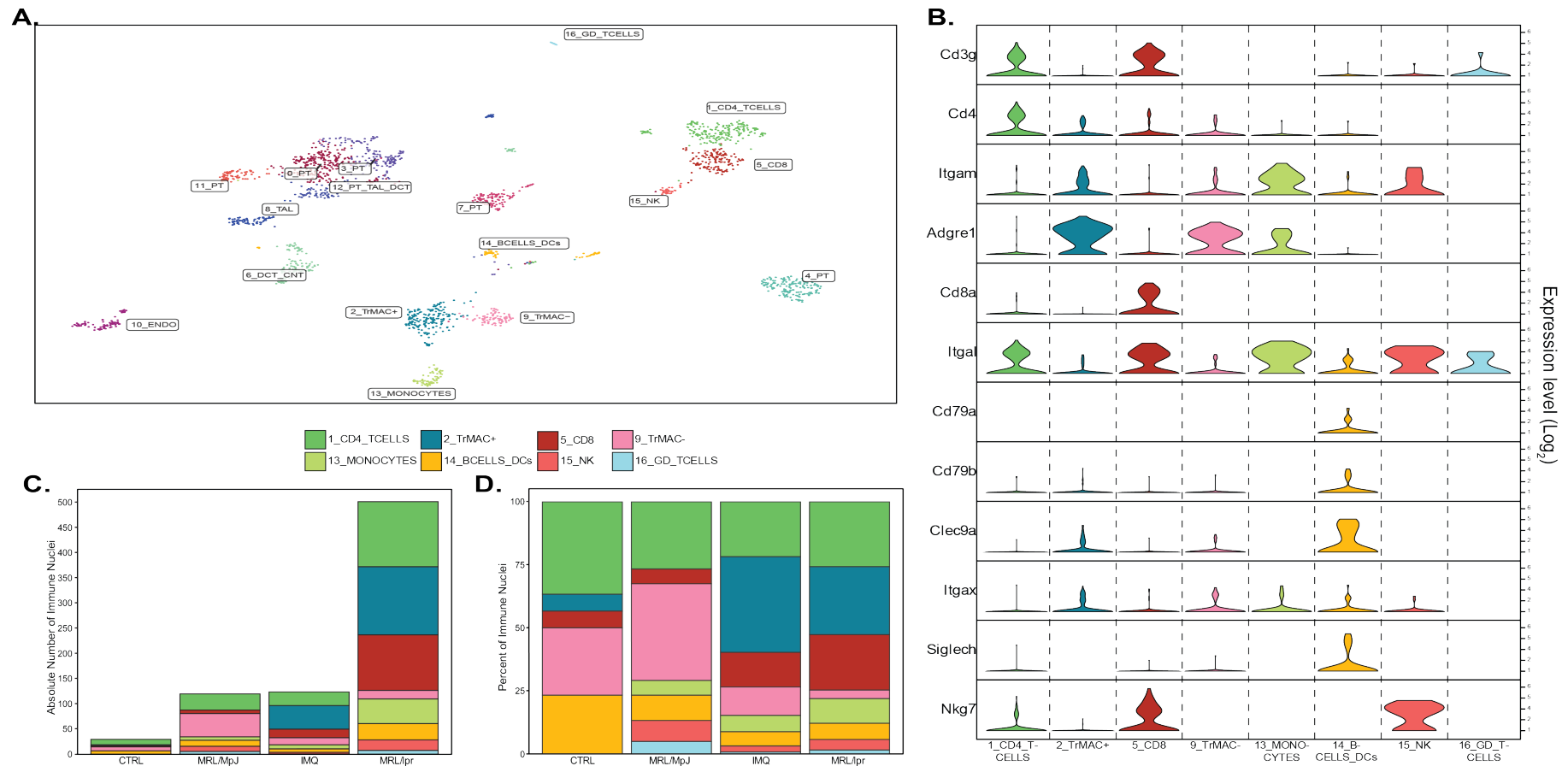


Figure 4.6: Focused analysis of immune clusters identifies intrarenal B.T and myeloid cells.

Nuclei within immune clusters (23_IMMUNE and 25_IMMUNE) were extracted from the integrated snRNA-seq dataset for further analysis (A) Unsupervised clustering of immune nuclei identified 17 clusters, visualised using dimensionality reduction method UMAP, nuclei were annotated by cluster identity (B) Visualisation of RNA expression of key markers genes used for annotation of immune cluster identity. The y-axis shows the log2 transformed normalised expression (C) Absolute number of nuclei per immune cluster within each group. (D) Proportion of immune nuclei per cluster within each group. **PT**: Proximal tubule; **TAL**: Thick ascending limb; **DCT**: Distal convoluted tubule; **CNT**: Connecting tubule; **ENDO**: Endothelial cells; **NK**: Natural killer cells; **TrMAC**: Tissue resident macrophages; **GD T cell**: Gamma delta T cells; **DCs**: Dendritic cells

4.4.3 NK and T cells from MRL/lpr mice express markers associated with exhaustion and active cytotoxicity

Characterisation of CD4 and CD8 T cell subsets identified functional signatures specific to MRL/lpr mice. CD4 T cells (1_CD4_TCELLS) were characterised by expression of markers such as *Eomes*, *Btla* and *Prdm1* (Figure 4.7A), comparable to markers expressed by exhausted CD4 T cells during chronic infection, however the effector functions of such cells remain unclear(494). A similar exhaustion signature was observed within CD8 T cells (5_CD8), characterised by expression of inhibitory molecules *Pdcd1* (PD1), *Tbx21* (Tbet) and *Havcr2* (TIM-3) (Figure 4.6B). *Pdcd1* and *Tbx21* upregulation mirrored the peripheral CD8 T cell phenotype observed by flow cytometry in these mice (Figure 3.6J and K). This similarity with circulating T cells suggests that the CD8 T cells are infiltrating and that the profile is systemic rather than renal intrinsic.

Cytotoxicity was evidenced by enhanced expression of cytokine genes *Ifng* and *Tnf* and cytotoxic granule proteins *Prf1* and *Gzmk* (Figure 4.7B). Calculation of an exhaustion score, defined as the average expression of a module of T cell exhaustion markers, for both T cell clusters, identified a significantly enhanced score in MRL/lpr mice (Figure 4.7C-D), though the presence of this signature in MRL/MpJ mice could indicate it reflects defective Fas mediated cell death rather than exhaustion per se. Gene ontology-based gene set enrichment analysis of differentially expressed genes from MRL/lpr mice in each T cell population identified GO terms associated with T cell activation in both clusters, leukocyte mediated cytotoxicity in 5_CD8 and positive T cell selection in 1_CD4_TCELLS (Figure 4.7E-F). Thus, the expanded CD8 population in MRL/lpr kidney was identified as exhausted-like cytotoxic effector CD8 T cells and the CD4 population as activated CD4 T cells(190). ScRNA-seq studies have identified cytotoxic CD8 T cells in the kidneys of LN patients, but these CD8 T cells did not demonstrate enhanced expression of exhaustion markers(190).

Cluster 15/15_NK had enhanced expression of *Ncr1* (NKp46), *Il2rb*, *Klrk1* (NKG2D) and *Itgam*, and low expression of *Cd27*, identifying these as mature NK cells(495). Enhanced expression of cytotoxic genes such as *Prf1*, *Gzma* and *Gzmb* in addition to MRL/lpr specific increased expression of *Lamp1* (CD107a) which is expressed by degranulating NK cells, suggested these were activated cytotoxic NK cells (Figure 4.7G)(496). Consistent with this gene ontology-based genes set enrichment analysis identified GO terms associated with NK cell activation and cytotoxicity (Figure 4.7H). Previous scRNA-seq studies have identified the presence of two distinct NK populations in the kidneys from patients with LN, with one cluster representing mature NK cells with enhanced expression of cytotoxic related genes, and another small population of CD16⁻ tissue-resident NK cells(190). Therefore, recognition of actively degranulating cytotoxic CD107a⁺ NK cell population which potentially enhances renal inflammation, represents a novel finding, though it is not yet clear if this is mirrored in human LN(496).

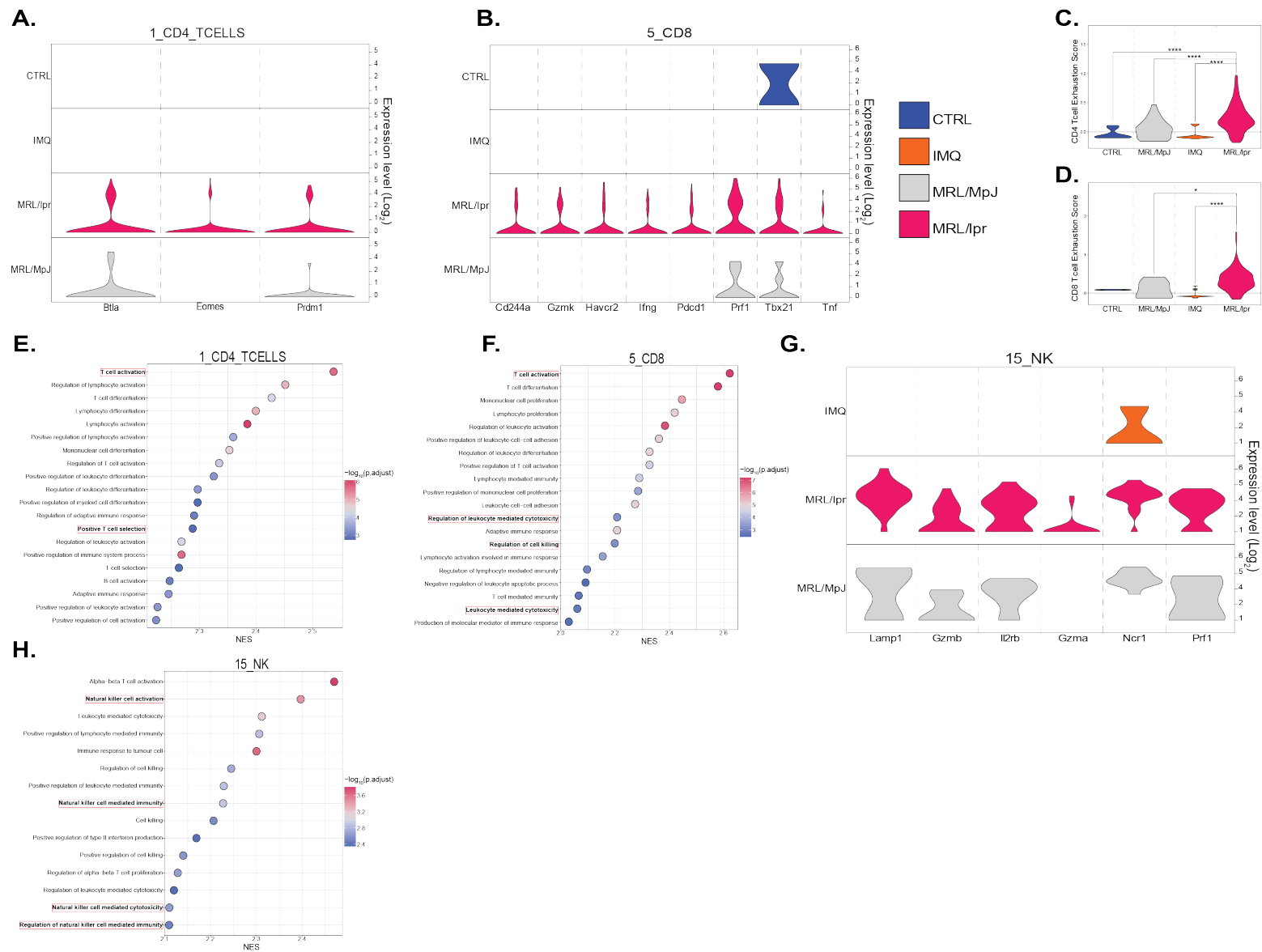


Figure 4.7: Cytotoxic NK and exhausted T cell signatures in nuclei from MRL/lpr mice.

Nuclei within immune clusters (23_IMMUNE and 25_IMMUNE) were extracted from the integrated snRNA-seq dataset for further analysis. **(A)** Expression of select markers associated with CD4 T cell exhaustion between groups within 1_CD4_TCELLS cluster. The y-axis shows the log2 transformed normalised expression. **(B)** Expression of select markers associated with CD8 T cell exhaustion and cytotoxicity between groups within 5_CD8 cluster. The y-axis shows the log2 transformed normalised expression. **(C)** Exhaustion score between groups within 1_CD4_TCELLS cluster visualised using a violin plot. Significance was calculated using Wilcoxon rank sum test with BH post-test where **** $P < 0.0001$. **(D)** Exhaustion score between groups within 5_CD8 cluster visualised using a violin plot. Significance was calculated using Wilcoxon rank sum test with BH post-test where * $P < 0.05$, **** $P < 0.0001$. GO ontology-based gene set enrichment analysis of differentially expressed genes in MRL/lpr mice when compared with remaining groups (MRL/MpJ, CTRL and IMQ) within **(E)** 1_CD4_TCELLS and **(F)** 5_CD8. Differentially expressed genes were defined as those with an absolute log2FC > 0.25 and p adjusted value < 0.05. GO terms that met the threshold of p adjusted value < 0.05 were sorted by the NES, which represents the degree to which gene sets of GO terms are represented in the list of differentially expressed genes from MRL/lpr nuclei per cluster. Dot plots represent the top 20 enriched GO terms per cluster and dot colour represents significance. **(G)** Expression of select markers associated with NK cytotoxicity between groups within 15_NK cluster. The y-axis shows the log2 transformed normalised expression. **(H)** GO ontology-based gene set enrichment analysis of differentially expressed genes as defined in **(E-F)** from MRL/lpr mice when compared with remaining groups (MRL/MpJ, CTRL and IMQ) within 15_NK. Dot plots represent the top 20 enriched GO terms per cluster and dot colour represents significance.

4.4.4 Identification of an altered tissue resident macrophage signature in the kidneys of diseased mice

Two renal tissue resident macrophage (TRM) clusters were identified from the immune sub clustering (2_TrMAC+ and 9_TrMAC-), defined based on expression of canonical markers *Itgam* (CD11b) and *Adgre1* (F4/80) and additional markers such as *C1qa*, *C1qb* and *Cd81* (Figure 4.6B and 4.8A)(497). These two clusters were primarily differentiated by the relative expression of *Itgam* and *Adgre1*, with the former observed to be higher in 2_TrMAC+, therefore defining it as CD11b^{hi}/F4/80^{int/high} and 9_TrMAC- as CD11b^{low}/F4/80^{high}. Two TRM populations have previously been described in the lupus kidney with the origins of CD11b^{low}/F4/80^{high} TRMs suggested to be prenatally seeded yolk sac derived and CD11b^{hi}/F4/80^{int/high} TRMs monocyte derived(359). The two TRM populations identified in this dataset are comparable to the MNP1 (CD11b^{low}/F4/80^{high}) and MNP2 (CD11b^{hi}/F4/80^{int/high}) groups identified in MRL/lpr kidneys in a study by Richoz et al, which enriched for renal mononuclear phagocytes prior to single cell sequencing(362).

2_TrMAC+ were observed in the kidneys in both LN models, with this population essentially absent in control kidneys (2 nuclei) (Figure 4.6D and Figure 4.8B). Conversely higher absolute numbers and proportions of 9_TrMAC- were observed in the kidneys of control mice, suggesting that 9_TrMAC- represents a steady-state TRM population in health (Figure 4.8B). These findings were confirmed by flow cytometry which identified increased proportions and absolute numbers of CD11b^{hi}/F4/80^{int/high} and decreased CD11b^{low}/F4/80^{high} macrophages in the kidneys of MRL/lpr mice (Figure 4.8C-E).

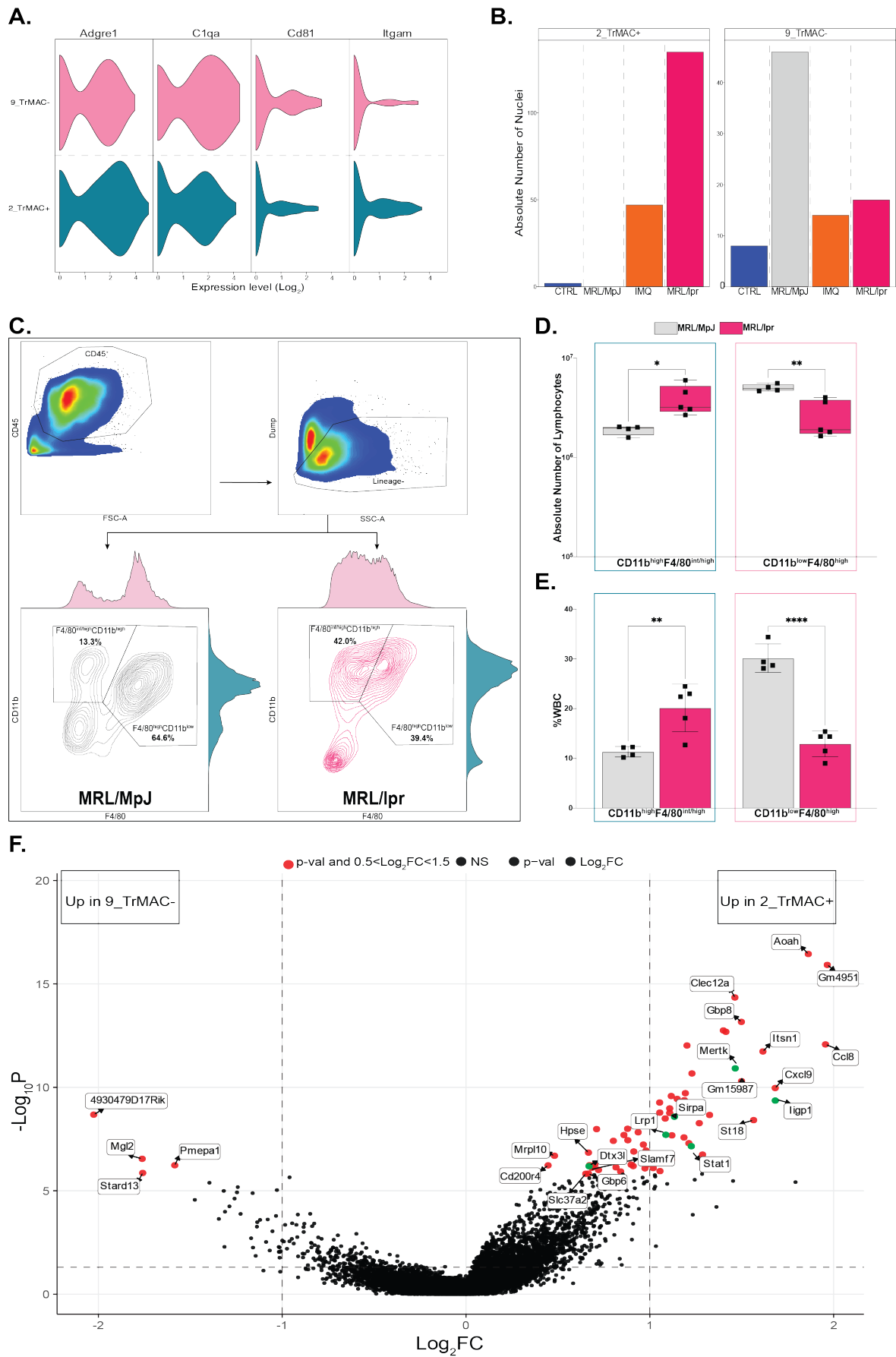


Figure 4.8: Shared altered tissue resident macrophage signature in the kidneys of diseased mice.

Nuclei within immune clusters (23_IMMUNE and 25_IMMUNE) were extracted from the integrated snRNA-seq dataset for further analysis. (A) Expression of select markers used for annotation of cluster identity. The y-axis shows the log2 transformed normalised expression. (B) Quantification of absolute number of nuclei per TRM cluster per group; 2_TrMAC+ (left panel) and 9_TrMAC- (right panel). After initial gating on singlets and dead cells as shown in Figure 3.3A (C) Renal immune cells were gated on CD45 expression after which B220+ and CD3+ were excluded prior to further analysis (top panel). Representative contour plots with adjacent histograms representative of CD11b (right y-axis) and F4/80 (top x-axis) expression on renal TRMs from MRL/MpJ (left) and MRL/lpr (right), the percentage of each TRM population is shown. (D) Absolute numbers of renal TRM populations, boxplot whiskers show min and max values, and line represents median. (E) Proportions of renal TRM populations, data are mean $\pm$ 95% CI. (F) Visualisation of Seurat FindMarkers derived differentially expressed genes between 2_TrMAC+ and 9_TrMAC-. Coloured dots (red or green) represent genes that met the threshold of log2FC <0.5 and p adjusted value <0.05, of these the top 20 genes based on absolute log2FC are labelled. Significance for (D/E) was determined by multiple t-tests using the Holm-Sidak method to correct for multiple comparisons *P $\leq$ 0.05, **P $\leq$ 0.01, ****P $\leq$ 0.0001 and data are representative of 2 independent experiments, each with 3-5 mice per group.

Markers defining the LN associated 2_TrMAC+ cluster relative to 9_TrMAC- included genes associated with phagocytosis, efferocytosis (*Mertk*, *Lrp1* and *Sirpa*) and interferon responses (*Stat1* and *ligp1*), alongside enhanced expression of activation associated gene *Slamf7* (Figure 4.8F). *Slamf7* expression is associated with “super activated” macrophages driving proinflammatory cytokine production in rheumatoid arthritis(256). Studies in haematopoietic tumour cells have suggested an important role for *Slamf7* in phagocytosis via interaction with CD11b(498). In conclusion, altered TRM populations in the kidneys of LN mice include expansion of CD11b^{hi}/F4/80^{int/high} likely monocyte derived macrophages, with enhanced expression of phagocytosis associated genes.

4.4.5 Increased monocytic infiltrate in LN kidneys represents a potential source for the increased TRM population

A single monocyte cluster was identified (13_MONOCYTES), which was increased in both absolute number and proportion in the kidneys of diseased mice in both LN models (Figure 4.6C-D). This cluster was characterised by canonical monocyte markers such as *Itgal* and *Itgam*, alongside high expression of *Nr4a1*, *Spn* (CD43), *Itgax* (CD11c), *Fcgr4* (CD16.2)

and low expression of *Ly6c2* (Figure 4.9A), identifying this cluster as patrolling non-classical monocytes(192).

A subpopulation of *Fcrl5*⁺ monocytes unique to IMQ treated mice was observed within this cluster representing a novel non-classical monocyte population (Figure 4.9B). Murine FCRL5 is expressed on B1 and MZ B cells as well as ABCs in aged mice, with a previous study reporting augmented IMQ induced autoimmunity in transgenic mice overexpressing *Fcrl5* specifically in B cells compared with WT mice(499). However, this study did not assess expression of *Fcrl5* on any other immune subsets or the impact of TLR7 agonism on *Fcrl5* expression in WT mice treated with IMQ(499). As expression of *Fcrl5* was observed in the 14_BCELLS_DCs (Figure 4.9B) in both groups of disease mice, this would suggest that differences in *Fcrl5* expression in monocytes was not due to ambient RNA contamination or misclustering of B cells. How TLR7 agonism drives monocyte *Fcrl5* expression, and whether *Fcrl5* monocytes represent a pathological population remains to be explored.

Differential expression between monocytes and each TRM cluster identified genes associated with tissue infiltration such as *Pecam1*, *Cd44* and *Itga4* to be upregulated in the monocytes, consistent with a renal infiltrating as opposed to a circulating monocyte population (Figure 4.9C-D). Whilst classical monocytes are usually considered to differentiate into macrophages, lung infiltrating non-classical Nr4a1⁺ monocytes can act as precursors to interstitial macrophages, differentiating in situ upon infiltration(193, 500). As the expression profile of disease enhanced TRM cluster 2_TrMAC⁺ was consistent with being monocyte derived, trajectory analysis was applied to computationally predict if disease enhanced infiltrating monocytes might differentiate into TRM. Inferred trajectory constructed a pathway from 13_MONOCYTES to the disease enhanced 2_TrMAC⁺ cluster (Figure 4.9E), suggesting infiltrating non-classical monocytes may serve as a precursor to a CD11b⁺ TRM population in the LN kidney. However, the effect of population abundance

on skewing trajectory inference cannot be discounted and must be considered when interpreting such an analysis(476).

4.4.6 Differential expression and ligand receptor analysis identifies pan-immune targets with therapeutic potential

To better understand interactions between the immune populations and renal cells, ligand receptor analysis was carried out across all nuclei. Assessment of interactions between immune and renal clusters identified differences in overall enhanced pathways between control and disease mice (Figure 4.10A). Pathways accounting for the highest proportion of interactions which were shared between immune clusters in disease mice and different from control mice included MHC-I and VCAM (Figure 4.10A). Within the MHC-I pathway all significant interactions were between immune clusters, with no interactions observed between immune and renal clusters (Figure 4.10B). Contrastingly, within disease enriched VCAM pathway a significantly enhanced probability of interaction between migration associated integrin ITGA4 and its ligand VCAM1 was observed, with both immune clusters serving as the receptor source (i.e., expressing ITGA4), and the targets (i.e., clusters expressing VCAM1) including endothelial clusters (4_ENDO and 20_ENDO) and parietal epithelial cell cluster (27_PECS) (Figure 4.10C). Integrin ITGA4 can heterodimerise with either ITGB1 or ITGB7, with binding to ITGB1 resulting in the formation of the VLA4 (very late antigen 4) complex, important for tissue homing and infiltration and increased on peripheral monocytes from patients with SLE(501). Heterodimerisation with ITGB7 forms the LPAM1 (Lymphocyte Peyer's patch adhesion molecule 1), implicated in leukocyte recruitment in ulcerative colitis(502).

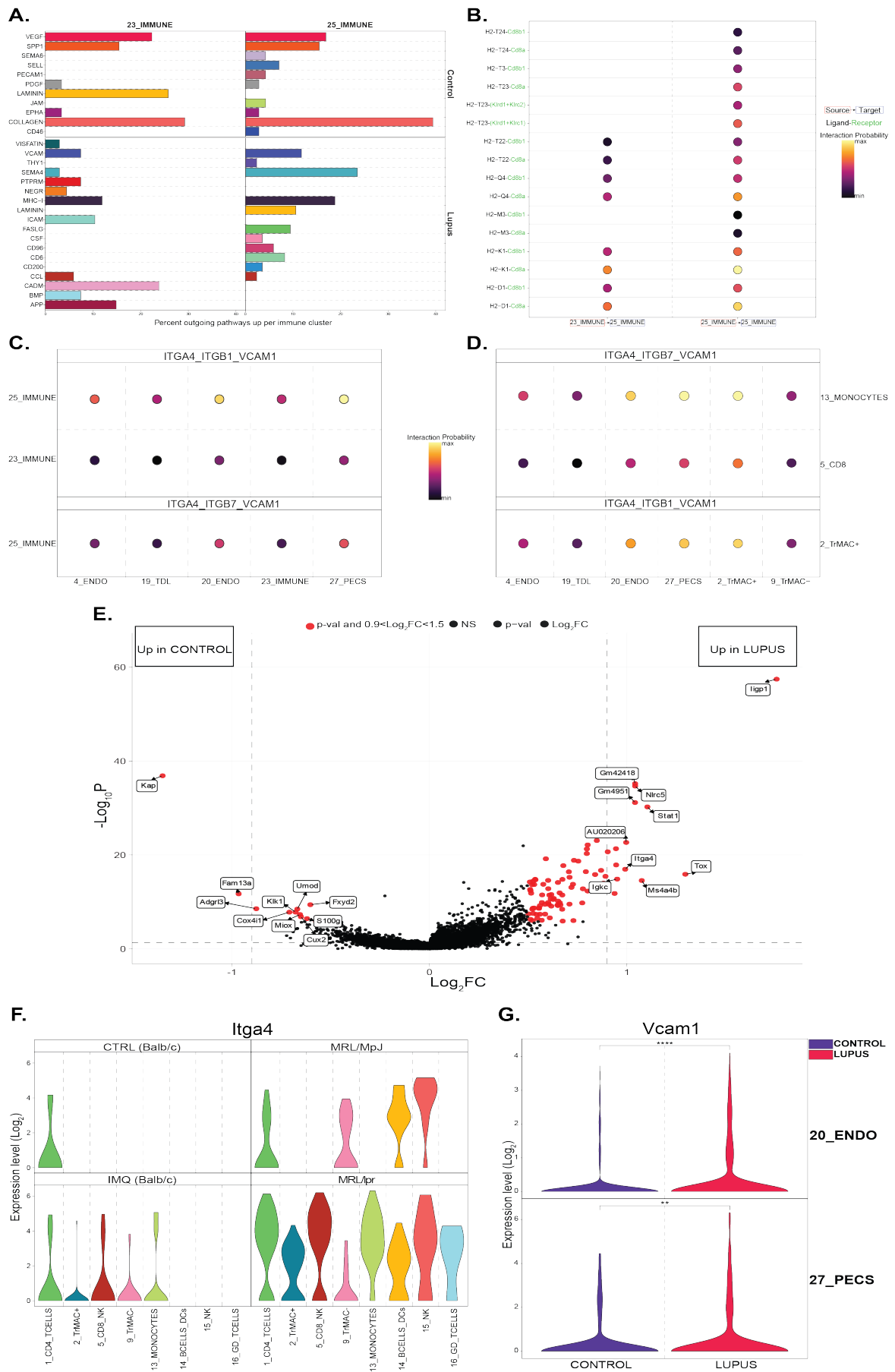


Figure 4.10: Identification of pan-immune targets with therapeutic potential.

(A) Percent contribution per pathway to upregulated signalling within 23_IMMUNE and 25_IMMUNE. (B) Significant ligand receptor interaction probabilities within the MHC-1 pathway, labels on the y-axis represent relevant ligand receptor pairs and labels on the x-axis represent directionality of interaction. Interactions are considered significant when the p value adjusted <0.05 of the interaction score. (C) Inferred probability of interaction between ITGA4/ITGB1 or ITGA4/ITGB7 in nuclei from Lupus mice with ligand Vcam1, labels on the y-axis represent the source and labels on the x-axis represent the targets, when ligand receptor analysis is carried on all nuclei. Interactions are considered significant when the p value adjusted <0.05 of the interaction score. (D) Inferred probability of interaction between ITGA4/ITGB1 or ITGA4/ITGB7 in nuclei from Lupus mice with ligand Vcam1, labels on the y-axis represent the source and labels on the x-axis represent the targets, when ligand receptor analysis is carried on all nuclei, with immune sub population annotations incorporated into analysis. Interactions are considered significant when the p value adjusted <0.05 of the interaction score. (E) DESeq2 derived differentially expressed genes between Lupus and Control mice across all immune sub populations. Coloured dots represent genes that met the threshold of $\log_2FC < 0.9$ and p adjusted value <0.05 , of these the top 10 genes based on absolute $\log_2FC$ are labelled. (D) Violin plot of *Itga4* expression across immune subpopulations per group; Control mice (top right and left panel) and Lupus mice (bottom right and left panel). (E) Violin plot of pseudobulk analysis derived differentially expressed *Vcam1* between Control and Lupus mice, within ligand receptor target clusters from (C/D) 20_ENDO (top panel) and 27_PECS (bottom panel).

To identify which immune subpopulations were involved in these interactions, immune subset annotations were mapped onto the ligand receptor data. This highlighted 5_CD8, 13_MONOCYTES and 2_TrMAC+ clusters as being the main sources of ITGA4 (Figure 4.10D). However, calculation of significant ligand receptor interactions can be skewed by differences in cell number. Therefore, to ascertain if these interactions were plausible, expression of ligand and receptor genes was also assessed by differential expression. Due to insufficient numbers of nuclei in some control immune clusters, differential expression analysis between disease and control mice at an individual cluster level was not possible, therefore this was assessed by comparing pooled immune clusters between lupus and controls. This confirmed that the highest expression of *Itga4* was on clusters identified by ligand receptor analysis in disease mice (Figure 4.10E-F). Consistent with ligand receptor analysis, differential expression identified enhanced *Vcam1* expression in clusters 20_ENDO and 27_PECS (Figure 4.10G).

4.4.7 Protein validation of enhanced expression of *Itga4* and ligand *Vcam1* in the kidneys of MRL/lpr mice.

Previous studies in NZB/NZW mice have reported enhanced renal *Itga4* and *Vcam1* expression with the onset of proteinuria(503). By immunohistochemistry nephritic MRL/lpr mice have increased expression of *Vcam1* in the endothelium and glomeruli and diffuse tubular expression, and increased numbers of VLA4 positive cells in the glomeruli, in comparison to MRL/MpJ, suggesting upregulation of *Vcam1* could mediate immune cell adhesion in nephritic kidneys via interaction with VLA4(504).

Therefore, to assess if the transcriptomically identified enhanced expression of *Itga4* was mirrored at the protein level in pre-nephritic mice, frozen kidney sections from MRL/lpr and MRL/MpJ mice were stained for *Itga4*. Immunofluorescence identified enhanced expression of *Itga4*, with staining localised primarily to the glomeruli, in the kidneys of MRL/lpr mice compared to MRL/MpJ (Figure 4.11A-B). Having identified *Vcam1* as being the primary ligand for *Itga4* (section 4.4.6), protein level expression was similarly assessed in the kidneys of pre-nephritic mice. *Vcam1* intensity was increased in the kidneys of MRL/lpr mice compared to MRL/MpJ, with brightest staining observed to be surrounding the glomeruli and some staining observed in the tubules of MRL/lpr mice (Figure 4.11C and D). In contrast in MRL/MpJ mice, staining was localised primarily to blood vessels, in line with constitutive endothelial expression of this protein, with very minimal staining observed in the glomeruli (Figure 4.11C and D). Images taken at 100X magnification (Figure 4.11C lower panel) suggested glomerular staining in MRL/lpr mice was localised around the capsule, and could therefore derive from parietal epithelial cells, however co-staining with a parietal epithelial cell specific marker would be needed to confirm this. Thus, enhanced protein expression of *Itga4* confirmed transcriptomic results, providing a basis for targeting this receptor in vivo.

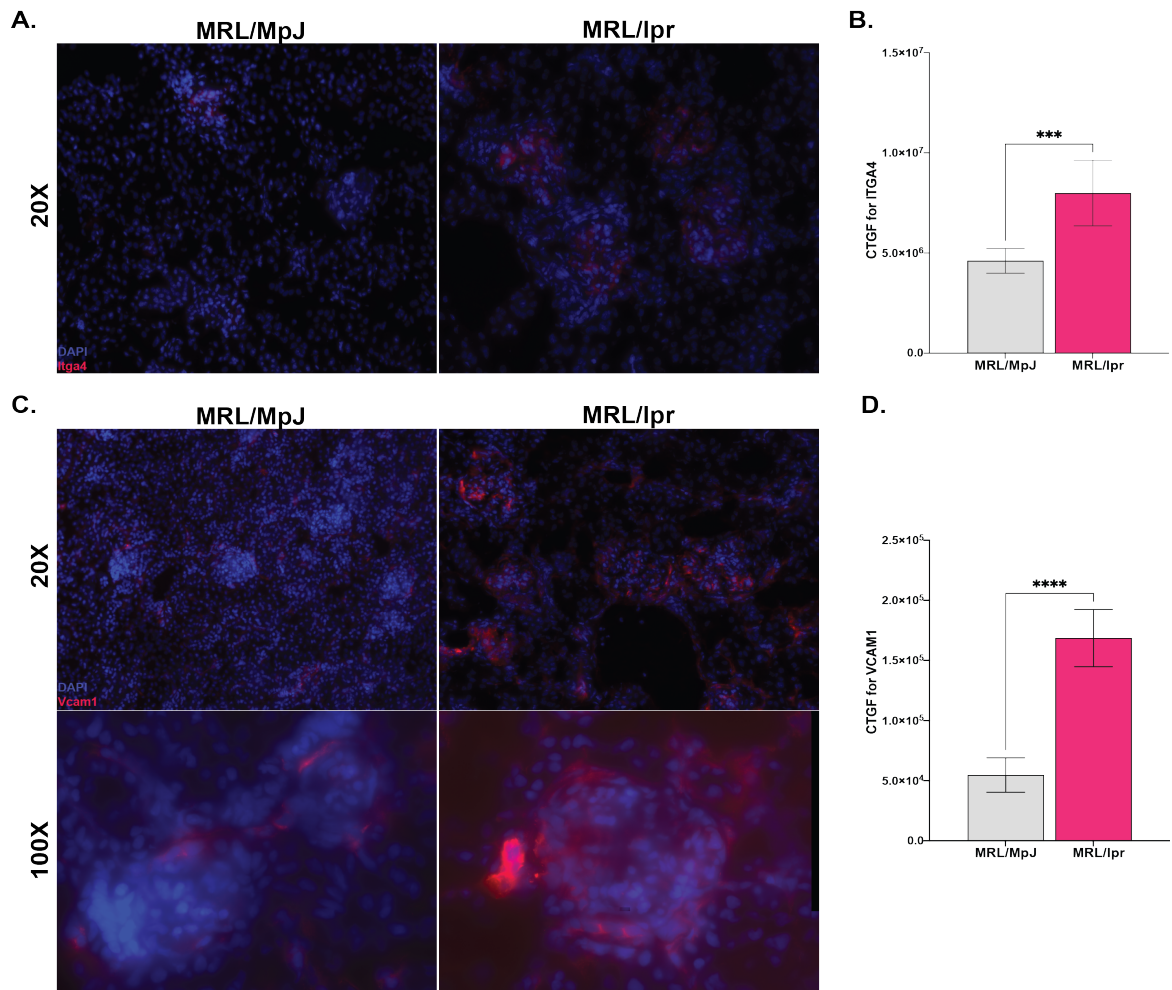


Figure 4.11: Immunofluorescence assessment of VCAM1 and ITGA4 expression in the kidneys 15-week-old MRL/MpJ and MRL/lpr mice.

MRL/lpr and background strain MRL/MpJ were aged for 15 weeks culled and kidneys snap frozen and embedded in OCT prior to being cut into 10µm sections. **(A)** Representative immunofluorescence staining of kidney sections from MRL/MpJ and MRL/lpr mice for *Itga4* (20X magnification). **(B)** Quantification of *Itga4* glomerular fluorescence intensity corrected for background. **(C)** Immunofluorescence staining of kidney sections from MRL/MpJ and MRL/lpr mice for *Vcam1*; 20X magnification (top panels) and 100X magnification (bottom panels). **(D)** Quantification of *Vcam1* glomerular fluorescence intensity after being corrected for background. In **(B/D)** intensities were measured for 20 glomeruli per mouse, for 5 MRL/MpJ and 3 MRL/lpr mice. CTGF is defined as the corrected total glomerular fluorescence and represents the glomerular fluorescence intensity after being corrected for background, calculated as per section 2.4.3.3. Data are mean ± 95% CI, analysed by a Welch's t-test *** $P < 0.001$, **** $P < 0.0001$ and representative of two independent experiments each with 3-5 mice per group.

4.5 Integration of human and murine LN immune cell transcriptomic profiles.

Currently available human LN datasets are restricted to tubular or immune populations, no existing human lupus dataset includes comprehensive renal and immune populations(190, 319). To better understand pathways overlapping with human disease and to assess human relevance of identified pathways, immune cell subpopulations were integrated with a scRNA-seq dataset comprised of CD45⁺ cells from the kidneys of patients with LN and controls(190).

4.5.1 Integration of murine and human immune renal transcriptomic data identifies overlapping pathways.

Integration and unsupervised clustering of human and murine immune data identified 19 clusters. Murine nuclei and human cells were observed to be contributing to most immune clusters, however tubular clusters were made up largely of murine nuclei, reflecting the purity of the CD45⁺ population isolated from human renal samples. IMQ nuclei were poorly represented in several immune clusters, but more consistent homology was observed between MRL/lpr and human data, therefore further analysis focused on the overlap between MRL/lpr and human kidney immune populations (Figure 4.12A-C).

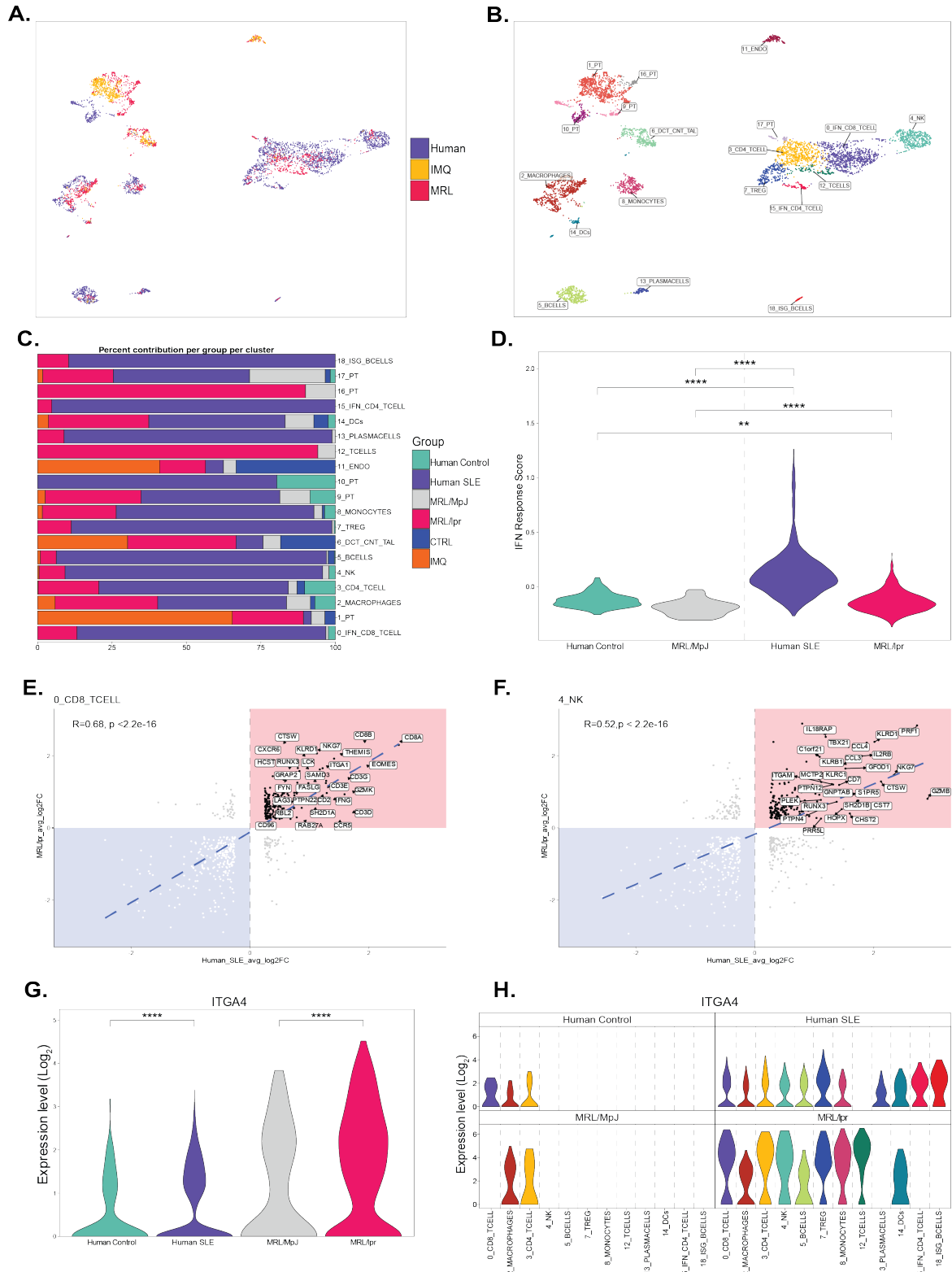


Figure 4.12: Integration of murine nuclei and human single cell data.

(A) Human CD45⁺ kidney single cell and mouse immune single nuclei were integrated using Harmony batch correction, efficiency of batch correction was visualised using dimensionality reduction method UMAP, with nuclei/cells coloured by batch of origin. (B) Unsupervised clustering of integrated data identified 19 clusters, visualised using dimensionality reduction method UMAP, with nuclei/cells annotated by cluster identity. (C) Percentage contribution to each cluster by group. (D) Comparison of interferon response score between human control, human SLE, MRL/lpr and MRL/MpJ visualised using violin plots. Significance was calculated using Wilcoxon rank sum test with BH post-test where $^{**}P < 0.01$, $^{****}P < 0.0001$. (E) Scatter plot comparing expression of marker genes from MRL/lpr mice (y-axis) and human SLE (x-axis) within 0_CD8_TCELL cluster, conserved genes are labelled in the upper right quadrant. Strength of correlation was quantified using Spearman's correlation test. (F) Scatter plot comparing expression of marker genes from MRL/lpr mice (y-axis) and human SLE (x-axis) within 4_NK cluster, conserved genes are labelled in the upper right quadrant. Strength of correlation was quantified using Spearman's correlation test. (G) Violin plot of *ITGA4* expression pooled across immune clusters compared between human Control and human SLE and MRL/MpJ and MRL/lpr. (H) Violin plot of *ITGA4* expression across clusters per group; Controls (top left and bottom left panel) and Lupus (top right and bottom left panel).

Overlapping features included a significantly enhanced interferon response score in disease, when both murine and human disease groups were compared with healthy controls (Figure 4.12D). Within the 0_CD8_TCELL and 4_NK clusters, shared human and mouse LN cytotoxic and activated signatures were observed, with expression of markers such as *IFNG*, *GZMK* and *ITGA1* in the CD8 T cells, *PRF1* and *GZMB* in NK cells and *FASLG* in both CD8 T and NK cells. These markers were positively correlated, in terms of fold change versus control, between LN patients and MRL/lpr mice (Figure 4.12E-F), indicating that the MRL/lpr model closely mimics the renal cytotoxic T cell response in LN.

The increased *ITGA4* signature observed in murine datasets was also found across CD45⁺ immune cells from human LN patients (Figure 4.12G), mapping of *ITGA4* expression by cluster identified enhanced expression in all immune clusters which included cells from LN patients (Figure 4.12H), supporting the putative relevance of this receptor in clinical disease.

4.6 Summary

Progress in multi-omic sequencing has enabled in-depth characterisation of cellular populations and modelling of complex interactions. However, no previous single cellular dataset characterising both renal and immune populations in lupus nephritis has been published, with existing data selective for immune cells(190). Whilst this has shed light on the heterogeneity and functionality of immune populations in the LN kidney, these approaches are unable to elucidate complex interactions between renal and immune cells. To do this requires adequate capture of both renal and immune populations.

ScRNA-seq of IMQ kidneys identified tubular and immune populations but inadequate representation of glomerular cells, with numbers insufficient to form discrete clusters(441). In contrast data from snRNA-seq experiments demonstrated sufficient capture of glomerular and immune populations, so this method was chosen for further experiments. Two murine models of LN with different aetiologies were chosen, to mimic some of the human heterogeneity seen in this disease, with the aim of identifying overlapping and model specific disease pathways.

IMQ treated mice had decreased numbers of mesangial cells and expansion of a transcriptomically similar fibroblast population. Phenotypic alterations in mesangial cells suggest acquisition of an activated phenotype characterised by enhanced expression of fibrotic mediators and production of proinflammatory mediators, accompanied by decreased expression of *Itga8*. I hypothesised that the reduction in mesangial numbers in the IMQ kidney was due to mesangial transition towards a more pro-fibrotic and inflammatory phenotype, contributing to the IMQ specific fibroblast cluster, as supported by trajectory analysis, and potentially driven by alterations in *Itga8* expression. Whilst trajectory analysis is indicative this does not definitively prove the relationship between these clusters, and these could represent unrelated populations. Spatial approaches and in vitro experiments would help to test this hypothesis. Better understanding of mesangial cell

contribution towards fibrosis may provide important insights into underlying fibrotic processes leading to the irreversible loss of renal function in disease.

Increased interferon response scores, particularly in proximal tubular clusters, were observed in the kidneys of MRL/lpr mice, with focused analysis of these clusters suggesting a role for these cells in antigen presentation via upregulation of MHC genes. Expression of chemokine and pro-inflammatory genes suggested a role for these cells in the renal inflammation, with the strongest signal localised to the S1 segment. The mesangial and proximal tubule signatures were model specific, showing that disease mechanism influences renal populations in LN. In other renal populations, the main differences related to interferon signalling, such as enhanced expression of STAT genes. As these are murine models of early renal disease such limited differences may reflect the mild extent of renal damage.

Discordance between renal populations may be influenced by spatial proximity to immune infiltrate and inflammatory stimuli, and insufficient numbers of nuclei in certain clusters may have also hampered detection of differentially expressed genes. Integrated analysis of datasets identified overlapping and model specific immune signatures in addition to differences in immune cell composition. T cell exhaustion-like and NK cytotoxic signatures were observed in the kidneys of MRL/lpr mice, identification of exhausted-like T cells in the periphery of these mice suggested this was a systemic phenotype, however previous studies have observed intact TCR signalling, expansion, and proliferation of these cells specifically in the kidney, raising the question of the role of the kidney environment in modulating such responses(375).

Overlapping model signatures included an increase in a tissue resident macrophage population expressing phagocytic markers. Trajectory analysis suggested this expanded TRM population to be non-classical monocyte derived, however the mechanism underlying this differentiation and whether this is a protective or damaging response is unknown(203).

Assessment of overlapping pan-immune signatures identified lymphocyte homing molecule *Itga4* as a target with upregulation of its ligand *Vcam1* observed in renal populations, with these findings confirmed via immunofluorescence at the protein level in the kidneys of MRL/lpr mice. Enhanced expression of this gene was also observed when murine immune data was integrated with human LN immune data suggesting relevance to human disease. Therefore, ITGA4 represents a putative immune cell specific target, with the potential to ameliorate immune cell infiltration and subsequent tissue damage.

In summary transcriptomic analysis has identified non-immune and immune targets, modelling human pathology, and their interactions, which warrant further functional investigation to better elucidate their role and relevance in lupus kidney disease.

Chapter 5 : Functional assays for mechanistic understanding of the role of targets identified from transcriptomic studies in disease

5.1 Introduction

Combining transcriptomic analysis with existing biological knowledge in chapter 4 identified immune targets potentially amenable to pharmacological modulation in vivo. In this chapter I aimed to assess the mechanistic and thus therapeutic relevance of these targets for disease. Renal snRNA-seq identified enhanced expression of *Itga4* in both murine models of LN, with higher expression on immune nuclei from MRL/lpr mice, in addition to enhanced expression of *Itga4* ligand *Vcam1* (Figure 4.9). Enhanced protein expression of both *Itga4* and *Vcam1* in MRL/lpr kidneys was confirmed via immunofluorescence (Figure 4.11), providing a rationale for targeting *Itga4* in vivo. Therefore, the first section of this chapter focuses on the results from in vivo intervention experiments in MRL/lpr mice, antagonising *Itga4*, to assess the relevance of this receptor in mediating immune cell infiltration into the kidney and the impact of this on disease phenotype. The second section focuses on in vitro data testing the effects of ITGA8 modulation on mesangial cells in the resting state and in the context of a proinflammatory stimulus, to further investigate the possible role of *Itga8* in driving mesangial alterations and adoption of a more fibrotic phenotype as observed in the kidneys of IMQ treated mice.

5.2 Pharmacological targeting of *Itga4* in MRL/lpr mice

ITGA4 is an α integrin subunit which associates with either ITGB1 to form the VLA4 complex or ITGB7 to form the LPAM1 complex, with heterodimerisation required for ligand binding and downstream functions such as trans endothelial migration(505). The biologics natalizumab (anti-VLA4) and vedolizumab (anti-LPAM1), are currently licensed for the

treatment of multiple sclerosis and ulcerative colitis respectively. Antagonism of these complexes results in inhibition of leukocyte transmigration into tissue, thus ameliorating tissue damage (Figure 5.1A)(506). Overlap between ligands which bind to VLA4 and LPAM1 makes it difficult to determine which of these might be more relevant for SLE pathogenesis. Therefore, to target Itga4 I selected clone PS/2, an antibody suitable for in vivo use that binds to and blocks residues on ITGA4 irrespective of heterodimerisation with either β subunit(507, 508).

5.2.1 Itga4 blockade is well tolerated in MRL/lpr mice

Administration of Itga4 antagonist PS/2 or isotype was well tolerated in MRL/lpr mice for the experimental period, with no adverse events observed. No differences in weight were observed between isotype and PS/2 MRL/lpr mice with disease progression (Figure 5.1C). Splenomegaly in MRL/Lpr mice, measured as a ratio of splenic weight to total body weight was not significantly altered by PS/2 treatment compared to isotype control (Figure 5.1D).

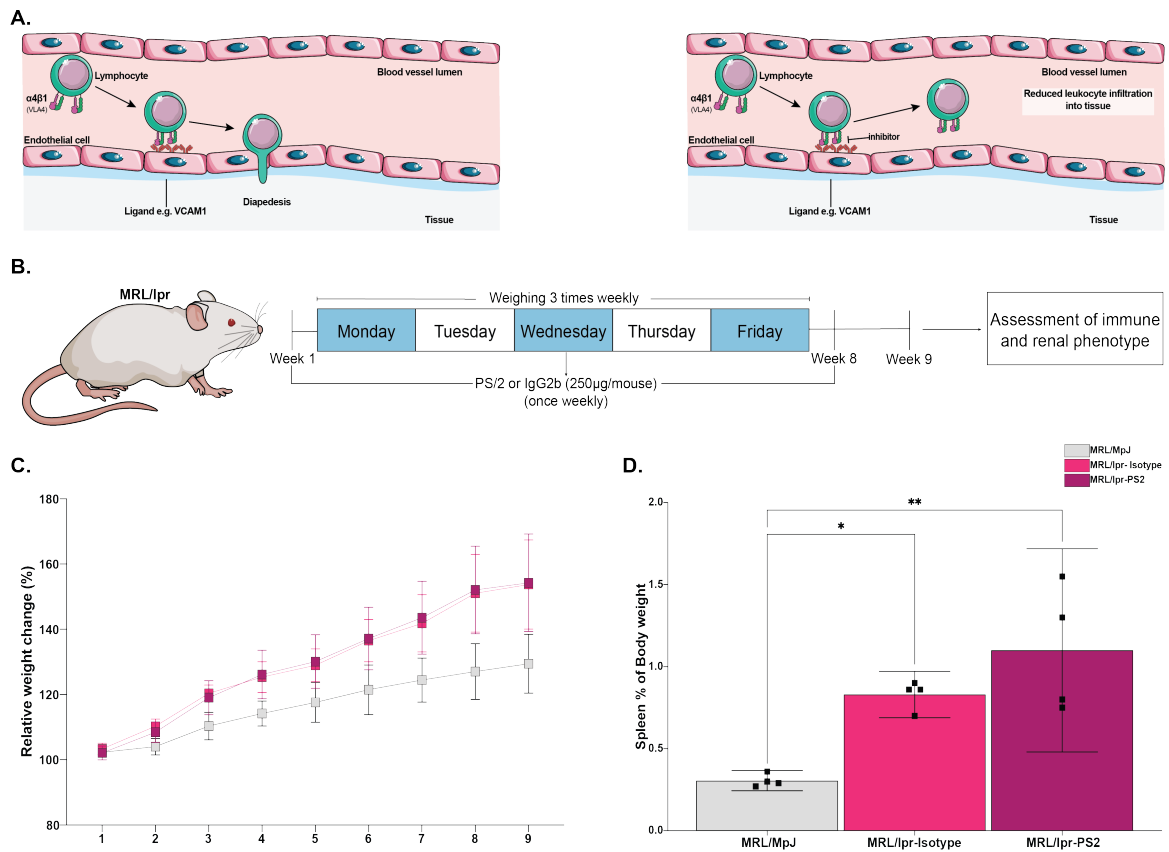


Figure 5.1: Itga4 blocking antibody PS/2 MRL/lpr model of SLE.

(A) Itga4 mediated lymphocyte homing in normal physiology (left panel) and with blockade (right panel). (B) Experimental protocol for MRL/lpr mice treatment. MRL/lpr mice were administered ITGA4 antagonist (PS/2) or isotype control (IgG2b) intraperitoneally at a dose of 250 μ g/mouse, once weekly for 8 weeks starting at 6 weeks of age. At 15 weeks of age MRL/lpr mice and age matched MRL/MpJ mice were culled, and immune organs and kidneys taken for analysis by flow cytometry. (C) Percentage change from starting weight for different treatment groups and MRL/MpJ mice aged 15 weeks. (D) Splenic weight as a percentage of final body weight in different treatment groups and MRL/MpJ mice aged 15 weeks. Data are mean $\pm$ 95% CI, analysed by a One-way ANOVA using the Holm-Sidak method to correct for multiple comparisons * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and representative of one experiment with 4 mice per group.

5.2.2 Itga4 blockade enhances lupus associated effector memory and activated CD4 populations in the spleen

VLA4 plays a role in thymic development, being highly expressed in double negative T (DNT) cells and essential for DNT interaction with thymic stromal cells, and onward thymocyte differentiation towards single positive and double positive populations(509, 510). However, Itga4 blockade did not alter the previously observed increase in absolute

numbers and proportions of thymic DNT cells and decrease in double positive (DP) T cells in MRL/lpr mice when compared to MRL/MpJ mice (Figure 5.2A-B and Figure 3.5E-F).

Within the spleen, absolute numbers of total T cells (denoted by CD3 positivity) were increased in both groups of MRL/lpr mice compared to MRL/MpJ control, and this effect was enhanced with Itga4 blockade, with T cell numbers in PS/2 MRL/lpr mice increased in comparison to isotype MRL/lpr mice (Figure 5.2C). Consistent with this, previous Itga4 blockade studies in the experimental autoimmune encephalitis model (EAE) also reported increased antigen specific T cells within the spleen(511, 512). While absolute numbers of splenic CD4 T cells increased in MRL/lpr mice when compared with MRL/MpJ controls, there was no modulation of this effect in the PS/2 MRL/lpr mice (Figure 5.2C), and no significant difference in CD4 T cells as proportions of splenic lymphocytes between groups. However within the CD4 T cell compartment significantly increased numbers and proportions of CD4 effector memory T cells were observed in both groups of MRL/lpr mice when compared to MRL/MpJ controls, and in both parameters a significant increase was identified in the PS/2 MRL/lpr mice in comparison to isotype MRL/lpr mice, this is in line with previous studies which have demonstrated a selective increase in CD4 effector memory populations in patients being treated with natalizumab (Figure 5.2E)(513). A similar pattern of a greater increase in proportions with Itga4 blockade compared to isotype occurred in activated CD4 T cells as denoted by CD69 expression, with majority of these cells identified to be CD69⁺ CD4 effector memory cells (Figure 5.2E-G). Proportions and numbers of Tregs were unchanged by Itga4 blockade (Figure 5.2E-F), consistent with previous studies demonstrating Tregs rely on lymphocyte function associated antigen 1 (LFA-1) for tissue homing and extravasation(514, 515). Additionally, proportions and absolute numbers of CD4⁺Tbet⁺ remained unchanged with Itga4 blockade (Figure 5.2E-F).

There was no difference in absolute numbers of CD8 T cells in MRL/lpr mice when compared to MRL/MpJ or between treatment groups (Figure 5.2C). CD8 T cell proportions

of total splenic lymphocytes were lower in MRL/lpr mice compared to MRL/MpJ controls, but this was not modulated by Itga4 blockade (Figure 5.2D). Likewise, in the CD8 T cell compartment a significant increase in the absolute numbers and proportions was observed in the effector memory population in isotype MRL/lpr mice versus MRL/MpJ, but this was unchanged by Itga4 blockade (Figure 5.2H-I). Itga4 blockade did not alter the increased numbers and proportions of CD8 T cells expressing markers of exhaustion in MRL/lpr mice compared to MRL/MpJ (Figure 5.2H-I, Figure 3.6J-K).

In summary, Itga4 blockade in lupus prone MRL/lpr mice resulted in enhanced expansion of activated and effector memory CD4 T cells. This data is in line with the T cell phenotype described in EAE autoimmune models and suggests that the dosing in this experiment is within the therapeutic range.

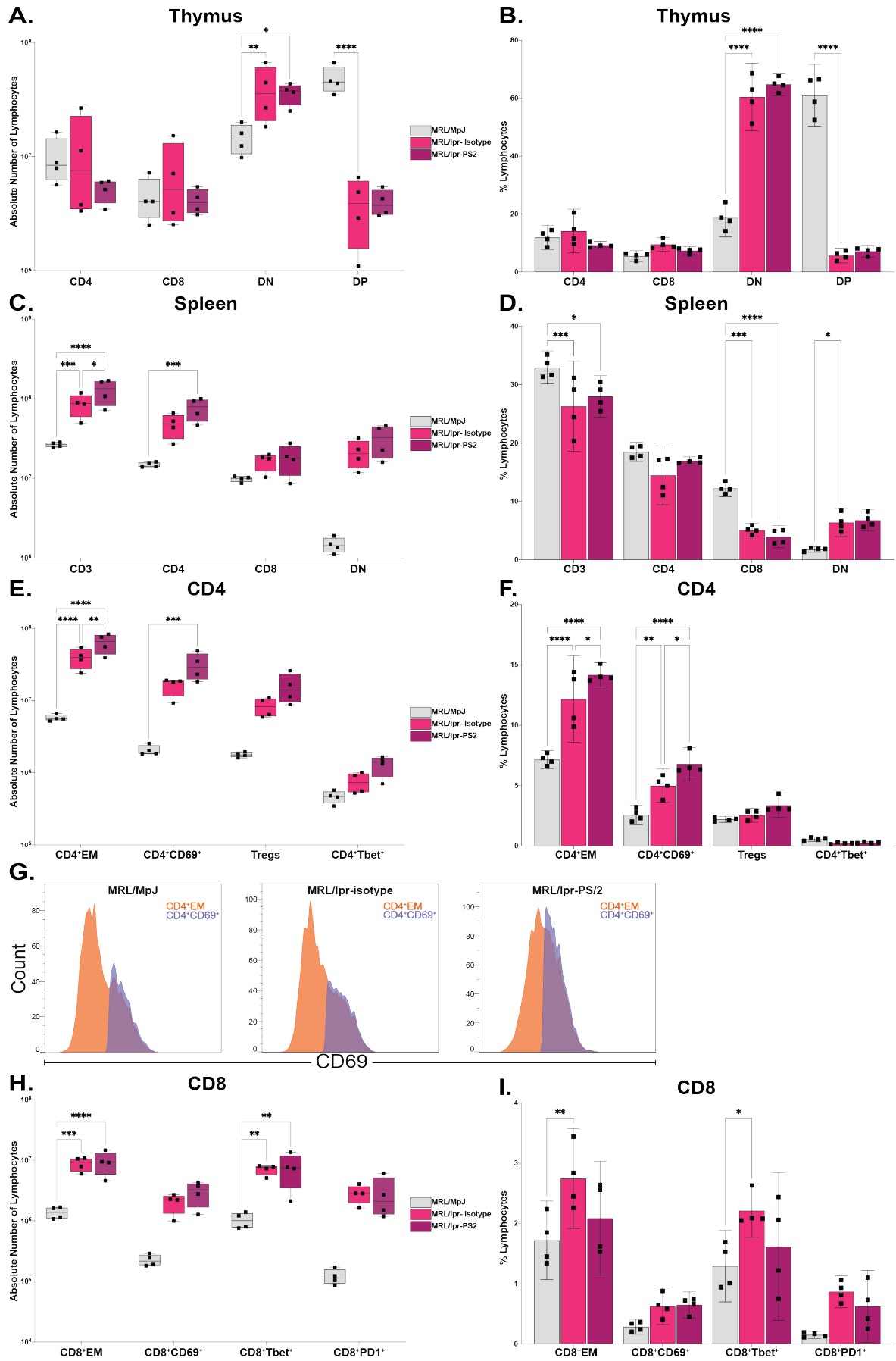


Figure 5.2: Effect of *Itga4* blockade on thymic and splenic T cells.

MRL/lpr were administered PS/2 (*Itga4* antagonist) or isotype (IgG2b) once weekly for 8 weeks, at 15 weeks treated mice and age matched MRL/MpJ were culled, and immune organs taken for analysis by flow cytometry. After initial gating as shown in **Figure 3.3A and 3.6B** splenic T cells were gated on CD3 expression and further gated as CD4⁺, CD8⁺ or DN. Effector Memory cells are defined as CD4⁺/CD8⁺CD44^{hi}CD62L^{low}; Tregs are defined as CD4⁺CD25⁺Foxp3⁺. Thymic cells were gated as CD4⁺, CD8⁺, CD4⁺CD8⁺ (DN) and CD4⁺CD8⁺ (DP). **(A-B)** Absolute numbers and proportion of lymphocytes for thymic T cell populations. **(C-D)** Absolute numbers and proportion of lymphocytes for splenic T cell populations. **(E-F)** Absolute numbers and proportion of lymphocytes for splenic CD4⁺ T cell subpopulations. **(G)** Representative overlaid histograms of CD4⁺EM on CD4⁺CD69⁺ population. **(H-I)** Absolute numbers and proportion of lymphocytes for splenic CD8⁺ T cell subpopulations. Boxplot whiskers in **(A/C/E/H)** show min and max values and line represents the median. Bar plots in **(B/D/F/I)** show means $\pm$ 95% CI. Significance was determined by a One-way ANOVA using the Holm-Sidak method to correct for multiple comparisons **P*<0.05, ***P*<0.01, ****P*<0.001 and data are representative of one experiment with 4 mice per group.

5.2.3 *Itga4* blockade does not alter the peripheral B cell phenotype in MRL/lpr mice

Absolute numbers of splenic B cells increased in both groups of MRL/lpr mice compared to MRL/MpJ, with significantly higher numbers when comparing isotype MRL/lpr with PS/2 MRL/lpr mice (Figure 5.3A). However, when looking at the proportions of B cells (Figure 5.3B), no significant difference was observed between groups, suggesting that the increase in absolute numbers was most likely due to the general lymphatic expansion in the spleen observed in PS/2 MRL/lpr mice (Fig 5.1D) as opposed to a specific expansion of B cells.

Itga4 blockade did not affect numbers or proportions of activated B cells as denoted by expression of CD86 and CD69, germinal centre like B cells or age associated B cells (ABCs). An increase in the absolute numbers of CD138⁺ plasma blasts was observed in both groups of MRL/lpr mice compared to MRL/MpJ, but there was no effect of *Itga4* blockade versus isotype control (Figure 5.3A). No significant differences were seen in mesenteric lymph node (MLN) B cell numbers or proportions with PS/2 compared to isotype control. In summary, *Itga4* blockade was not shown to specifically modulate the peripheral B cell phenotype seen with Fas deficiency.

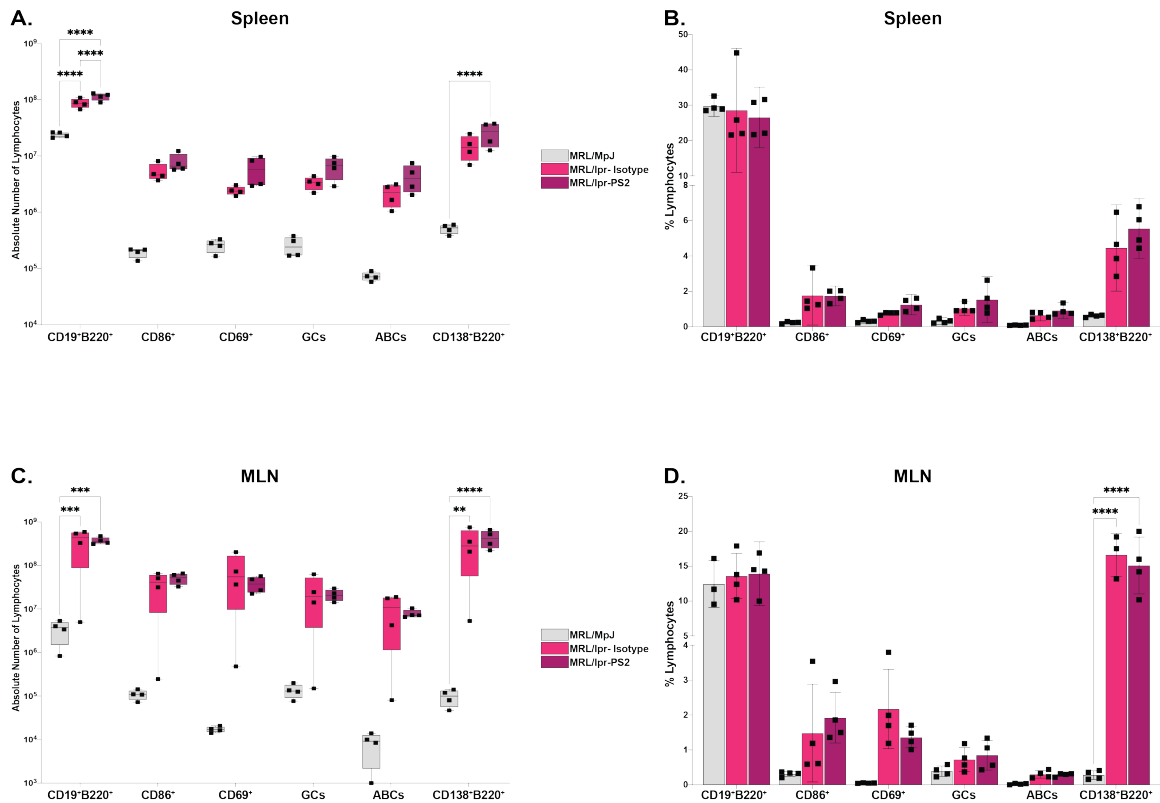


Figure 5.3: Effect of *Itga4* blockade on splenic and MLN B cells.

After initial gating as shown in **Figure 3.3A and 3.4A**. Splenic/MLN B cells were identified as B220⁺CD19⁺ and used for further gating of subpopulations. Germinal centre B cells are defined as GL7⁺PNA⁺IgD^{low}; ABCs are defined as CD19⁺ B220⁺CD21⁺CD11c⁺CXCR5⁺. **(A-B)** Splenic B cell absolute numbers and proportions of lymphocytes. **(C-D)** Absolute numbers and proportion of lymphocytes for MLN B cell populations. Boxplot whiskers in **(A/C)** show min and max values and line represents the median. Bar plots in **(B/D)** show means $\pm$ 95% CI. Significance was determined by a One-way ANOVA using the Holm-Sidak method to correct for multiple comparisons * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and data are representative of one experiment with 4 mice per group.

5.2.4 Effect of *Itga4* blockade on circulating immune populations

Alterations in circulating blood immune populations have been demonstrated in humans with *Itga4* blockade for treatment of MS(513). Circulating immune cells were identified based on CD45 expression and further gated for identification of subpopulations (Figure 5.4A). Whilst isotype treated MRL/lpr mice had increased proportions of CD45⁺ cells compared to MRL/MpJ congenic controls, there was no difference between PS/2 and isotype treated MRL/lpr proportions of circulating B, T, NK, or dendritic cells (Figure 5.4B and C). PS/2 treated MRL/lpr mice had decreased proportions of CD11b⁺ cells compared to MRL/MpJ mice, but no effect of *Itga4* blockade was observed when compared with

isotype control mice (Figure 5.4C). These findings contrast with the increased proportions of circulating immune populations observed in MS patients treated with natalizumab, with a specific enhancement observed in T, B and NK cells(516).

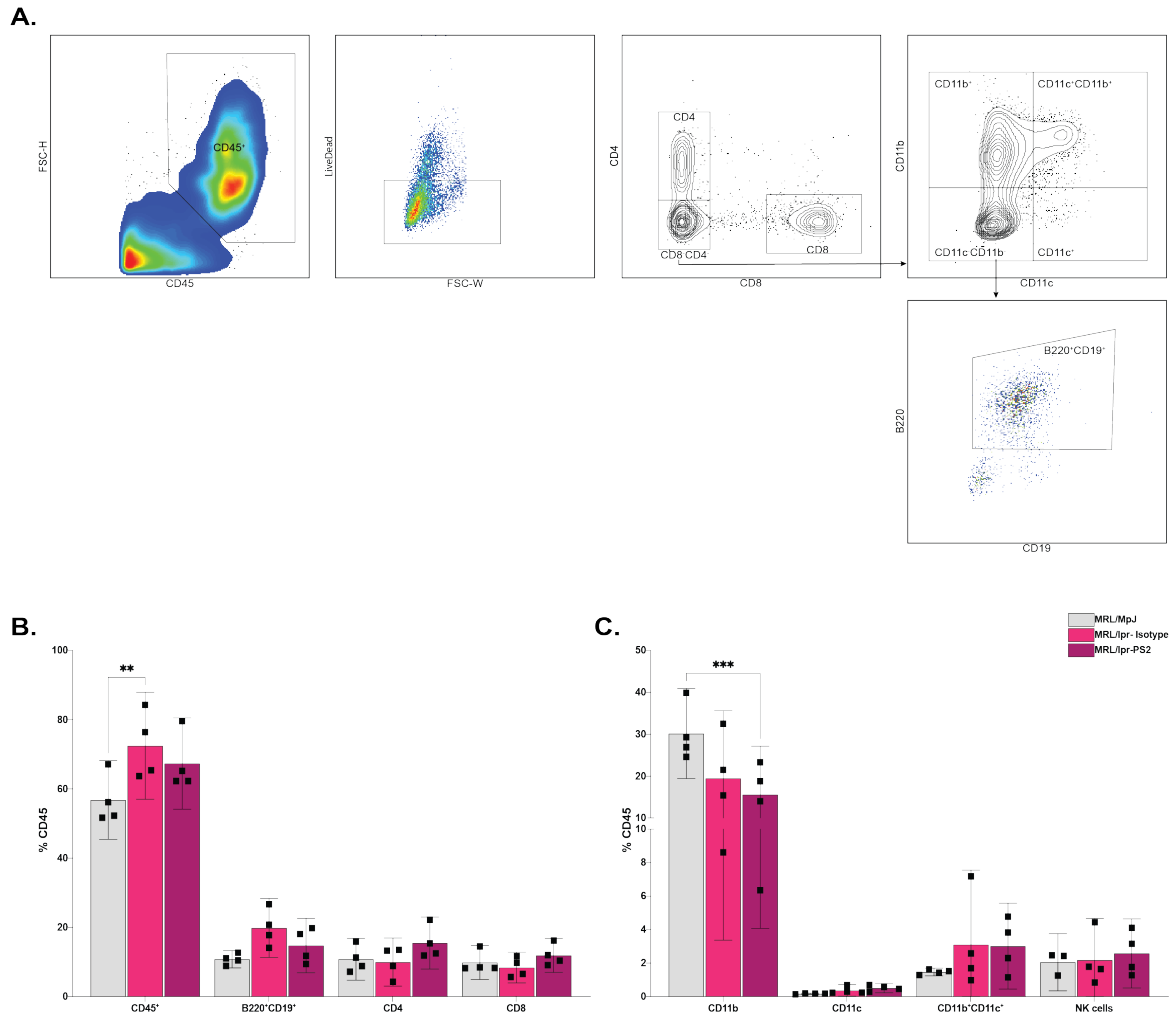


Figure 5.4: Effect of *Itga4* blockade circulating immune populations.

(A) Gating strategy used for circulating immune populations in the blood. **(B)** B cells and T cells as proportion of CD45⁺ cells in the blood. **(C)** Innate populations in the blood as proportion of CD45⁺ cells. Data are mean $\pm$ 95% CI, analysed by a One-way ANOVA using the Holm-Sidak method to correct for multiple comparisons ** $P < 0.01$, *** $P < 0.001$ and representative of one experiment with 4 mice per group.

5.2.5 Effect of *Itga4* blockade on kidney infiltrating immune populations

Previous studies and our own analysis (data not shown) have demonstrated very little B cell infiltrate in the kidneys of MRL/lpr mice with the infiltrate at 15 weeks predominantly

comprised of T and myeloid cell, therefore T cell and myeloid populations in the kidneys of MRL/MpJ mice, and MRL/lpr mice treated with PS/2 or isotype, were compared(264).

5.2.5.1 No effect of Itga4 blockade on renal T cells in MRL/lpr lupus kidney

While the proportion of CD45⁺ cells appeared to increase in the kidneys of MRL/lpr mice, compared to MRL/MpJ, there was no effect on the size of the immune expansion detected with Itga4 blockade when compared with isotype control mice (Figure 5.5A). There was a significant increase in total T cell proportions in PS/2 MRL/lpr mice, with a similar trend increase observed in isotype MRL/lpr when compared with MRL/MpJ controls, again suggesting no overall effect on T cells with Itga4 blockade (Figure 5.5B). No significant differences were found in CD4 or CD8 T cells as a percentage of CD45⁺ cells in the kidney between Itga4 blockade and isotype (Figure 5.5B). Within both CD4 and CD8 T cell compartments, effector memory cells were expanded in both MRL/lpr mice compared with MRL/MpJ controls but no effect of Itga4 blockade was detected on memory or other T cell subsets (Figure 5.5C).

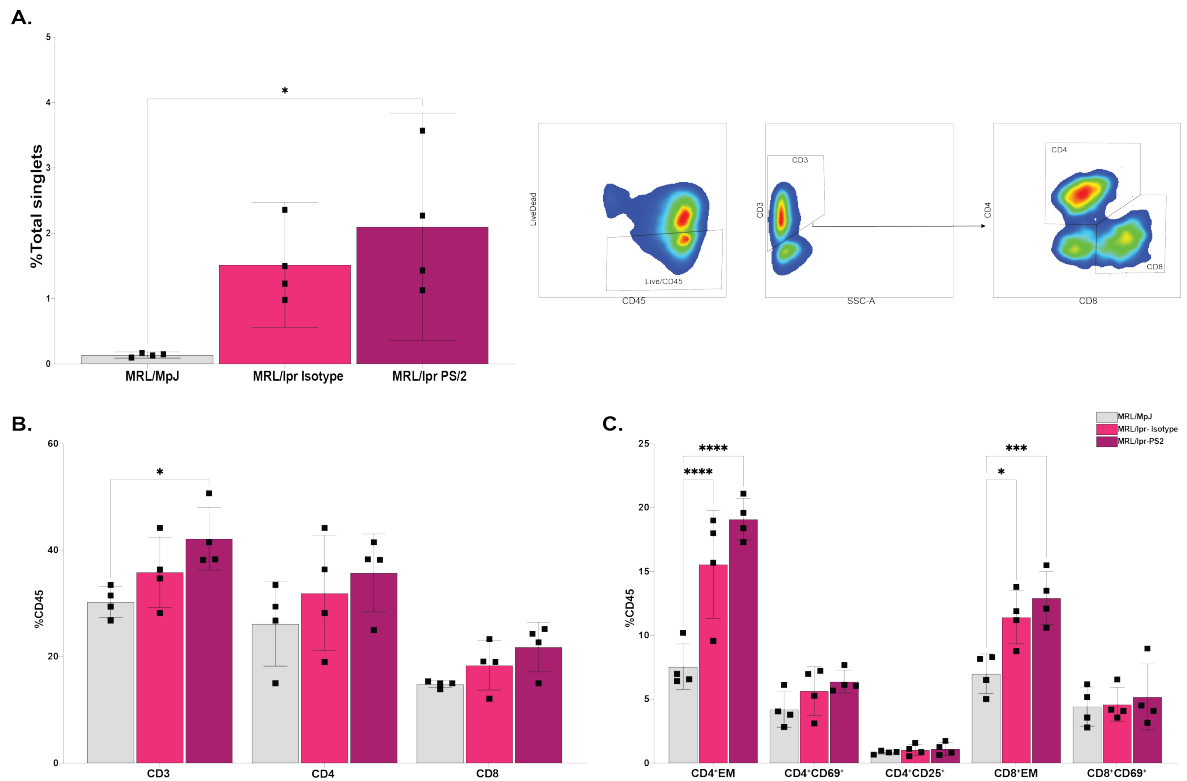


Figure 5.5: Effect of *Itga4* blockade on renal T cells.

(A) CD45⁺ cells as proportion of all cells in the kidney. (B) T cells as percentage of CD45⁺ in the kidney. (C) Proportion of CD4/CD8 subpopulations in the kidney. Doublets and dead cells were gated out and cells were gated first on CD45 expression thereafter, T cells were gated on CD3 expression and further gated on CD4 and CD8 expression. Effector Memory cells are defined as CD4⁺/CD8⁺CD44^{hi}CD62L^{low}. Data are mean $\pm$ 95% CI, analysed by a One-way ANOVA using the Holm-Sidak method to correct for multiple comparisons * P <0.05, ** P <0.01, *** P <0.001 and representative of one experiment with 4 mice per group.

5.2.5.2 *Itga4* blockade selectively reduces non-classical monocyte infiltration

There were no significant differences between isotype and *Itga4* antibody treated MRL/lpr renal CD11b⁺ dendritic cells, neutrophils, or NK cells as a proportion of CD45⁺ renal immune cells (Figure 5.6A). Given the non-classical monocyte signature observed from renal transcriptomic data (Figure 4.6C-D and 4.9), focused flow cytometric phenotyping was carried out on monocytic populations. Cells from B, T, NK cell and granulocyte lineage were gated out, thereafter monocytic populations were divided into classical, non-classical and intermediate subpopulations based on several well described markers (Figure 5.6B).

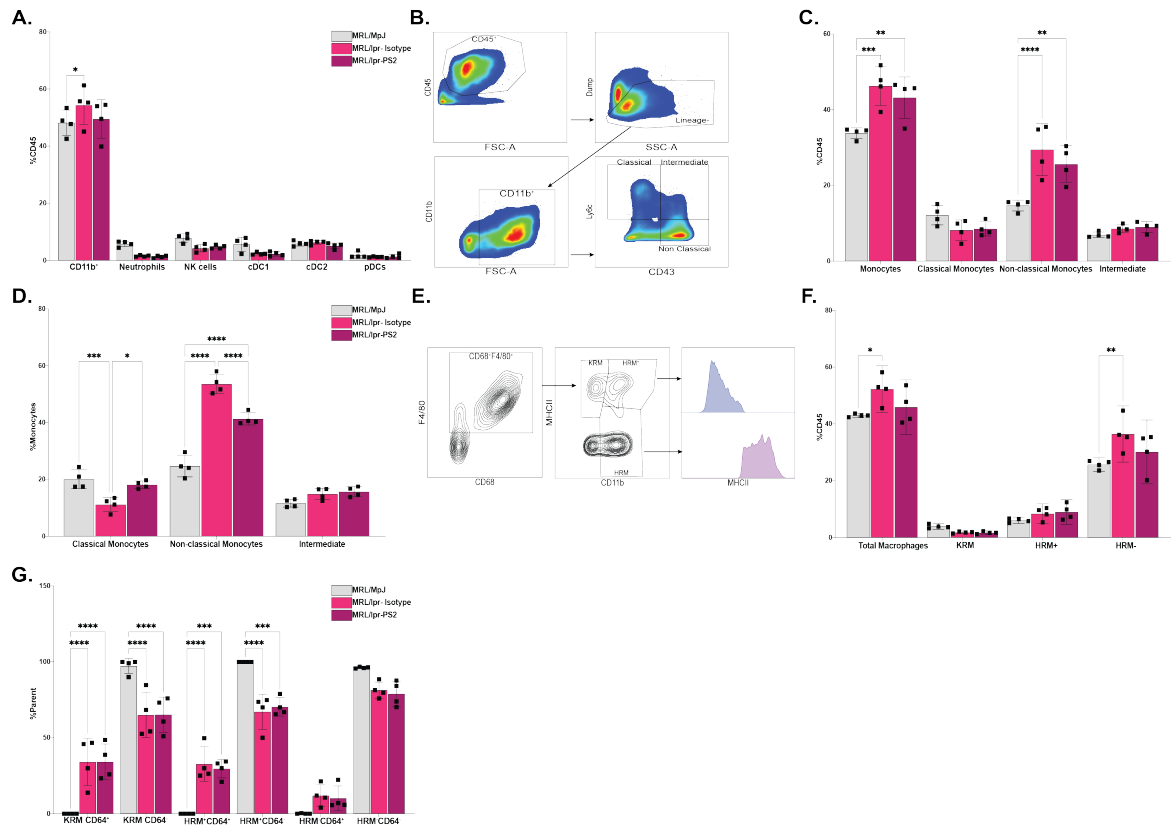


Figure 5.6: Effect of *Itga4* blockade on renal myeloid cells.

(A) Renal myeloid cell populations as a proportion of CD45⁺ cells in the kidney. (B) Gating strategy for renal monocytes. (C) Proportion of monocytes and monocyte subpopulations in the kidney. (D) Monocyte subpopulations as a proportion of total monocytes. (E) Gating strategy for renal macrophages. (F) Proportion of macrophage and macrophage subpopulations in the kidney. (G) Macrophage subpopulations as a proportion of parent gate. Data are mean $\pm$ 95% CI, analysed by a One-way ANOVA using the Holm Sidak method to correct for multiple comparisons * P <0.05, ** P <0.01, *** P <0.001 and representative of one experiment with 4 mice per group.

Non-classical monocytes increased in both groups of MRL/lpr mice when compared with MRL/MpJ controls, confirming the transcriptomic data, but with no significant effect of *Itga4* inhibition when measured as a proportion of CD45⁺ renal cells, although a trend towards lower numbers of non-classical monocytes was observed with PS/2 compared to isotype (Figure 4.9 and Figure 5.6C). To identify changes in monocyte subsets without confounding by any changes in other CD45⁺ populations, I assessed monocyte subpopulations as a proportion of total monocytes. This revealed a decrease in the percentage of monocytes classified as 'classical' in isotype MRL/lpr mice, when compared with MRL/MpJ, which was fully rescued in PS/2 MRL/lpr mice (Figure 5.6D). Mirroring the snRNA-seq data (Figure

4.6C-D and 4.9), the proportion of non-classical monocytes increased in both groups of MRL/lpr mice, however this expansion was attenuated by Itga4 blockade (Figure 5.6D). These observations are in line with the different roles played by non-classical and classic monocytes; classical monocytes have patrolling capacity in homeostatic conditions, including in glomerular capillaries, from where they extravasate readily into tissue under inflammatory conditions in an integrin dependent manner. Whereas classical monocytes are recruited in a CCL2 dependent manner into the kidney during periods of inflammation and may participate in perpetuating inflammatory responses via production of cytokines such as TNF- α (203, 517). Flow cytometry showed that Itga4 is required for the relative increase in non-classical renal monocyte proportions in LN and suggests that Itga4 blockade may reduce the lupus driven infiltration of non-classical monocytes into the kidney, expansion due to altered proliferation or survival cannot be excluded.

Transcriptomic data suggested a role for monocyte differentiation into CD11b^{hi}F4/80⁺ renal resident macrophages (Figure 4.9E). To assess if Itga4 mediated suppression of the lupus induced expansion of non-classical monocytes had an impact on this, renal macrophage subsets were analysed. Cells from B, T, NK cell and granulocyte lineage were gated out prior to gating of macrophage populations based on positivity for pan macrophage markers F4/80 and CD68, which were further divided into resident yolk sac derived (KRM) and haematopoietic derived resident (HRM) populations based on CD11b and MHCII expression, HRMs were further divided into two groups based on MHCII expression termed HRM⁺ (MHCII⁺) and HRM⁻ (MHCII⁻) (Figure 5.6E) (362). Validating the snRNA-seq data, total macrophages were increased as a proportion of CD45⁺ cells, accounted for by increased HRM⁻ macrophages, in isotype MRL/lpr mice, compared to MRL/MpJ, with a trend towards lower proportions of total and HRM⁻ macrophages, similar to non-lupus MRL/MpJ, in PS/2 treated mice (Figure 5.6F). The HRM⁻ population most likely represents a circulating or infiltrating mixed monocyte-macrophage mixed population as denoted by low MHCII expression, similar to the MNP2⁻ population observed in a study by Richoz et al,

which was shown to express markers consistent with both monocytes and macrophages by scRNA-seq(362).

Markers such as MertK and CD64 (*Fcgr1*) have been widely used to identify resident macrophages in tissues such as the lung and spleen, however MertK has been shown to be poorly expressed within kidney macrophage populations(518). CD64 is a marker known to be expressed by cells from the macrophage lineage and previous studies have demonstrated the ability of CD64 in distinguishing tissue macrophages from dendritic cells, further supporting its use as a phenotypic marker(517, 519). When looking at CD64 positive populations as a proportion of each macrophage subpopulation, a significant increase in CD64^{hi} populations was seen in MRL/lpr mice groups in the KRM and HRM⁺ populations but not HRM⁻ cells (Figure 5.6G), with no CD64^{hi} cells observed in the MRL/MpJ mice suggesting this to be a disease modulated change. CD64 encodes a receptor which is integral for internalisation of IC, therefore enhanced expression in MRL/lpr mice compared to MRL/MpJ may be in response to IC deposition in the kidneys, this is further supported by studies in *FcyR1*^{-/-}(CD64) MRL/lpr mice in which numbers of renal infiltrating immune cells including monocytes were reduced in conjunction with attenuated renal disease, characterised by improved histological scores and reduced proteinuria(520). These observations would suggest that HRM⁻ represent a monocyte population that infiltrate the kidney in LN and may subsequently acquire MHCII and CD64 expression to become HRM⁺, similar to findings in nephrotoxic serum nephritis models(517).

Overall, the renal myeloid data validated the snRNA-seq evidence of expansion of non-classical monocytes and CD11b⁺ tissue resident renal macrophages in murine LN and demonstrated upregulation of the Fcγ receptor CD64 in SLE renal macrophage populations. Further, the expanded proportion of non-classical monocytes in SLE was reduced, suggesting that *Itga4* blockade can limit renal monocyte infiltration in LN. Whilst there was

a trend decrease in the HRM⁺ population in PS/2 MRL/lpr mice, high inter-mouse variability limited interpretation of any Itga4 mediated effect on this population.

5.2.5.3 No significant impact of Itga4 blockade on LN renal histology or function.

Consistent with the MRL/lpr phenotype (Figure 3.8), light microscopy imaging of sections identified immune cell infiltration, endocapillary proliferation, mesangial hypercellularity, crescent formation, karyorrhectic debris and interstitial inflammation, mirroring active human class III LN, however these features were unchanged between isotype and Itga4 blockade treated groups (Figure 5.7A). Urinary protein was unchanged between isotype and PS/2 MRL/lpr mice, with both higher than MRL/MpJ controls, no differences were observed in creatinine, a marker for renal function, or albumin, which is low in nephrotic range proteinuria (Figure 5.7B-D).

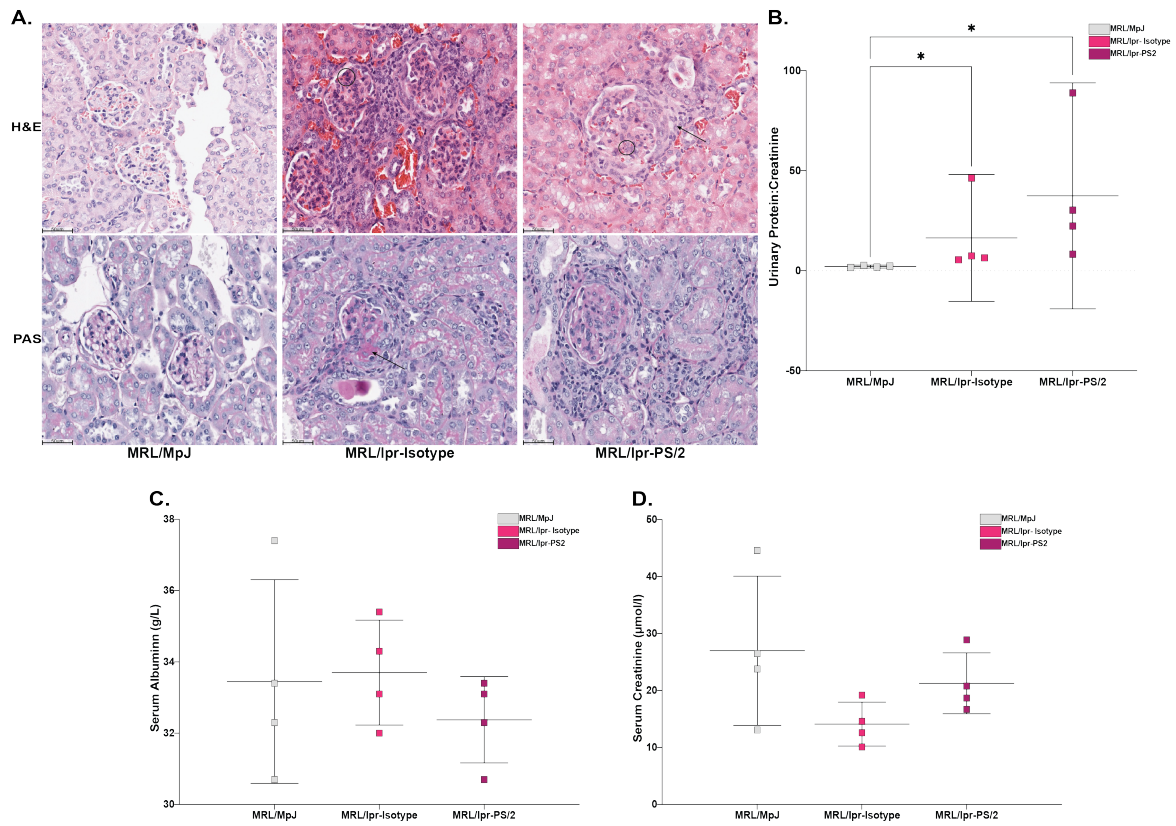


Figure 5.7: Effect of *Itga4* blockade on renal histology.

MRL/lpr were administered PS/2 (*Itga4* antagonist) or isotype (IgG2b) once weekly for 8 weeks, at 15 weeks treated mice and age matched MRL/MpJ, were culled and kidneys fixed in 10%NBF for 24 hours, processed and embedded into paraffin, prior to being cut into 3µm sections. **(A)** Stained kidney sections from MRL/MpJ and PS/2 or isotype treated MRL/lpr mice aged 15 weeks (40X magnification). Haematoxylin and Eosin (H&E) (top panel) showing endocapillary hypercellularity (black circle-middle image) and karyorrhectic debris (red circle- right image) and Periodic-acid Schiff (PAS) (bottom panel) showing segmental crescent formation (black arrow-middle image) and immune cell infiltration (red arrow-right image). Bar = 50µm. **(B)** Urinary protein:creatinine ratio for MRL/MpJ and PS/2 or isotype treated MRL/lpr mice aged 15 weeks. **(C)** Serum albumin levels for MRL/MpJ, PS/2 and isotype treated MRL/lpr mice aged 15 weeks. **(D)** Serum creatinine levels in for MRL/MpJ, PS/2 and isotype treated MRL/lpr mice aged 15 weeks. Data are mean ± 95% CI, analysed by a One-way ANOVA using the Holm Sidak method to correct for multiple comparisons *P<0.05 and representative of one experiment with 4 mice per group.

5.3 Role of ITGA8 in mesangial cell function in vitro

The transcriptomic data showed an association of loss of *Itga8* with acquisition of a fibrotic phenotype in mesangial cells (Figure 4.2H-J). To test the functional role of *Itga8* in mesangial response to inflammation, I considered both in vivo and in vitro models. Due to the heterogenous nature of stromal populations and overlap in gene expression patterns, specific mesangial cell targeting, and manipulation is challenging in vivo. *Itga8* is critical for ureter formation and epithelialisation during neonatal development, thus in *Itga8*^{-/-} mice,

most animals are non-viable with renal agenesis and dysgenesis(521). Targeted knock out with cre-recombinase systems to circumvent developmental lethality are unable to specifically target mesangial cells(522).

By contrast cell lines provide an easily modifiable system to study cell intrinsic responses at homeostasis and in disease without systemic interference. Therefore, a conditionally immortalised human mesangial cell line was used to study the role of ITGA8 in mesangial function. This cell line is immortalised using a temperature sensitive simian virus 40 large T antigen (tsSV40) and human telomerase (hTERT)(445, 523). Cells proliferate at 33°C, while switching to 37°C results in inactivation of tsSV40, stopping proliferation as hTERT alone is insufficient, and allowing cells to differentiate into their native phenotype(445, 523). This cell line was used to study the relevance of ITGA8 in the development of a profibrotic mesangial phenotype.

5.3.1 Cytokine stimulation of cultured mesangial cells mimics LN mesangial complement activity and matrix production

To study the effect of disease on mesangial cell phenotype I required a reproducible assay to mimic the inflammatory environment in vitro. A cytokine stimulation assay using a combination of inflammatory mediators ($\text{TNF}\alpha$, $\text{IFN}\gamma$, $\text{IFN}\alpha$ and $\text{IL1}\beta$) implicated in LN, or sera from patients with LN, results in similar patterns of expression of key extracellular matrix and chemokine genes, suggesting mesangial cell cytokine stimulation can mimic aspects of the inflammation seen in disease(384).

Therefore, this approach was first piloted in wild type mesangial cells (Figure 5.8A), which were passaged at 33°C and then transferred to 37°C for 10 days to allow for full inactivation of tsSV40 and differentiation of cells into native phenotype. Subsequently, cells were stimulated with the cytokine combination for a period of 24 hours, with this process followed for all subsequent experiments unless stated otherwise. Changes in expression patterns of

genes (*FN1*, *COL4A1*, *PDGFRB*, *CXCL12*, *ACTA2*, *CCN2* and *C7*), as measured by qPCR, were used as readouts for the assay.

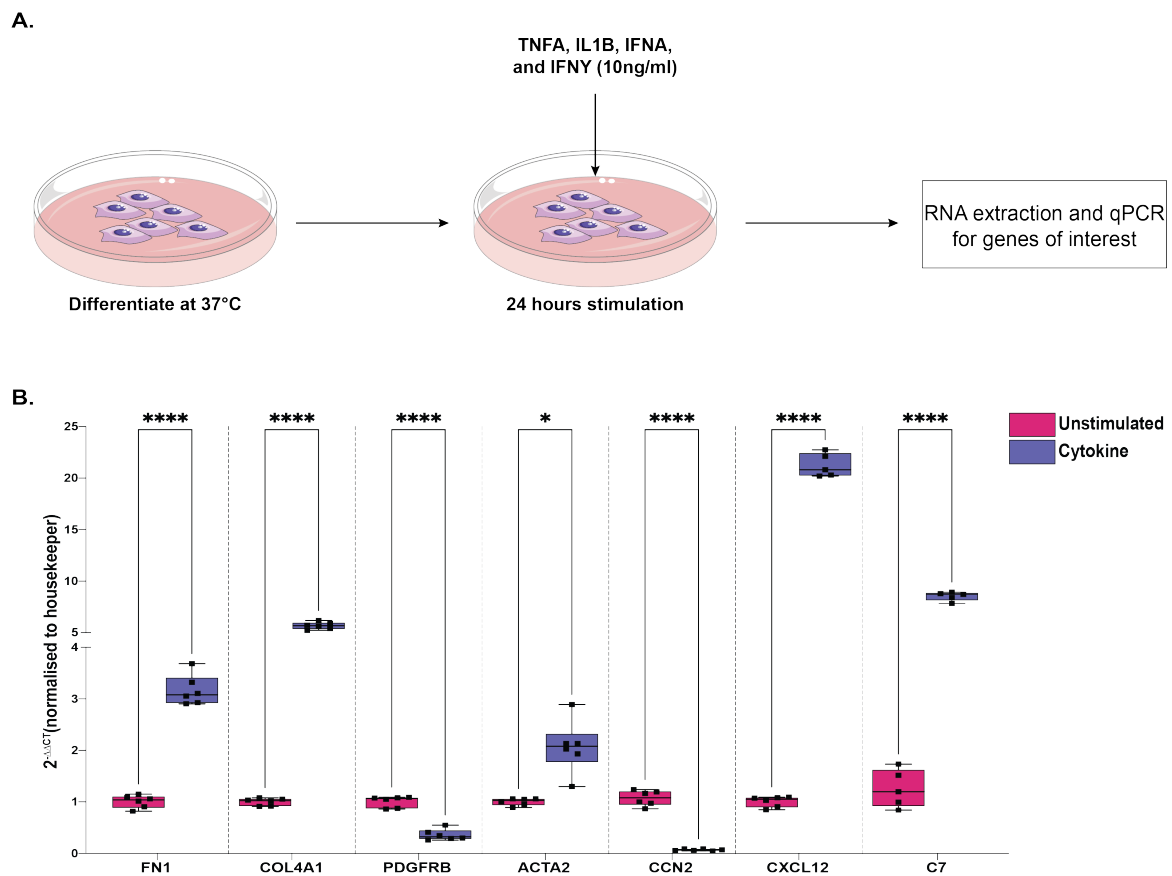


Figure 5.8: Effect of cytokine stimulation on culture mesangial cells.

Differentiated mesangial cells were stimulated with TNF α , IFN γ , IFN α and IL1 β for 24h and thereafter RNA was extracted for qPCR. **(A)** Experimental protocol for mesangial cell cytokine stimulation. Mesangial cells were differentiated for 7-10 days at 37°C prior to stimulation with TNF α , IFN γ , IFN α and IL1 β for 24h and thereafter RNA was extracted for qPCR. **(B)** mRNA expression as assessed by qPCR for *FN1*, *COL4A1*, *PDGFRB*, *CXCL12*, *ACTA2*, *CCN2* and *C7*. *TUBB* was used as housekeeping control. Data are mean $\pm$ 95% CI, analysed by multiple *t*-tests using the Holm-Sidak method to correct for multiple comparisons **P*<0.05, ***P*<0.01, ****P*<0.001 and representative of 4 independent experiments.

Chosen genes were based on markers of mesangial dysfunction from existing literature and findings from the transcriptomic data (Figure 4.2F), and encompassed extracellular matrix genes (*FN1* and *COL4A1*) associated with mesangial expansion, genes associated with fibrosis and activation (*PDGFRB*, *CCN2* and *ACTA2*) and genes associated with immune modulation (*CXCL12* and *C7*)(48, 384).

Cytokine treatment resulted in increased expression of extracellular matrix genes *COL4A1* and *FN1* as well as *ACTA2* suggesting enhanced matrix production and adoption of an activated phenotype, and consistent with observations from human and in vivo LN data (Figure 5.8B)(384, 524). Stimulation led to decreased expression of *PDGFRB* and *CCN2* (Figure 5.8B). Consistent with transcriptomic data, the cytokine stimulation also promoted complement activity with significantly increased expression of *C7* and *CXCL12* (Figure 5.8B) with stimulation compared to normal media.

5.3.2 Genetic modulation of *ITGA8* in mesangial cells with CRISPR-Cas9

Establishment of a reproducible assay in vitro provided a controlled system which could then be used to assess the effect of *ITGA8* downregulation in mesangial cells. CRISPR-Cas9 editing was used to knockdown *ITGA8* in mesangial cells (Figure 5.9A), briefly a dual guide approach was utilised in which two single guide RNAs (sgRNA), targeting exons 17 and 27 respectively, were electroporated into cells alongside Cas9 ribonucleoprotein, resulting in a deletion of 59,320bp.

Individual mutations in these two exons, mapping to the thigh and calf2 domains of *ITGA8* respectively, prevent the conformational change required by *ITGA8* for ligand binding and have been associated with bilateral renal agenesis (BRA) in humans(525).

Single transfected cell clones were screened for genomic evidence of deletion (Figure 5.9B), identifying 19 edited clones, an unedited clone (E3) was used as a control. A forward primer from cut site 1 (CR1-F1) and the reverse primer from cut site 2 (CR2-R1) were designed such that only when a deletion had occurred would the PCR produce a product, while a perfect deletion would result in no products using F and R primers from the same cut site. For 18/19 clone's products were observed from genomic PCRs using primers around cut site 1 except for clone F9 (Figure 5.9B), with products observed for all clones around cut site 2 in addition to a PCR product for the CR1-F1/CR2-R1 reaction, indicating a successful large deletion but also the suggesting presence of unedited alleles. By

Western blot 5 clones demonstrated decreased, though not absent, protein expression (Figure 5.9C). Of the 5 clones, genomic evidence of complete deletion at cut site 1 and of 59,320bp deletion, coupled with reduced protein expression in clone F9, made this the most suitable clone for subsequent functional assays.

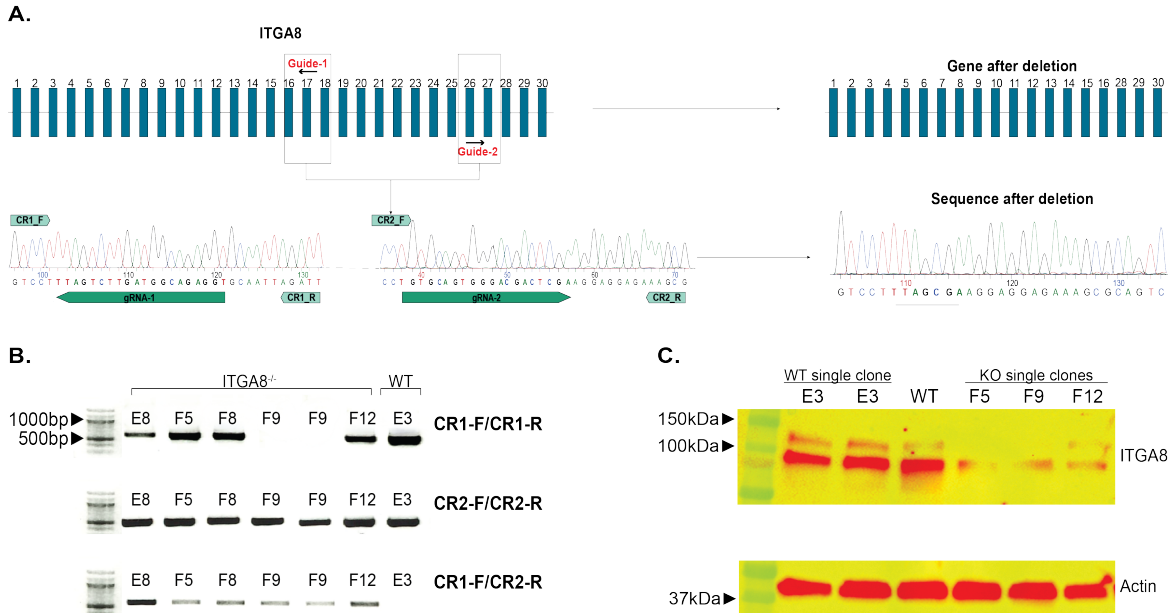


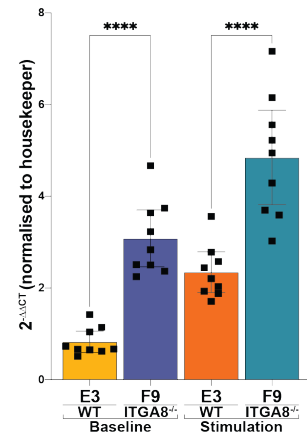
Figure 5.9: CRISPR/Cas9 approach for knocking out ITGA8 in mesangial cells.

A dual guide approach was used to knockout ITGA8 from mesangial cells. **(A)** Binding site for guide RNA and primers used for screening of deletion of ITGA8 and sequence of successful deletion. **(B)** DNA gel of PCRs from single clone genomic screening, run with 100bp ladder as reference for size, primers used for PCRs are indicated on the right-hand side of each image. **(C)** Representative Western blot of protein lysate from mesangial cell single clones. Beta actin was used as a loading control for protein quantification. **(B-C)** are representative of 4 independent experiments.

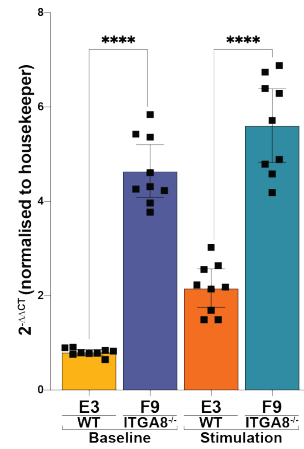
5.3.3 Effect of ITGA8 knockdown on mesangial cells at homeostasis and in response to cytokine stimulation

Increased baseline expression of extracellular matrix proteins *FN1* and *COL4A1* and increased *CXCL12* and *PDGFRB*, was observed in ITGA8 knockdown F9 (*ITGA8*^{-/-}) (Figure 5.10A-D). There were no significant differences observed in baseline expression of *CCN2*, *ACTA2* and *C7* (Figure 5.10E-G) between *ITGA8*^{-/-} F9 and wild type control under normal culture conditions.

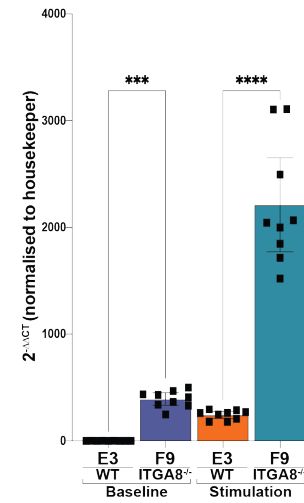
A. FN1



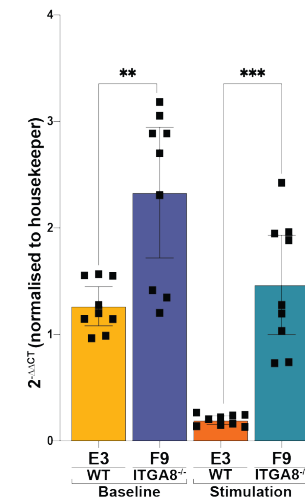
B. COL4A1



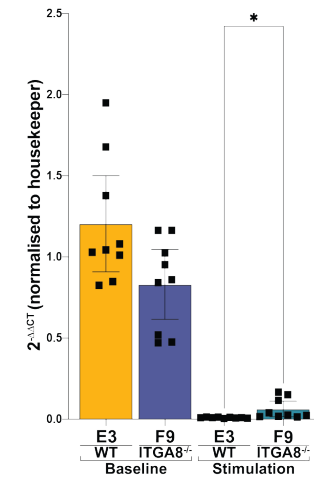
C. CXCL12



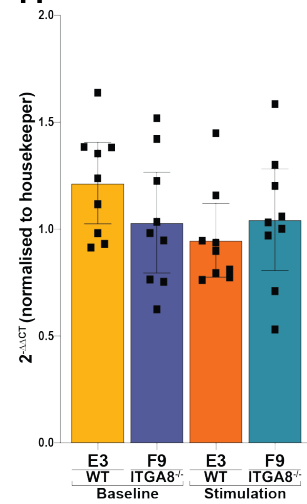
D. PDGFRB



E. CCN2



F. ACTA2



G. C7

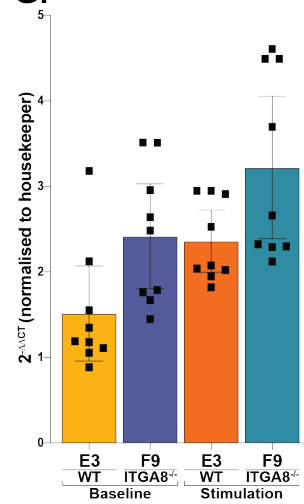


Figure 5.10: Effect of cytokine stimulation on ITGA8 knockout mesangial cell single clones.

*Differentiated mesangial cells were stimulated with TNF α , IFN γ , IFN α and IL1 β for 24h and thereafter RNA was extracted for qPCR to assess expression of (A) FN1. (B) COL4A1. (C) CXCL12. (D) PDGFRB. (E) CCN2. (F) ACTA2. (G) C7. TUBB was used as housekeeping control. Data are mean $\pm$ 95% CI, analysed by a One-way ANOVA using the Holm Sidak method to correct for multiple comparison * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and representative of 4 independent experiments. The following comparisons were used for calculation of statistical significance E3-Baseline vs F9-Baseline and E3-Stimulation vs F9-Stimulation.*

Cytokine stimulation significantly increased expression of FN1, COL4A1, CXCL12, PDGFRB and CCN2 in ITGA8^{-/-} F9 when compared to stimulated control (Figure 5.10A-E), whereas expression of ACTA2 and C7 were unchanged (Figure 5.10F-G). Taken together these data suggest increased extracellular matrix and chemokine production at baseline with ITGA8 knockdown, with enhanced upregulation of matrix and chemokine genes compared in the context of cytokine stimulation in ITGA8 knockdown clone F9 compared to unedited control, suggesting loss of ITGA8 potentiates an activated and profibrotic mesangial phenotype.

5.3.4 Enhancement of ITGA8 expression rescues mesangial cell phenotype

To confirm whether the observed activated profibrotic phenotype seen in ITGA8^{-/-} F9 was due to decreased ITGA8 expression and not a consequence of a non-specific off-target or clonal effect, a rescue experiment was carried out in which ITGA8 was reintroduced into ITGA8^{-/-} F9, alongside overexpression in an unedited cell line (E3), to explore the effects of rescuing ITGA8 expression and ITGA8 overexpression in mesangial cells.

A construct consisting of ITGA8 under the control of a ubiquitously expressed promoter and a puromycin resistant cassette was introduced into wildtype E3, and ITGA8^{-/-} F9 cells, Successful transfection of the expression cassette at the protein level was confirmed in both clones (Figure 5.11A), thereafter clones E3 and the ITGA8 overexpression E3 cells, (henceforth known as E3oe) and F9 and the ITGA8 rescue F9 cells (henceforth known as F9oe) were differentiated and stimulated with cytokine as previously.

At baseline there was increased expression of *FN1*, *COL4A1*, *PDGFRB*, *CXCL12* and *ACTA2* in *ITGA8*^{-/-} F9 compared with unedited E3 control, and these changes were rescued by overexpression of *ITGA8* in the F9oe cells to the levels observed in unedited cells (E3) (Figure 5.11B-F). Baseline expression of *CCN2* was significantly decreased in F9 KO in comparison to wild type control, with a further decrease observed in F9oe (Figure 5.11G). No significant differences were observed between groups in baseline expression of *C7* (Figure 5.11H). These results indicate that in vitro loss of *ITGA8* in mesangial cells results in increased matrix production (*FN1*, *COL4A1*), signalling and proliferation (*PDGFRB*, *CXCL12*) and activation (*ACTA2*)(48, 526).

To explore whether therapeutic measures to increase *ITGA8* could reduce the pro-inflammatory and profibrotic mesangial phenotype in SLE, the *ITGA8* manipulated cell lines were subjected to cytokine stimulation. There were no significant reductions in expression of any of the measured genes in the E3oe cells compared to E3 (unedited) (Figure 5.11I-O), however similar to the unstimulated experiment, expression of *FN1*, *COL4A1*, *PDGFRB* and *CXCL12* was increased in F9 *ITGA8*^{-/-} compared with wild type E3, with full rescue for *FN1*, *COL4A1* and *CXCL12* and partial rescue of *PDGFRB* with *ITGA8* overexpression in F9oe (Figure 5.11I-L). Expression of *CCN2* was significantly decreased in *ITGA8*^{-/-} F9 and F9oe when compared with wildtype controls, with no effect of *ITGA8* overexpression observed between *ITGA8*^{-/-} F9 and F9oe (Figure 5.11N). No significant differences were observed in the expression levels of *C7* and *ACTA2* between groups with cytokine stimulation (Figure 5.11M and Figure 5.11O).

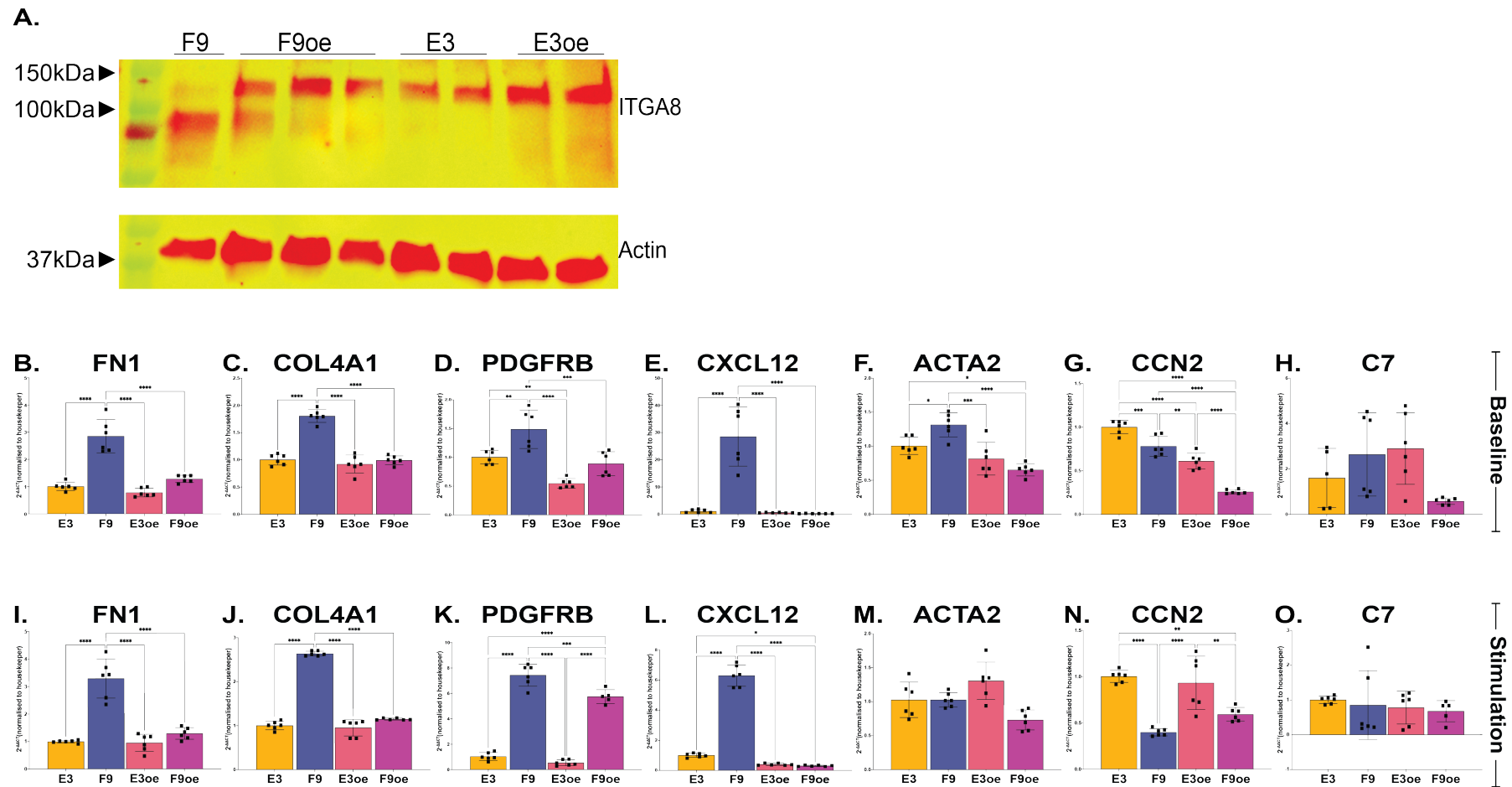


Figure 5.11: Effect of cytokine stimulation on ITGA8 knockout and overexpressed mesangial cell single clones.

(A) Western blot of protein lysate from mesangial cell single clones. Beta actin was used as a loading control for protein quantification. Differentiated mesangial cells were stimulated with $\text{TNF}\alpha$, $\text{IFN}\gamma$, $\text{IFN}\alpha$ and $\text{IL1}\beta$ for 24h and thereafter RNA was extracted for qPCR to assess expression of (B/I) FN1. (C/J) COL4A1. (D/K) PDGFRB. (E/L) CXCL12. (F/M) ACTA2. (G/N) CCN2. (H/O) C7. (B-H) baseline expression; (I-O) cytokine stimulated expression. TUBB was used as housekeeping control. Data are mean $\pm$ 95% CI, analysed by a One-way ANOVA using the Holm Sidak method to correct for multiple comparisons * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$ and representative of 2 independent experiments.

The cytokine stimulation data showed that while there was no effect of ITGA8 overexpression in E3oe versus the control WT E3 cell line, in mesangial cells with lower levels of ITGA8 expression (F9), restoration of ITGA8 expression (F9oe) normalised expression of extracellular matrix and chemokine genes. However, the partial effect on *PDGFRB* and *CCN2* with cytokine stimulation suggests that mediation of such pathways may change in the context of inflammation compared to the basal cellular state. Regarding *CCN2*, *Itga8* mediated disassembly of actin fibres has been shown to inhibit expression, however the further decrease observed in *ITGA8* overexpressing cells makes it more likely that this observation is due to experimental variables, and whilst consistent over 2 replicate experiments, further repeat experiments may better clarify this result(527, 528). Previous studies have demonstrated transient increase in *CCN2* mRNA with stimuli making it difficult to interpret any impact of *ITGA8* expression on *CCN2* in the context of cytokine stimulation(529).

In conclusion, ITGA8 is required to limit profibrotic, proliferative and inflammatory processes in cultured human mesangial cells, similar to renal disease changes observed in the in vivo lupus murine model in association with downregulation of *Itga8*. Therapeutic targeting of this integrin signalling pathway may be a novel approach to reduce mesangial proliferation, hypercellularity and matrix expansion in LN.

5.4 Summary

To assess the therapeutic relevance of immune molecular signatures identified in the previous chapter, an interventional functional experiment for modulating the immune compartment in vivo was carried out utilising a drug which blocked *Itga4*. For assessment of non-immune signatures identified from transcriptomic data, in vitro studies targeting ITGA8 in mesangial cells were carried out to understand if downregulation of this gene was indeed relevant to profibrotic changes.

The benefit of preventing immune infiltration into tissue by ITGA4 antibody, without broad spectrum immune suppression, has been demonstrated in MS and inflammatory bowel disease, however ITGA4 blockade has not been assessed in the context of SLE(502, 530). Increased expression of *Itga4* and its ligand *Vcam1* in renal immune cells in murine lupus nephritis suggested relevance in LN, with this increased expression confirmed at the protein level (Figure 4.9 and Figure 4.10)(504). Therefore, assessment of *Itga4* blockade in the higher penetrance MRL/lpr model was carried out.

The systemic phenotype with *Itga4* blockade was in concordance with previous studies, with increased numbers of activated immune cells in the periphery, suggesting that drug dosage was within a therapeutically relevant window(513). Whilst a reduction was observed in proportions of potentially pathogenic nonclassical monocytes in the kidney, no amelioration of histological or clinical phenotype was seen in mice with *Itga4* blockade(203).

The role of ITGA8 in stromal populations is unclear with seemingly different roles dependent on the cell type it is expressed in, however expression of this gene in mesangial cells has been suggested to be crucial for homeostatic mesangial functions(531, 532). In transcriptomic data, decreased expression of *Itga8* was seen in association with alterations in mesangial phenotype in disease, however it was unclear if this was just a marker of mesangial depletion or a functional mediator of mesangial cell dysregulation.

To assess the role of this gene in an isolated system without potential confounders, and for ease of genetic manipulation, an in vitro approach was chosen with a human mesangial cell line. Alterations in the baseline and cytokine stimulated expression of extracellular matrix proteins, fibrosis, and chemokine genes in *ITGA8* knockdown cells, demonstrated a profibrotic and inflammatory phenotype, with or without stimulation to model systemic inflammation. Rescue experiments with an overexpression construct confirmed that this was an ITGA8 specific effect and suggest a possible therapeutic benefit by targeting this pathway in SLE. Both pro and anti-fibrotic roles for ITGA8 have been reported which differ

by stromal cell type, the results from these experiments suggest an anti-fibrotic role for this gene in mesangial cells and warrant further investigation(478).

Chapter 6 : Discussion

This thesis aimed to explore the cellular pathology underlying LN to advance our current understanding of altered immune and non-immune pathways and the interplay between immune and renal cells in disease. A better understanding of the role of renal resident cells in autoimmune disease, and their interactions with immune cells, could lead to better predictions of the response to treatment and the development of more targeted therapies, both for lupus and potentially translatable to other immune complex mediated renal disease.

In this work I characterise the immune and systemic phenotype of two murine models of early LN with different aetiologies, generating a renal transcriptomic dataset consisting of both immune and renal cells to measure disease related changes at the level of single cells, and to identify, and test, pathways amenable to modulation. The following chapter will discuss the relevance of the findings presented and directions for future work.

6.1 Murine models of SLE and translation to human disease

In depth renal and immune phenotyping was carried out on two murine models of LN with differing aetiologies, the first a TLR7 agonist (imiquimod) inducible model and the second Fas deficient MRL/lpr model.

An inherent limitation of murine models is their translatability to human disease. However, gain of function mutation in TLR7 has been shown to cause SLE-like disease in a female patient, demonstrating the relevance of overactivation of TLR7 as seen in the IMQ model as a monogenic human disease driver, albeit in a rare case(115). Whilst disease in MRL/lpr mice mimics several aspects of human SLE, mutations in human *FAS* are associated with development of autoimmune lymphoproliferative syndrome (ALPS), which has features of SLE but is rarely characterised by renal disease(124, 533). Nonetheless, mechanistic findings from MRL/lpr mice have been integral to the development of several clinically

relevant B cell depletion therapeutics such as anti-CD20 monoclonal antibody rituximab and BAFF inhibitor belimumab(534, 535).

Alternative approaches to improve translatability include the use of humanised models, in which PBMCs from SLE patients are injected into immunodeficient mice, resulting in disease phenotype mimicking the donor; however, the phenotypes are influenced by the engraftment efficiency and the strain of immunodeficient mouse used(536, 537). Immunodeficient mice given human SLE PBMCs typically only survive for 4 weeks, with CD3⁺ cell reconstitution but very few detectable myeloid, NK or B cells (537). In the kidney, graft versus host is disease likely to predominate, since renal disease also develops in mice given PBMCs from healthy donors, limiting the scope and relevance of the model(538).

The study of human SLE renal biopsy tissue overcomes concerns about clinical relevance; however, the acquisition of fresh human biopsy samples is a limiting factor, since renal biopsy carries significant bleeding risks, and is often carried out later in the disease course when signals for early disease drivers may be replaced by non-specific fibrosis and scarring. Despite this, paraffin preserved, and biobanked samples represent an important resource, and the advent of newer transcriptomic and proteomic technologies is making the molecular study of these samples increasingly possible, reducing reliance on fresh tissue, and permitting direct study of human renal samples. Such an approach has been applied to the study of T cell mediated renal transplant rejection using paraffin embedded biopsy samples and has identified heterogeneity in response across the transplanted tissue, which could lead to the better understanding of clinically relevant pathways implicated in acute rejection, with such an approach applicable to other renal diseases(539, 540)

Murine models will remain a crucial complement to the human experiments however, because of the ability to manipulate target genes or proteins and thus test functional mechanisms and explore potential new therapeutic approaches, as demonstrated in Chapter 5 targeting *Itga4* in MRL/lpr mice.

For translation it is advantageous that overlap in immune and renal signatures between murine and human datasets demonstrate homology in disease pathways. However, certain comparisons can be challenging - for example in the case of innate markers, where fundamental differences in the identification of innate immune populations may limit comparisons defined by gene expression. Furthermore, the same gene may have differing functions and patterns of cellular expression in humans versus mice, further limiting interpretation.

6.2 Application of transcriptomic methods for interrogating cellular pathology of disease

Transcriptomic methods represent a key tool for discovery-based unbiased interrogation of dysregulated disease pathways. The composition of the kidney biases the findings from bulk approaches towards tubular signatures, therefore single cell studies are necessary to attribute signals to specific cell populations, particularly glomerular cell types, and to characterise communication between populations. Recently, spatial approaches in murine and human renal samples have begun to validate these interactions based on the colocalization of cells and markers in the context of diseases such as diabetic nephropathy and acute kidney injury(541-543). This is particularly relevant in LN, where an understanding of the dysregulated interactions between renal and immune populations is lacking. The spatial field is rapidly evolving to address issues of resolution, depth, and cell segmentation(544).

Another consideration is that current sc/snRNA seq datasets are biased towards characterisation of immune populations in disease. To address this, chapter 4 presents a novel single nuclei dataset comprised of both immune and renal cells(190, 440). Results from this chapter identified overlapping and unique signatures which may be modifiable targets for further functional validation.

6.3 Single nuclei RNA sequencing is able to adequately capture renal populations

Information acquired from single cell studies is increasingly moving towards potential clinical application, for example in cancer biology, mapping the tumour microenvironment and identifying differentially expressed genes which may correlate with disease progression(468). A similar approach could be applied to SLE, with findings from transcriptomics studies enabling stratification of patients into groups based on molecular signatures which determine response rates to therapy, predict disease progression and guide future therapeutic options for patients. The feasibility of such an approach needs to be validated, and well-defined murine models on a defined genetic background are a tool for this.

Renal populations are heterogeneous, and some single cell studies do not adequately capture glomerular populations, which is particularly problematic in the context of a glomerular disease such as LN(439, 441). In line with this, a pilot single cell study using kidneys from IMQ mice demonstrated inadequate representation of glomerular populations, therefore, snRNA-seq was carried out on nuclei isolated from kidneys of the chosen murine models. This approach has been shown to be superior for capture of populations from tissue that does not readily dissociate into a single cell suspension, with studies in the kidney demonstrating representation of glomerular and tubular populations, whilst minimising transcriptomic changes associated with dissociating tissue to a single cell suspension(441). SnRNA-seq successfully captured glomerular, tubular, and immune populations, validating this approach as far as population representation.

Studies comparing scRNA-seq with snRNA-seq have observed cellular biases, with scRNA-seq biased towards immune cells and snRNA-seq towards epithelial and stromal cells(545). However, results from flow cytometry experiments in chapter 5 indicate that in

our dataset, transcriptomically identified immune populations were representative of those identified by a protein-based method.

6.4 Evidence for mesangial phenotypic changes in LN

Mesangial cell proliferation and increased production of extracellular matrix are observed in renal diseases including LN and are key features of the ISN/RPS classification of LN(306). Although mesangial cells account for approximately 40% of the total cells in the glomerulus, understanding of the pathways leading to mesangial dysfunction is limited(42).

Analysis of stromal populations in the kidneys of IMQ mice in this thesis points to mesangial cell transition into a more fibroblast like phenotype, characterised by decreased mesangial cell numbers and an increase in transcriptomically closely related fibroblast-like population in the kidneys of diseased mice. An 'activated' phenotypic change termed myofibroblast trans differentiation (MFT) has been described in mesangial cells in models of diabetic nephropathy, characterised by a production of pro-inflammatory and fibrotic mediators akin to the observations in mesangial cells from the kidneys of IMQ treated mice(546). The pathways leading to these mesangial changes are not clear, but one possible explanation came from the observation of decreased *Itga8* expression in mesangial cells from IMQ mice.

Mesangial cell expression of *Itga8* is conserved between mouse and humans and is essential for homeostatic functions, for example through the control of proliferation and regulation of activation, with decreased expression associated with a more activated and fibrotic phenotype(478, 547). Furthermore, a snRNA-seq study from patients with diabetic nephropathy describes a similar mesangial phenotype characterised by downregulation of *ITGA8*, suggesting this as an important marker of mesangial dysfunction(548). An important question raised by the results in this thesis is whether downregulation of *Itga8* is mechanistically important in driving a profibrotic phenotype, or simply a marker of mesangial dysregulation, in LN and potentially in other glomerular renal pathologies.

On the other hand, if this indeed is a pathway shared across different renal diseases, it is not clear why this change was not observed in the MRL/lpr model. This may be explained by differences in disease drivers or disease stage. One possible explanation may be altered fibrotic and healing pathways in MRL/lpr mice, with enhanced regenerative capacity enabling faster healing of wounds such as ear punches without the presence of fibrosis or scarring compared to C57BL/6 mice, and lower fibrotic activity following acute kidney injury(549-551). The molecular basis for this phenotype is not well understood but studies suggest a strong genetic component, and alterations in extracellular matrix breakdown characterised by enhanced expression of matrix metalloproteinases (MMPs) may be important(552). Particularly as MMPs break down basement membrane formed over a site of injury allowing blastema formation and scarless healing(553). It is conceivable that this enhanced healing trait might be altering the mesangial fibrotic response in MRL/lpr mice. MMP9 knockout on the MRL/lpr background is associated with more severe disease and higher burden of IC and studies have suggested that MMP9 selectively cleaves autoantigens in ICs and may therefore be protective in disease(554).

6.5 In vitro modulation of ITGA8

The mesangial cell line provides a valuable tool to modulate the expression of ITGA8 and assess the downstream impact of this in an isolated system. Although the impact of interactions with other cell types is not tested, this method can define cell intrinsic pathways and the interactions between the mesangial cell clones.

Knockdown of ITGA8, under standard culture conditions, resulted in increased expression of *COL4A1*, *FN1*, *CXCL12* and *PDGFRB* compared to WT cells. Previous studies of mesangial cells from *Itga8* deficient mice have demonstrated enhanced mesangial cell specific expression of $\alpha 2$ and $\alpha 6$ integrins, which regulate collagen and fibronectin binding, and may explain the enhanced baseline expression of these extracellular matrix components with ITGA8 knockdown(528, 532).

In murine diabetes *CXCL12* upregulation reduces mesangial expansion and fibronectin production, therefore the increased baseline *CXCL12* could reflect a compensatory mechanism to limit aberrant matrix production and ultimately fibrosis(555). Conversely, increased baseline *PDGFRB* could be a consequence of a switch towards a more profibrotic phenotype.

With cytokine stimulation and knockdown of ITGA8, I observed increased expression of extracellular matrix and chemokine genes compared to unedited cells. Correction of both basal and cytokine stimulation induced responses in ITGA8 knockdown cells through the reintroduction of ITGA8 confirms that the effects are mediated via ITGA8 and not due to off-target or clonal effects.

These findings show that ITGA8 loss is not only a marker of mesangial transition to a more fibroblast phenotype, as shown by the snRNA-seq data, but that lack of ITGA8 promotes inflammatory, profibrotic and matrix production pathways, at least in vitro, such that modulation of ITGA8 may regulate fibrotic processes and therefore could be a novel therapeutic target for kidney disease.

There are several limitations of this experiment, notably incomplete knockout of ITGA8 in clones studied, though arguably this may mimic the IMQ treated mice, which demonstrated reduced expression of mesangial *Itga8*. CRISPR/Cas9 editing can lead to off-target effects resulting in non-specific editing; however, rescue by reintroduction of ITGA8 into the knockdown cells demonstrated that the observed effects were gene specific.

Further work is needed before extrapolating these results to human kidney, particularly as previous studies have demonstrated a discordance between in vitro and in vivo findings in *Itga8* deficient models in the lungs, with a protective effect in fibroblasts in vitro which could not be replicated in vivo(522). This could be due to differential cell/organ reliance on ITGA8

or differences in the role of ITGA8 in models of chronic versus acute fibrosis but reflects the need for in vivo translation of findings in this thesis.

An additional disease relevant role for ITGA8 not investigated in this thesis but relevant to LN is in regulation of phagocytosis by mesangial cells(390). Downregulation of ITGA8 and consequent impairment of mesangial cell mediated phagocytosis could lead to the build-up of IC, matrix components and autoantigens in the renal environment. Experiments comparing the phagocytosis of fluorescently tagged ICs, apoptotic cells and matrix coated latex beads, between ITGA8 knockdown and wild type mesangial cells would be able to test the relevance of this hypothesis in vitro(390).

Assessment of this phenotype in vivo may be facilitated through the use of RGD binding integrin antagonists such as CWHM-12, which has been successfully used in murine models and may be an alternative, more translational way to assess the impact of inhibiting ITGA8 signalling(556). However, ITGA8 is not the only integrin able to bind to RGD motifs (556).

ITGA8 is expressed in non-mesangial cell types including vascular smooth muscle and some fibroblasts. Whilst systemic conditional deletion of *Itga8* in stromal populations in murine models circumvents the developmental problems seen in mice with a germ line deletion, this may still result in non-cell type specific effects. On the other hand, use of a mesangial cell specific recombinase remains challenging due to lack of a specific marker, with several of the key markers used to identify mesangial cells (e.g., *Pdgfrb*, *Pdgfra*, *Gata3*), expressed by other populations within the kidney.

A sequential dual recombinase approach such as *Pdgfrb*-Cre; *Gata3*-LSL-DreER; *Itga8*-rx, in which cre activation results in deletion of loxP sites in *Gata3* and subsequent tamoxifen induced Dre-rox recombination and deletion of *Itga8* in *Pdgfrb*⁺*Gata3*⁺ cells could target mesangial cells in a more specific manner, however low level *Gata3* expression in vascular smooth muscle cells may result in some “leakiness”(475, 557). Another key consideration

is the impact of disease on expression of chosen markers, as this may impact the deletion efficiency.

6.6 Proximal tubular inflammation in LN

Histological evidence of tubular injury in LN is clinically correlated with poorer outcomes, and in patients with LN, tubular IFN response scores correlate with response to therapy(319). However, differentiation of injury by tubular segment during standard histological classification is inaccurate and therefore not used(306, 311). Mirroring human data, increased IFN response scores were observed in MRL/lpr mice compared to MRL/MpJ controls, particularly in the proximal tubule compartment.

Differentially expressed genes pertaining to antigen presentation and leukocyte chemotaxis suggested a proinflammatory role for proximal tubules with interactions between proximal tubule cells and immune components potentially perpetuating the cycle of inflammation. For the first time I identify proximal tubule S1 segment cells as being most sensitive to interferon mediated damage. This is the segment that immediately follows the glomerulus and therefore enhanced susceptibility might be explained by spatial proximity to glomerular or periglomerular immune cells. In chronic kidney injury signalling from the S1 segment has been suggested to orchestrate responses to inflammatory stimuli in the S2/S3 segments, and damage and maladaptive repair of the S1/S2 segments has also been observed in a diphtheria toxin induced sub-lethal injury model(491, 558). In LN chronic inflammatory stimuli might have a similar impact on the S1 segment, promoting a strong IFN response. As the S1 segment cells are the most abundant of proximal tubular cells, a caveat to this finding is that underrepresentation of cells from other segments could bias the results and contribute to the observed effect.

Joint analysis of transcriptomic data from both models identified enhanced response to interferon in several clusters in the kidneys of diseased mice. Type 1 interferon has a well-established role in SLE, but these data suggest that renal interferon mediated responses occur early in disease, in the absence of severe histological changes or clinical evidence of LN. Therefore, future experiments assessing the correlation between tissue interferon signature and urinary secretion of interferon may offer a non-invasive method for monitoring responses to therapy.

6.7 Transcriptomic identification of immune and renal interactions in disease

Observation of active, cytotoxic and exhaustion signatures in T and NK cells in the kidneys of MRL/lpr mice is keeping with reported roles for these cells in disease pathology(264). As a similar exhausted-like profile was observed by flow cytometry in the periphery, particularly amongst the CD8 T cells, this appears to be a systemic response, not localised to the kidney, perhaps due to chronic antigen stimulation. The enhanced expression of cytotoxic genes in this population suggests a mix of actively cytotoxic and exhausted CD8 T cells, which may be able to induce damage in the renal space(375).

Peripheral CD8 T cell exhaustion correlates with better prognosis in autoimmune diseases(559). Immune checkpoint pathways such as PD1 and BTLA are upregulated in exhaustion, and the ability of immune checkpoint inhibitors to induce an exhaustion like phenotype in activated cells is being actively explored(559, 560). However, the consequence of exhausted-like cells within the renal tissue is not known. Impaired cytotoxicity due to CD8 T cell exhaustion could result in accumulation of injured cells, able to secrete proinflammatory mediators and acting as a source of self-antigen. Whilst a cytotoxic signature was observed in the kidneys of LN patients, immune scRNA- seq did not identify an exhaustion signature in human CD8 T cell populations, raising the possibility this is a unique murine signature(190). However, 67% (16/24) of patients in the Arazi et al study

were taking some form of immunosuppressive treatments, some of which may deplete exhausted CD8 T cells, for example cyclophosphamide treatment in tumours resulted in the depletion of exhausted CD8 T cells, furthermore this CD8 cluster was primarily derived from two patients(561).

Actively degranulating cytotoxic NK cells represent another proinflammatory and potentially pathogenic population identified in this thesis. CD8⁺ T cells with CD107a co-expression have been observed in biopsy samples from patients with LN, with numbers correlating with proteinuria(374). However, a proportion of the CD107a⁺ cells were CD8⁻, thus it is likely that NK cells contributed to this population. Systemic ablation of NK cells in lupus prone mice has been shown to worsen disease, conversely tissue infiltrating NK cells in murine models of SLE have been shown to acquire an activated and cytotoxic phenotype with potential for tissue damage(562). The impact of this in the context of renal damage requires further investigation, potentially through drug-based modulation of cytotoxic pathways since genetic knockouts of cytotoxic genes have been associated with development of autoimmunity(563).

Alterations in kidney resident macrophages (KRM) described in this thesis have been previously reported in the context of LN, however the functions of these cells in LN remain unclear(362). Numbers of CD11b^{hi}/F4/80^{mid/high} tissue resident macrophages (2_TrMAC+) were increased in the kidneys of both LN models compared to controls, and these cells showed enhanced expression of phagocytic, interferon mediated and activation markers. This population has similarities to an infiltrating, alternatively activated and pathogenic M2b subset of M2 macrophages, described in the kidneys of NZM2328 lupus prone mice, characterised by expression of CD11b and PDL1 (*Cd274*)(187). M2b macrophages can be induced by exposure to IC and are proposed to be monocyte derived and present in active disease(564). Consistent with this in our snRNA-seq data, a renal monocyte population was observed in diseased mice, with trajectory analysis suggesting this could serve as a

precursor to 2_TrMAC+ population. This monocyte population most closely resembles non-classical monocytes, pathogenic in murine and human LN(200, 203). The overlap of these findings between several models indicates the importance of this signature and suggests that blockade of this monocyte-macrophage axis could be therapeutic.

ITGA4 is an α integrin subunit which associates with the β chain in VLA4 and LPAM1 complexes and is important in facilitating immune cell migration into tissues(502, 530). Given the evidence for non-classical monocyte infiltration, the findings of immune upregulation of *ITGA4* and interaction between *Itga4* and its ligand *Vcam1*, specifically in LN, suggested it as a possible target to reduce monocyte infiltration. Mapping identified disease enhanced macrophage, nonclassical monocyte and CD8 T cell clusters as the main sources of *Itga4*, with *Vcam1* expressed in parietal epithelial and endothelial cell clusters. Assessment of ITGA4 expression in the murine-human integrated immune dataset showed enhanced expression in clusters consistent with infiltrating immune cells, supporting the clinical relevance of this signature.

The VLA4 and LPAM1 complexes have been linked to leukocyte trafficking in autoimmune diseases such as multiple sclerosis and ulcerative colitis respectively, with therapeutics targeting these complexes already in clinical use(502, 530). Blockade of VLA4 with the monoclonal antibody natalizumab in patients with MS, has been shown to reduce clinical relapses and brain lesions; however, an association with “rebound” upon the cessation of therapy and the development fatal progressive multifocal leukoencephalopathy (PML) has limited its use to patients with repeated relapses(565). In ulcerative colitis, LPAM1 was observed to be more relevant for leukocyte trafficking and blockade and the LPAM1 antibody vedolizumab can induce remission in refractory and non-refractory patients(502, 566).

The potential role of targeting ITGA4 to inhibit immune cell trafficking into tissue in SLE has not been investigated. Targeting of a receptor mediating immune cell trafficking is an

attractive option, to prevent or ameliorate tissue damage, however the impact of accumulation of activated and dysregulated immune cells in the periphery is unknown, with potential for deleterious consequences.

6.8 Itga4 blockade as an approach for modulating immune cell infiltration in SLE

Itga4 was blocked with an antibody (PS/2) which binds the B2 epitope on Itga4, as opposed to an epitope present upon heterodimerisation with either Itgb1 or Itgb7. Therefore PS/2 can inhibit effects mediated by either VLA4 or LPAM1, via binding to the B2 epitope, relevant for ligand binding and homotypic cellular aggregation(507, 508).

Peripheral phenotyping identified an additive effect of Itga4 blockade combined with Fas deficiency on CD4/CD8 effector memory populations, in line with studies demonstrating sequestration and activation of effector memory T cells in the periphery of patients being treated with natalizumab(513, 567). This suggests that the dosing of PS/2 was within an adequate therapeutic range. Decreased CD11b⁺ cells in the peripheral circulation in PS/2 MRL/lpr mice, may have been in line with the trend towards lower levels observed in isotype MRL/lpr mice in the same population, but could also reflect an additive effect of Itga4 blockade, as seen with natalizumab in human studies(568). Additional myeloid flow cytometry panels would be required to conclusively identify individual CD11b⁺ populations.

Total kidney immune infiltrate was not reduced with Itga4 blockade; however, alterations were observed in some subpopulations. Itga4 blockade led to a reduction in non-classical monocytes as a proportion of total monocytes, compared to untreated MRL/Lpr, consistent with ex vivo experiments in muscle tissue, where blockade of Itga4 in the presence of inflammation prevented adhesion of these monocytes to blood vessel wall(569). Previous studies have demonstrated disease amelioration in lupus prone B6.Sle.Yaa mice by inhibiting non-classical monocytes, via genetic deletion of transcription factor Nr4a1(203). However, in this study, despite some modulation of renal non-classical monocytes, there

was no improvement in renal histology or proteinuria. This could be because blockade of Itga4 did not completely block infiltrating monocytes, perhaps due to differential ligand use for leukocyte extravasation, but a more likely explanation is that monocyte blockade in the context of MRL/lpr model may not be sufficient to ameliorate renal disease, particularly as T cells represent the majority of infiltrate(264).

In rat models of anti-GBM disease, both Itga4 blockade and anti-Vcam1 antibodies are protective, and blockade of Vcam1 on renal tissue sections from MRL/lpr mice incubated with immune cells inhibited T cell and macrophage adhesion to renal tissue. *Vcam1* was also shown to be increased at disease onset in microarray data when comparing pre and post nephritic NZB/W mice. Taken together these studies suggest the importance of VCAM1 in disease across several other species and models.(504, 570-572).

Inhibition of Itga4 has been associated with enhanced secretion of proinflammatory cytokines in the periphery including $\text{TNF}\alpha$, $\text{IFN}\gamma$ and IL17, and polarisation towards a Th1 response(571). The implications of this in the context of the systemic inflammation seen in SLE are unclear, but it is conceivable that such an environment may give rise to populations such as Th17 cells which are able to circumvent Itga4 blockade, and cause tissue damage(513, 573).

A role for VLA4 has been proposed in inhibition of apoptosis. It is possible that VLA4 antagonism could further enhance cell death in MRL/lpr mice, already deficient for one apoptotic mediator, hence increasing the availability of systemic autoantigens and further driving autoimmunity. Thus, Itga4 blockade's therapeutic effects might be masked in a model of defective apoptosis(574). To test this hypothesis, the same approach could be applied to an alternative model of SLE such as NZM2410 mice in which renal disease onset is well characterised, more rapid than its parental precursor NZB/W mice and does not require any complex breeding for colony maintenance(575). Alternatively, to assess if blockade of ligand and integrin mediating tissue extravasation are relevant targets for SLE,

specific inhibition of relevant ligands such as Vcam1, could be performed in vivo with a Vcam1 blocking antibody such as M/K-2.7(576). Such an experiment in MRL/lpr mice could determine if the effect of Itga4 blockade was indeed masked by Fas deficiency. On the other hand, to address the possibility of alternate pathways for tissue homing, blockade of LFA-1 (CD11A/ITGB2) or dual blockade might be an approach to assess in future experiments.

6.9 Limitations of this approach

Important limitations to single cell or nuclei transcriptomics remain. One key challenge is the adequate and consistent representation of renal and immune populations, especially when this technology is applied to a human biopsy sample where tissue is limited. Previous studies using human samples have often obtained samples from patients undergoing nephrectomy or tumour removal, therefore attaining a much larger quantity of tissue(438). Tissue dissociation can impact the sample quality, however increased use of these technologies has gone hand in hand with the development of more standardised protocols to enhance consistency across experiments. For example, in tissue dissociation, improving the reproducibility between experiments and cell/nuclei yield(577).

Transcriptomic expression does not always correlate with protein expression, this is exemplified in this thesis by the increased transcriptomic expression of *Fas* in a deficient model. Particularly in snRNA-seq, which consists of both pre-mRNA and mature RNA, alignment with protein expression may be poor, however even in scRNA-seq correlation between protein and gene expression remains incomplete. Transcript capture is a stochastic event and therefore sequencing of higher numbers of cells is likely to give better transcriptomic representation, however this might not always be economically or practically feasible.

A further major limitation of single cell modalities is the loss of spatial information, which is vital for contextualising interactions between cell types. Currently available spatial

approaches have a trade-off between resolution and transcript capture, for example, with in situ capture based methods such as 10X Visium the size of each spot is 55µm, and is therefore composed of multiple cell types with approximately 2,500 unique genes detected per spot; however at subcellular resolution, as in the case of Stereoseq, in which each spot size is 0.22µm, the number of unique genes captured per spot is only 5-10, requiring aggregation of transcripts across spots for cell type annotation(578, 579). Computational methods for imputation of cell type identity such as robust decomposition of cell type mixtures (RCTD) and Tangram, rely on single cell/nuclei datasets for supervised annotation, and cell deconvolution is challenging due to difficulties defining cell boundaries, exacerbated by RNA diffusion(580, 581).

Proteomic approaches such as laser capture microdissection combined with mass spectrometry are beginning to generate protein information at close to the level of single cells, but this is not currently available at high throughput, and detection of low abundance proteins is challenging(582). Combining single cell /nuclei transcriptomics with additional proteomic, spatial, and histopathological data will ultimately provide a more representative picture of disease pathology(583).

6.10 Application of this pipeline in interrogating cellular pathology and next steps

Improvements in prognostic ability, therapeutics and management of renal immune complex disease will depend upon advancing our mechanistic understanding of pathology. Whilst histological observations remain indispensable for diagnosis, precedent for further incorporation of complementary molecular information comes from studies such as those carried out in renal transplant patients which have identified genes predictive of allograft loss(584). The advent of new “omics” technologies has made interrogation of heterogeneous disease more achievable by enabling the individual characterisation of multiple cell

populations as well as the interactions between these cell populations, as demonstrated in Chapter 4.

It is likely that in future histological information will be increasingly complemented by screening for targets identified by discovery-based modalities, such as snRNA-seq, proteomics and spatial transcriptomics. Whilst discovery-based methods may not be yet well suited for direct integration into clinical practices, panels of select molecular signatures identified via these experiments can be interrogated utilising hypothesis-driven high throughput approaches.

Such an approach will facilitate better stratification of patients into groups based on prognosis or anticipated therapeutic response, which will enable more personalised clinical management, and more targeted recruitment to optimise power in interventional trials. In addition, rich cellular datasets in tissue will be a powerful tool to identify candidate novel targets for treatment and modifiable disease signatures, with potential relevance to wider disease, which can be validated functionally using in vitro and in vivo models as exemplified in Chapter 5 for ITGA8 and ITGA4.

Whilst 'agnostic' discovery methods generate a plethora of data to be mined, this thesis could only focus on a select few targets for functional validation, and additional differences in key populations observed in the transcriptomic data remain to be explored, such as changes in endothelial cells with LN. Further study of these differences using functional assays may shed light on questions pertaining to the pathogenic versus protective roles of kidney resident and immune populations in disease.

To circumvent questions of translatability to human disease, future experiments could utilise archived human tissue samples, applying spatial and single cell transcriptomics. A pipeline moving from these findings to functional assays in vivo and in vitro for validation will be applicable to multiple renal diseases.

6.11 Conclusion

Utilising discovery-based approaches this thesis has generated a novel transcriptomic dataset consisting of renal and immune populations from two distinct murine models of LN, enabling identification of dysregulated pathways in disease.

Findings included alterations in tissue resident macrophage profile characterised by increased non-classical monocyte derived tissue resident macrophages in disease, enhanced proximal tubular interferon responses localised to the S1 segment, and a putative role for ITGA8 in acquisition of a fibrotic phenotype in mesangial cells. The latter was validated in vitro, representing a pathway with therapeutic promise.

Further analysis of crosstalk between immune and renal populations identified alterations in integrin-ligand interactions required for tissue homing in LN. However in vivo blockade of Itga4 reduced the expanded proportion of non-classical renal monocytes in MRL/lpr mice but did not alter clinical disease parameters. This could reflect the choice of model or redundancy with other integrins for immune tissue infiltration, and additional experiments are suggested that could further assess the possible therapeutic benefit of such an approach.

The approach used in this thesis provides a broadly applicable strategy that can be further developed to address questions about the role of renal environment in perpetuating immune dysfunction and factors contributing to immune migration into the kidneys in early disease, as well as for interrogation of potential therapeutic targets. With the advent of technologies enabling in-depth characterisation of archived human samples, the strategy demonstrated in this work will be applicable to directly characterise human pathology, advancing current understanding of renal disease pathways.

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Appendix

Appendix A: Antibodies used for flow cytometry, antibodies in yellow denote intracellular stains

Antigen	Clone	Fluorochrome	Company and catalogue number	Titration
IgD	11-26c.2a	FITC	Biolegend 405704	400
		APC-Cy7	Biolegend 405716	400
PD-1	RMP1-30	FITC	eBioscience 11-9981-82	100
PNA		FITC	Vector labs FL-1071	1500
CD44	IM7	FITC	Biolegend 103006	100
		PerCP-Cy5.5	Biolegend 103032	100
		Pacific Blue	Biolegend 103020	100
CD62L	MEL-14	FITC	Biolegend 104406	100
		APC	Biolegend 104412	100
CD11c	N418	FITC	Biolegend 117396	100
		PE	Biolegend 117307	100
CD1d	1B1	PE	Biolegend 123510	400
CD138	281-2	PE	Biolegend 142504	100
CXCR5	L138D7	PE	Biolegend 356904	100
CD25	PC61	PE	Biolegend 12008	100
		Pacific Blue	Biolegend 102022	100
FoxP3	MF-14	PE	Biolegend 126404	100
CD8a	53-6.7	PE	Biolegend 100708	400
		PerCP-Cy5.5	Biolegend 100734	400
		Alex Fluor 700	Biolegend 100730	200
B220	RA3-6B2	PerCP-Cy5.5	Biolegend 103236	400
		APC-Cy7	Biolegend 103224	400
		Brilliant Violet 605	Biolegend 103244	400
		Alex Fluor 700	Biolegend 103232	400
		PE-Cy7	Biolegend 103222	400
ICOS	C398.4A	PerCP-Cy5.5	Biolegend 313518	100
Ly6c	HK1.4	PerCP-Cy5.5	Biolegend 128012	400
CD23	B3B4	PE-Cy7	Biolegend 101614	250
CD3e	145-2C11	PE-Cy7	Biolegend 100320	100
		APC-Cy7	Biolegend 100330	100
		Pacific Blue	Biolegend 100334	100
CD3e	17A2	Alex Fluor 700	Biolegend 100216	100
CD95	Jo2	PE-Cy7	BD Biosciences 557653	200
CD4	GK1.5	PE-Cy7	Biolegend 100422	200
		Alex Fluor 700	Biolegend 100430	200
		APC-Cy7	Biolegend 100414	200
F4/80	BM8	PE-Cy7	Biolegend 123114	1000

CD93	AA4.1	APC	Biolegend 136510	100
CD86	GL-1	APC	Biolegend 105046	100
GL7	GL7	APC	Biolegend 144618	100
CD11b	M1/70	APC	Biolegend 101212	500
		PB	Biolegend 101223	100
		Alexa Fluor 488	Biolegend 101217	100
CD19	6D5	Alex Fluor 700	Biolegend 115528	400
Ly6g	1A8	Alex Fluor 700	Biolegend 127622	200
CD21	7E9	APC-Cy7	Biolegend 123418	400
Zombie NIR Live/Dead		APC-Cy7	Biolegend 423106	200
IgM	eB121-15F9	eFluor 450	eBioscience 48589082	200
Zombie Aqua Live/Dead		Brilliant Violet 510	Biolegend 423102	200
CD69	H1.2F3	Brilliant Violet 605	Biolegend 104530	200
IgG1	A85-1	Brilliant Violet 605	BD Biosciences 563285	500
CD43	S7	PerCP-Cy5.5	BD Biosciences 562865	100
		Brilliant Violet 421	BD Biosciences 752957	100
CD49b	DX5	Alex Fluor 700	eBioscience 56-5971-80	100
		PE-Cy7	Biolegend 108922	100
Siglec-H	551	PE	Biolegend 129605	100
CD45	30-F11	APC	Biolegend 103111	100
		Brilliant Violet 605	Biolegend 103139	100
		Brilliant Violet 510	Biolegend 103137	100
CD68	FA-11	PE	Biolegend 137013	100
CD64	X54-5/7.1	PerCP-Cy5.5	Biolegend 139307	100
CD192	SA203G11	Brilliant Violet 605	Biolegend 150615	100
CD115	AFS98	PE	Biolegend 135505	100
CX3CR1	SA011F11	Brilliant Violet 605	Biolegend 149027	100
CD24	M1/69	Brilliant Violet 605	Biolegend 101827	400
BP-1	Lg-51	PE	BD Biosciences 553735	100
MHC11 (I-A/I-E)	M5/114.15.2	Brilliant Violet 421	Biolegend 107631	100
Tbet	4B10	APC	eBioscience 17-5825-82	100

Appendix B: Genes used for calculation of interferon score in Chapter 4

Interferon Score genes		
<i>Gm36028</i>	<i>Trim30d</i>	<i>Gbp2b</i>
<i>Ifi44</i>	<i>Rec8</i>	<i>Gbp2</i>
<i>Ifi44l</i>	<i>Parp10</i>	<i>Rbck1</i>
<i>Phf11a</i>	<i>Mov10</i>	<i>Herc6</i>
<i>Phf11b</i>	<i>Unc93b1</i>	<i>Tnfsf10</i>
<i>Phf11d</i>	<i>Ccl12</i>	<i>Zc3hav1</i>
<i>Phf11c</i>	<i>Ccl8</i>	<i>Lap3</i>
<i>Ifit3</i>	<i>Isg20</i>	<i>Eif2ak2</i>
<i>Ifit3b</i>	<i>Oas3</i>	<i>Bst2</i>
<i>Srbd1</i>	<i>Trim75</i>	<i>Parp9</i>
<i>Rsad2</i>	<i>Asprv1</i>	<i>Adar</i>
<i>Ifit2</i>	<i>Oasl1</i>	<i>Sp100</i>
<i>Tap2</i>	<i>Gbp7</i>	<i>Stat1</i>
<i>Cpt1b</i>	<i>Gbp3</i>	<i>Otof</i>
<i>Trim38</i>	<i>Sp110</i>	<i>Ifih1</i>
<i>Acta2</i>	<i>Irf9</i>	<i>Chmp5</i>
<i>Ly6e</i>	<i>Dhx58</i>	<i>Ifitm6</i>
<i>Psmb9</i>	<i>Parp12</i>	<i>Xaf1</i>
<i>Tap1</i>	<i>Parp14</i>	<i>Timm10</i>
<i>Tymp</i>	<i>Dynlt1a</i>	<i>Gadd45b</i>
<i>Cmpk2</i>	<i>Dynlt1b</i>	<i>Oas1c</i>
<i>Trim6</i>	<i>Dynlt1c</i>	<i>Oas1b</i>
<i>Mx1</i>	<i>Dynlt1f</i>	<i>Oas1f</i>
<i>Mx2</i>	<i>Batf2</i>	<i>Oas1h</i>
<i>Fbxo6</i>	<i>Tmem140</i>	<i>Oas1g</i>
<i>Gbp8</i>	<i>Ifi35</i>	<i>Oas1a</i>
<i>Gbp9</i>	<i>Ido1</i>	<i>Oas1d</i>
<i>Gbp4</i>	<i>Trank1</i>	<i>Serping1</i>
<i>Gm43302</i>	<i>Rhbdf2</i>	<i>Irf7</i>
<i>Gbp10</i>	<i>Nt5c3</i>	<i>Nbn</i>
<i>Gbp6</i>	<i>Hsh2d</i>	<i>Zbp1</i>
<i>Gbp11</i>	<i>Rnf213</i>	<i>Ddx58</i>
<i>Socs1</i>	<i>Dhrs9</i>	<i>Tcn2</i>
<i>Apol6</i>	<i>Ddx60</i>	<i>Tnfaip6</i>
<i>Ncoa7</i>	<i>Stat2</i>	<i>Znfx1</i>
<i>Trim5</i>	<i>Helz2</i>	<i>Casp1</i>
<i>Trim12a</i>	<i>Spats2l</i>	<i>Aim2</i>
<i>Trim12c</i>	<i>Cxcl10</i>	<i>Ube2l6</i>
<i>Trim30b</i>	<i>Oas1e</i>	<i>Trim21</i>
<i>Trim30c</i>	<i>Galm</i>	<i>Trim56</i>
<i>Trim30a</i>	<i>Oas2</i>	<i>Trafd1</i>
<i>Abca1</i>	<i>Nmi</i>	

Appendix C: Genes used for calculation of exhaustion score in Chapter 4

Exhaustion score genes			
<i>Klf5</i>	<i>Gcm2</i>	<i>Cnih2</i>	<i>Wfs1</i>
<i>Ptger2</i>	<i>Hoxc6</i>	<i>Phldb2</i>	<i>Adamdec1</i>
<i>Nap1l2</i>	<i>Spred2</i>	<i>Igf1r</i>	<i>Arid3b</i>
<i>Sephs1</i>	<i>Ciz1</i>	<i>Syt1</i>	<i>St6galnac2</i>
<i>Mocos</i>	<i>Ercc5</i>	<i>Ddit4</i>	<i>Sec14l1</i>
<i>Msx1</i>	<i>Serping1</i>	<i>Scamp1</i>	<i>Polrmt</i>
<i>Ada</i>	<i>Aff4</i>	<i>Kctd12</i>	<i>Lima1</i>
<i>Brs3</i>	<i>Tro</i>	<i>Tnks1bp1</i>	<i>Irf6</i>
<i>Nrk</i>	<i>Gpld1</i>	<i>Spock1</i>	<i>Fmo3</i>
<i>Htr2c</i>	<i>Pnrc1</i>	<i>Gdap1</i>	<i>Myo6</i>
<i>Serpinb2</i>	<i>Cotl1</i>	<i>Clca1</i>	<i>Egr2</i>
<i>Slc35e4</i>	<i>Ncan</i>	<i>Slc12a2</i>	<i>Rgs16</i>
<i>Six1</i>	<i>Tnfsf10</i>	<i>Dusp6</i>	<i>Neurod1</i>
<i>Mrc1</i>	<i>Fgf1</i>	<i>Abcd2</i>	<i>Cit</i>
<i>Cellf4</i>	<i>Gapdhs</i>	<i>Rab33b</i>	<i>Inpp1</i>
<i>Snai1</i>	<i>Tm4sf1</i>	<i>Il1a</i>	<i>Dazap1</i>
<i>Nkx2-2</i>	<i>Pold2</i>	<i>Polr2c</i>	<i>Gpx2</i>
<i>App</i>	<i>Mrps18a</i>	<i>Tm2d3</i>	<i>Trpc1</i>
<i>Eya2</i>	<i>Bop1</i>	<i>Carm1</i>	<i>Atp13a2</i>
<i>Twsg1</i>	<i>Cxcl14</i>	<i>Atp5j2</i>	<i>Rnf123</i>
<i>Sall4</i>	<i>Acs1</i>	<i>Hcfc1r1</i>	<i>Hinfp</i>
<i>Tgm2</i>	<i>Reln</i>	<i>Cadm1</i>	<i>Canx</i>
<i>Rprd1b</i>	<i>Nr1h4</i>	<i>Crybb2</i>	<i>Sash1</i>
<i>Tcf4</i>	<i>Polr1b</i>	<i>Arg1</i>	<i>Dbp</i>
<i>Tug1</i>	<i>Gabrr2</i>	<i>Cd22</i>	<i>S100a8</i>
<i>Efs</i>	<i>Ctla4</i>	<i>Sars2</i>	<i>C1qc</i>
<i>Ogn</i>	<i>Chl1</i>	<i>Gtf3c4</i>	<i>Gmppb</i>
<i>Ahnak</i>	<i>Osbpl9</i>	<i>Mllt1</i>	<i>Csf1</i>
<i>Kctd9</i>	<i>Bcl3</i>	<i>Rorc</i>	<i>Gata2</i>
<i>Klrk1</i>	<i>Itih1</i>	<i>F2r</i>	<i>Krt27</i>
<i>Mrps2</i>	<i>Mrps36</i>	<i>Slc4a7</i>	<i>Tnni1</i>
<i>Rgs10</i>	<i>Surf4</i>	<i>Fgf6</i>	<i>Hoxd10</i>
<i>Etv1</i>	<i>Nsdhl</i>	<i>Ctsk</i>	<i>Ifih1</i>
<i>Tspan13</i>	<i>Cpsf2</i>	<i>Phf7</i>	<i>Rpa2</i>
<i>Hmx3</i>	<i>Gem</i>	<i>Terf1</i>	<i>Prl</i>
<i>Fkbp10</i>	<i>Coch</i>	<i>Csk</i>	<i>Tapbp</i>
<i>Smad7</i>	<i>Serpinh1</i>	<i>Ccdc93</i>	<i>Dync1i1</i>
<i>Adam7</i>	<i>Myo1f</i>	<i>Slc11a1</i>	<i>Rps14</i>
<i>Tln1</i>	<i>Cplx2</i>	<i>Litaf</i>	<i>Wls</i>
<i>Bnip2</i>	<i>Rasgrf2</i>	<i>Tnfrsf1a</i>	<i>Cxcl13</i>
<i>Elk1</i>	<i>Znrf1</i>	<i>S100a13</i>	<i>Per2</i>
<i>Baat</i>	<i>Perp</i>	<i>Htra2</i>	<i>Twf1</i>
<i>Ptpro</i>	<i>Nfib</i>	<i>Pou2f1</i>	<i>Selp</i>
<i>Tlr7</i>	<i>Smad1</i>	<i>Lclat1</i>	<i>Gabrd</i>
<i>Ahr</i>	<i>Pik3c2g</i>	<i>Rpp21</i>	<i>Plekha1</i>
<i>Ccl11</i>	<i>Hcn1</i>	<i>Id2</i>	<i>Tbx15</i>
<i>Pclo</i>	<i>Efnb3</i>	<i>Dffa</i>	<i>Nr4a2</i>
<i>Gpm6b</i>	<i>Clic4</i>	<i>Pxmp2</i>	<i>Tpk1</i>
<i>S1pr3</i>	<i>Lhcgr</i>	<i>Cps1</i>	<i>Sag</i>
<i>Ddrgk1</i>	<i>Sim1</i>	<i>Pou2f2</i>	<i>Xcr1</i>
<i>Foxo4</i>	<i>Stim1</i>	<i>Trappc5</i>	<i>Tyrobp</i>

<i>Cxcr3</i>	<i>Bcar1</i>	<i>Irs1</i>	<i>Slc15a2</i>
<i>Col19a1</i>	<i>Eomes</i>	<i>Cish</i>	<i>P4ha2</i>
<i>Stra6</i>	<i>Fstl1</i>	<i>Phlda3</i>	<i>Atf6</i>
<i>Stx3</i>	<i>Epcam</i>	<i>Rbm15</i>	<i>Ctse</i>
<i>Lrp10</i>	<i>Anxa3</i>	<i>Ptprj</i>	<i>Dmp1</i>
<i>Sf3a1</i>	<i>Vmp1</i>	<i>Prrc1</i>	<i>Vcam1</i>
<i>Comtd1</i>	<i>Ndufa13</i>	<i>Bhlhe40</i>	<i>Padi1</i>
<i>Ndufa1</i>	<i>Spic</i>	<i>Ccr5</i>	<i>Hao2</i>
<i>Gsto1</i>	<i>Dpp7</i>	<i>Ikzf4</i>	<i>F2rl1</i>
<i>Scgb1a1</i>	<i>Mmp14</i>	<i>Tmem150a</i>	<i>Dpy30</i>
<i>Hspa4l</i>	<i>Eea1</i>	<i>Mcam</i>	<i>Lamc1</i>
<i>Pawr</i>	<i>Agap1</i>	<i>Tjp1</i>	<i>Pam</i>
<i>Lcn2</i>	<i>Six2</i>	<i>P2rx4</i>	<i>Parp1</i>
<i>Auh</i>	<i>Gdnf</i>	<i>Hmga2</i>	<i>Mitf</i>
<i>Lgmn</i>	<i>Scrg1</i>	<i>Efhd2</i>	<i>Csrp1</i>
<i>Coro2b</i>	<i>Pla2g10</i>	<i>Pcdha12</i>	<i>Gstm3</i>
<i>Rin2</i>	<i>Tnfrsf4</i>	<i>Prr15</i>	<i>Arl8b</i>
<i>Meox1</i>	<i>Hsd3b7</i>	<i>Pdlim7</i>	<i>Spock2</i>
<i>Nefh</i>	<i>Zfp28</i>	<i>Ptpn12</i>	<i>Scn7a</i>
<i>Gja1</i>	<i>Dusp8</i>	<i>Pgr</i>	<i>Mrpl17</i>
<i>Yap1</i>	<i>Tm2d1</i>	<i>B3galt4</i>	<i>Cryz</i>
<i>Pdgfra</i>	<i>Frk</i>	<i>Acadvl</i>	<i>Gmcl1</i>
<i>Sfrp2</i>	<i>Dmap1</i>	<i>Apobec2</i>	<i>Gmcl1</i>
<i>Frmd5</i>	<i>Gnptab</i>	<i>Plscr1</i>	<i>Lin9</i>
<i>Inca1</i>	<i>Slc4a8</i>	<i>Ly86</i>	<i>Dpt</i>
<i>Crisp2</i>	<i>Fgf10</i>	<i>Pla2g4a</i>	<i>Wiz</i>
<i>Zranb1</i>	<i>Pax1</i>	<i>Map1s</i>	<i>Kcnab1</i>
<i>Clrn3</i>	<i>Atg2a</i>	<i>Tcta</i>	<i>Atp2a2</i>
<i>Ptk6</i>	<i>Igf1bp1</i>	<i>Txnrd3</i>	<i>Immt</i>
<i>Ercc5</i>	<i>Ercc1</i>	<i>Khk</i>	<i>Shox2</i>
<i>Ercc5</i>	<i>Tnfrsf9</i>	<i>Aloxe3</i>	<i>Nop2</i>
<i>Igf2</i>	<i>Itih4</i>	<i>Scn1a</i>	<i>Evi5</i>
<i>Htra1</i>	<i>Ckmt2</i>	<i>Rhag</i>	<i>Nkiras1</i>
<i>Scand1</i>	<i>Magel2</i>	<i>Eif2ak2</i>	<i>Wnt2b</i>
<i>Ace2</i>	<i>Mtrf1l</i>	<i>Map2</i>	<i>Ssbp2</i>
<i>Trpc6</i>	<i>Cp</i>	<i>Figla</i>	<i>Vamp5</i>
<i>Mdn1</i>	<i>Cacna1g</i>	<i>Spp1</i>	<i>Cpa3</i>
<i>Slc7a11</i>	<i>Ephb6</i>	<i>Ccl2</i>	<i>Cldn11</i>
<i>Zdhhc9</i>	<i>Mrpl48</i>	<i>Slc30a1</i>	<i>Gstm1</i>
<i>Afp</i>	<i>Exosc8</i>	<i>Zkscan3</i>	<i>Ovgp1</i>
<i>Slc6a4</i>	<i>Ifng</i>	<i>Ccdc47</i>	<i>Gzmb</i>
<i>Cmb1</i>	<i>Sub1</i>	<i>Gabrb2</i>	<i>Il10ra</i>
<i>Fjx1</i>	<i>Nqo1</i>	<i>Dppa2</i>	<i>Entpd7</i>
<i>Shmt2</i>	<i>Tgm3</i>	<i>Dock7</i>	<i>Enpp2</i>
<i>Prdm1</i>	<i>Metap2</i>	<i>Cela1</i>	