Investigating the antibody-independent functions of B lymphocytes

Candidate name	Akimichi Inaba
College	Girton College, University of Cambridge
Date of submission	April 2019
Supervisor	Dr Menna Clatworthy, Department of Medicine, University of Cambridge

This dissertation is submitted for the degree of Doctor of Philosophy

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee

_____

Akimichi Inaba

1 April 2019

Summary

Title Investigating the antibody-independent functions of B lymphocytes

Name Akimichi Inaba

B cells are critical for the generation of antibody, but there is increasing evidence that they have a broader functional remit: For example, innate B1a cells are a critical source of granulocyte macrophage colony-stimulating factor (GM-CSF) during Gram-negative sepsis, inducing neutrophil expansion and mobilisation to limit bacteraemia. B cells can also regulate immune responses via the production of cytokines such as IL-10, and this can inhibit deleterious autoimmune and alloimmune responses in murine models.

Here I investigated whether B cells may play a pathogenic role in the sterile inflammation associated with acute kidney injury (AKI), coordinating a systemic response in which neutrophils and inflammatory monocytes are mobilised from the bone marrow to the injured kidney. We found that during murine models of AKI, neutrophils exit the bone marrow and increase in the blood and kidneys. This neutrophil mobilisation was tightly correlated to the severity of AKI. B cells were recruited to the kidney in a CD11b-dependent manner and produced CCL7 to attract inflammatory monocytes. The absence of Siglec-G, an inhibitory receptor expressed in innate B1a cells, exacerbates AKI in murine models. Conversely, concomitant administration of a Siglec-G agonist, sialic acid (Neu5AC), ameliorated AKI and reduced renal neutrophil infiltration. These data suggest that manipulation of innate B cells may be a viable therapeutic strategy in AKI.

In the second part of the thesis, I investigated whether IL-10-producing regulatory B cells could be induced in humans *in vivo*. Transcriptomic analysis demonstrated that IL-10-producing human B cells expressed transcripts of all components of the IL-2 receptor (CD25, CD122 and CD132). We found that surface CD25 was upregulated on a subset of mouse and human B cells following stimulation with toll-like receptor (TLR) agonists and CD40L, rendering these cells receptive to IL-2. The addition of IL-2 to these activated B cells significantly augmented IL-10 production, whilst pro-inflammatory cytokines such as IL-6 and tumour necrosis factor alpha (TNF-α) were unchanged, resulting in a skewing of B cells towards a regulatory phenotype. Consequently, co-culture of IL-2-treated B cells with activated CD4 cells led to a reduction in T cell production of TNF-α. *In vivo*, in mice and patients treated with low dose IL-2, we observed a significant increase in IL-10-producing B cells. Together, our data suggest that low dose IL-2 may be a useful strategy to promote the generation of regulatory B cells *in vivo*.

Acknowledgements

There are a countless number of people without whose kindness this thesis would not have been possible. I would like to thank my colleagues in the Clatworthy lab and in the Laboratory of Molecular Biology including Tomas Castro-Topico, John Ferdinand, John James, Lawrence Lok, Kevin Loudon, Rebeccah Mathews, Alexandra Riding and Kirsten Scott. In addition, the contributions of my collaborators including Natalie Burrows, Mahzuz Karim, Jane Kennet, Lucy Truman, Kourosh Saeb-Parsy, John Todd and Frank Waldron-Lynch have been invaluable for expanding our studies. During the whole of the PhD, my supervisor Menna Clatworthy has been always been consistently enthusiastic and supportive of my project even when the experiments did not appear to be making much progress. My partner, Jenny Ogilvie, has since the beginning of my time in research given me balanced advice and emotional support without which I would not have arrived where I am now.

Most of all, I would like to mention the organ donors and their families. Without recently deceased human organs – which were selflessly donated in times of loss and deep sadness – a large portion of the research presented in this thesis would not have been possible. Thank you.

Table of Contents

Abbreviations

Ab	Antibody
AKI	Acute kidney injury
APC	Antigen-presenting cell
B cell	B lymphocyte cell
B-reg	Regulatory B cell
BAFF	B cell activating factor belonging to the tumour necrosis factor family
BCR	B cell receptor
BM	Bone marrow
BTLA	B And T lymphocyte associated protein
CAT	Catalase
CIA	Collagen-induced arthritis
CD	Cluster of differentiation
CpG	5'-cytosine-phosphate-guanine-3'
CXCL-	C-X-C motif ligand (followed by an identifying number)
DC	Dendritic cell
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
D region	Diversity region
DSS	Dextran sulphate sodium
EAAT1	Excitatory amino acid transporter 1
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FCGR2B	Low affinity immunoglobulin gamma Fc region receptor II-b
fDC	Follicular dendritic cell
FOXP3	Forkhead box P3
GSH-Px	Glutathione peroxidase
GSK3	Glycogen synthase kinase 3
GvHD	Graft-versus-host disease

H&E	Hematoxylin and eosin
IBD	Inflammatory bowel disease
IFN-γ	Interferon γ
Ig	Immunoglobulin
ILC	Innate lymphoid cell
IL-	Interleukin (followed by an identifying number)
IL-2Rα	Interleukin 2 receptor α subunit (CD25)
IL-2Rβ	Interleukin 2 receptor β subunit (CD122)
IL-2Rγ	Interleukin 2 receptor γ subunit (CD132)
IRA	Innate response activator
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITGAM	Integrin Subunit Alpha M
J region	Joining region
LDL	Low density lipoprotein
LT	Lymphotoxin
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
MAC-1	Macrophage-1 antigen
µMt	µ chain transmembrane region
Neu5AC	N-Acetylneuraminic acid
NK	Natural killer
NMDA	N-methyl D-aspartate
PC	Phosphorylcholine
PCR	Polymerase chain reaction
PD-1	Programmed death protein 1
PirB	Paired immunoglobulin-like receptor B
PMA	Phorbol myristate acetate
RA	Rheumatoid arthritis
RNA	Ribonucleic acid

Siglec-g	Sialic acid binding Ig like lectin G
SIGLEC-10	Sialic acid binding Ig like lectin 10
SLC1A3	Solute carrier family 1 member 3 (glial high affinity glutamate transporter)
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
T1	Transitional 1 B cell
T2	Transitional 2 B cell
TGF-β	Tumour growth factor β
Th17	T helper 17 cell
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
T-reg	Regulatory T cell
V region	Variable region
VEGF-A	Vascular endothelial growth factor A

Chapter 1: Introduction

To contextualise current efforts to understand the role of B cells and plasma cells in immune responses and disease, it is worth considering the scientific work of the both named and anonymous pioneers in the field of B cell biology particularly over the past 150 years. Contrary to the timelines of discoveries that imply a clean linear historical narrative, even casual investigation of the discovery of B cells reveals a story full of unintended diversions which plot a meandering path to scientific knowledge. Though within an experimental medical doctoral thesis a rigorous and original history derived from primary sources is unfeasible, a less ambitious epistemological exercise considering how different forces shaped the early scientific knowledge about B cells may provide perspective on how we as research immunologists approach our work today.

The early history of B cells is defined as the history leading up to three major discoveries: the identification of B cells, the identification of plasma cells and the isolation of antibodies. The history presented here, is how an understanding of the *functions* of B cells – and not the B cell in and of itself – was propelled by the search for antitoxins or antibodies that could be life-saving during an infection. The identification of the B cell was a secondary priority. Even after speculation that B cells existed in the early twentieth century, there is no evidence that a concerted effort to search for the antibody's source ever existed. Indeed, I show that in order for the discovery of B cells to take place, it was necessary for the intellectual atmosphere to change from one driven by the pragmatic needs of saving lives to

one in which science could be conducted in order to provide a better understanding of the basic tenets of biology.

1.1 Inoculation and the early history of B cells

While there is a rich history of the study of immunisation and therefore a study of the functional consequence of B cells before the nineteenth century, these discoveries will be only briefly considered here. Smallpox, a disease which has at least a 16,000 year-long history was by the 18th century killing 400,000 Europeans annually (Esposito, et al. 2006) (Hays 2005).

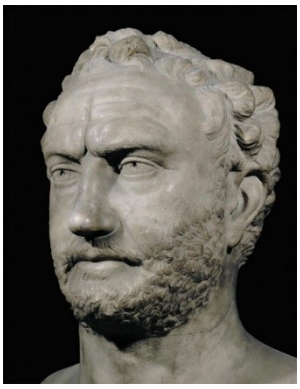


Figure 1.1 (left): Bust of Thucydides, Louvre Museum, Paris, France (Artist and date of artwork unknown). Figure 1.2 (right). *Plague of Athens* (as reimagined in the 17th century) by Michiel Sweerts (1618-1664) (The Plague of Athens, Michiel Sweerts 1652-1654).

A preventative treatment for the disease was documented as early as 429 BCE, by the Athenian historian and general Thucydides (*circa* 460-400 BCE, figure 1.1) who noted in his book, *History of the Peloponnesian War,* that during the Plague of Athens (430-427 BCE, figures 1.2 and 1.3) those Athenians who survived the disease once were unlikely to acquire smallpox again, marking perhaps the very first mention, consciously or not, of adaptive immunity:

> "Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice- never at least fatally. And such persons not only received the congratulations of others, but themselves also, in the elation of the moment, half entertained the vain hope that they were for the future safe from any disease whatsoever." (Thucydides 431 BCE)

Subsequently, a more directed therapy called variolation was developed. Variolation derives its name from the variola family of viruses which contains the smallpox virus. This inoculation which involved either exposing skin to scabs or insufflating scabs from smallpox patients became widely practiced from about 1000 AD first in India and subsequently in China, Western Asia and Africa (Macgowan 1884) (Needham 1980). The practice eventually reached Europe and North America in the 18th century. In England, Mary Wortley Montagu (1689-1762) reputedly imported variolation following her family's diplomatic deployment to Constatinople. A development on this was illustrated by Edward Jenner (1749-1823, figure 1.3) along with a number of other lesser known contemporaries such as Jobst Bose in Göttingen, Germany, Benjamin Jesty (circa 1736-1816) in Dorset, England and others who noted that exposure to cowpox, which gives a relatively mild viral disease in humans, conferred protection from subsequent exposure to smallpox.

Figure 1.3. Edward Jenner conducting experiments into smallpox. (Artist and date of artwork unknown).

1.2 Speculation on the existence of B cells

It is possible, perhaps somewhat arbitrarily, to demarcate the formal start of the search for B cells with the genesis of the documented *idea* of a B cell which was posited as early as 1908 by Paul Ehrlich (1854-1915). At the end of the nineteenth century, the German physician Emil von Behring (1854-1917, figure 1.4) and the Japanese physician Kitasato Shibasaburō (1853-1931) hypothesized the existence of 'antitoxin,' or what later became known as antibodies in response to bodily exposure to filtrates of diphtheria culture containing no bacterial cells. Ehrlich went on to hypothesize in his Nobel lecture " . . . I believe that I may now pronounce it as a definite fact that certain specific groupings must in fact exist in the cells which fix the poison." (Ehrlich 1967). The question then remained how these cells could be identified.

Figure 1.4: Emil von Behring (far right) experimenting with immunisation of a horse (Artist unknown 1894).

1.3 Early developments in antibodies

Arne Tiselius (1902-1971), a Swedish biochemist, initially developed the technique of protein electrophoresis in a buffer in 1930 and subsequently with his colleague Elvin A Kabat (1914-2000) showed that the gamma fraction of serum had within it large amounts of

immunonoglobulins (Black 1997) (Tiselius and Kabat 1939). This was the first antibody to be isolated.

1.4 Identification of B cells

First described by Hieronymus Fabricius (1537-1619) of Padua, the Bursa of Fabricius, is an outpouching of the proctodaeum, which is located in the back ectodermal segment of the alimentary canal of a hen (figures 1.5 and 1.6). The function of this bursa was not completely clear at that time.

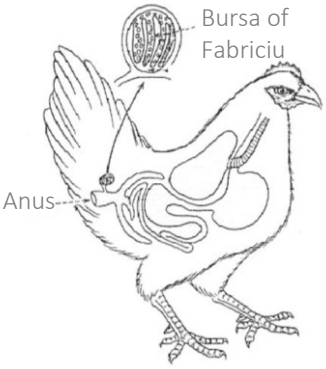


Figure 1.5 (left). Engraving of Hieronymus Fabricius (Artist and date of artwork unknown). Figure 1.6 (right). Diagram of position of bursa of Fabricius (Sivakumar 2018).

Little progress was made on the function of this structure, though some hypothesized an endocrine function (Perek and Eilat 1960) (Newcomer and Connally 1960). Almost four centuries after Fabricius's initial descriptions, in 1956, a post-graduate student named Bruce Glick (1927-2009, figure 1.7) at the University of Ohio surgically removed the bursa of newly-hatched white leghorn chicks to investigate their phenotype. Crucially, the decision to do so was not based on a strong hypothesis of the function of the structure. There was no evidence of suspicion the organ was responsible for antibody production. When Glick observed no outward differences, he returned the birds to the farm where Timothy Scott Chang (1925-2011, figure 1.8) another student from the same laboratory used them along with other non-

bursectomised hens for an immunisation demonstration with *salmonella*-type O. A number of bursectomised birds died and the blood of the surviving birds showed no agglutination which would have been suggestive of effective immunisation. Disappointment was followed by the realisation that cells within the bursa may be responsible for antibody production.



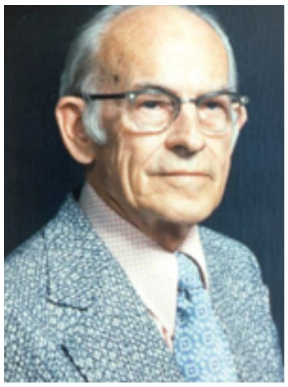


Figure 1.7 (left). Bruce Glick pictured with the subject of his life-long research (Kincade 2003). Figure 1.8 (middle). Timothy Scott Chang (American Association of Avian Pathologists 2018). Figure 1.9 (right). George Japp (American Poultry Historical Society, Inc. 2013).

With excitement, Glick and Chang's supervisor, George Jaap (1905-1992, figure 1.9) suggested the work, "The Role of the Bursa of Fabricius in Antibody Production" be submitted to the journal *Science*. The article was turned down because of the lack of mechanistic understanding (Davison 2012) (Glick 1987). Eventually the work was published in *Poultry Science* where it remained largely forgotten until the mid-1960s when there was an acceleration in interest in antibody biochemistry (Glick, Chang and Jaap 1956).

Glick and Chang's discovery was fortuitous because gallinaceous birds such as chickens, turkeys, pheasants, grouse, partridges, and quails have a discrete organ which contains the majority of their B cell population. Such a discovery would not have been possible in mammals such as mice in which migratory B cells are scattered in the bone marrow and secondary lymphoid organs such as spleen and lymph nodes.

1.5 Identification of plasma cells

Astrid Fagraeus (1913-1997, figure 1.10) observed the histological features of a rabbit spleen before and after antigen immunisation. The changes were subtle but the increase of plasma cells in the red pulp suggested these cells may be the source of antibodies (Fagraeus 1948). *In vitro* assays measuring supernatant immunoglobulins of carefully dissected splenic tissue suggested the red pulp to indeed be the source of antibodies. Subsequently, antibody production was confirmed in these cells in one of the first descriptions of immunofluorescent microscopy (figure 1.11) (Coons, Leduc and Connolly 1955).

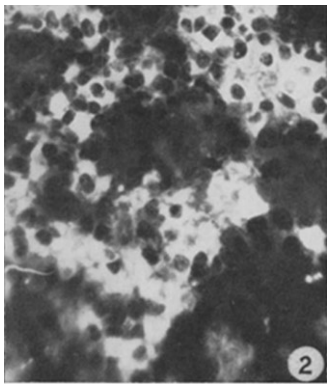
A  B 

Figure 1.10 (left). A. Astrid Fagraeus (Norberg, Biberfeld and Wigzell 1998). Figure 1.11 (right). Originally published microscopy image image of "Rabbit . . . Spleen. Stained for anti-human γ-globulin. Higher power of similar islands of cells in the red pulp. X 560" (Coons, Leduc and Connolly 1955).

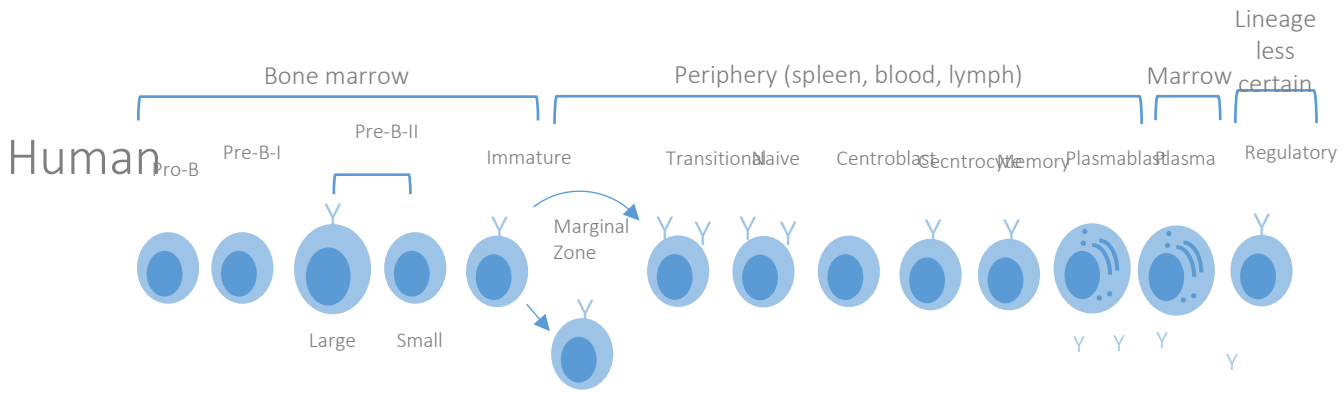
1.6 Current understanding of the development of B cells

Following these early discoveries, the developments in understanding of B cells have accelerated greatly since the 1960s. Presented here is a basic review of our understanding of B cells largely from a developmental perspective. For further historical perspectives of more recent discoveries, see Edward Moticka's *A Historical Perspective on Evidence-Based Immunology* (Moticka 2015).

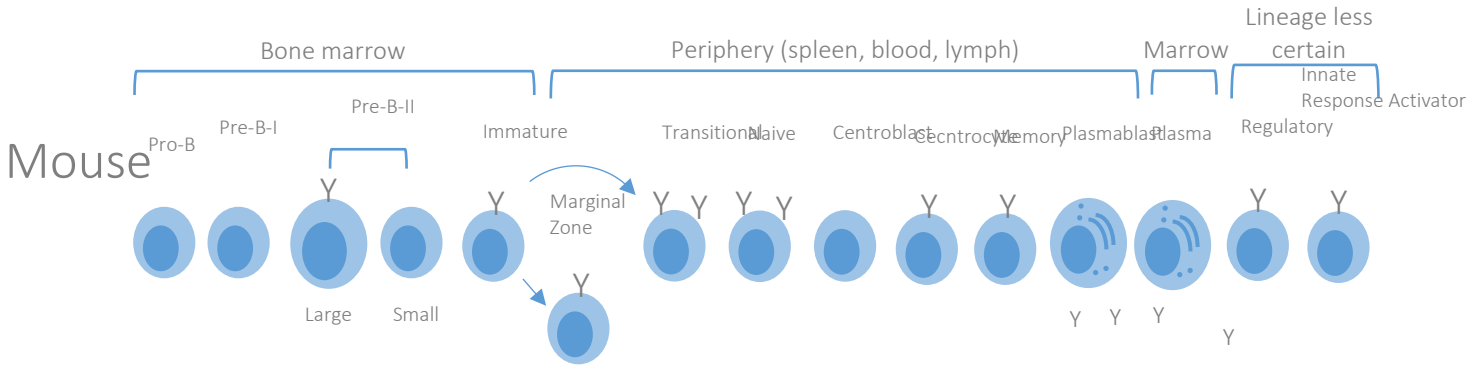
B cells, also known as B lymphocytes, are a type of white blood cell which develop in the bone marrow from haematopoietic stem cells via a common lymphoid progenitor. Other

cells with a lymphoid lineage include T cells, natural killer cells and innate lymphoid cells. B cells, unlike other lymphoid cells however, are unique in their expression of a B cell receptor (BCR), a surface bound antibody (Ab) or immunoglobulin (Ig). In addition to the BCR, B cells also transiently express a range of surface markers such as CD10, CD19, CD20, CD25, and B220 which reflect the cell's developmental stage, activation status and function (Tables 1.1 and 1.2). The primary function of a B cell has been defined as a precursor to Ab-producing plasma cells. However, as explored later, this perception of B cells has undergone revision lately given the wide range of additional functions that B cells also exhibit.

Tables 1.1 and 1.2 (over the next two pages). Human and murine B cell surface markers during development. Where I have found no evidence of a surface marker's expression on certain B cells, the box has simple been left blank.



BAFFR					+	+	+	+	+	+	+			
BCMA											+	+	+	
CD1c						+								hi
CD1d														+
CD5							+							
CD9														+
CD10	+	+	+	+	+		lo							
CD19	+	+	+	+	+	+	+	+	+	+	+	+	lo	+
CD20		+	+	+	+	+	+	+	+	+	+	-	-	
CD21					+	hi	+							+
CD24	+	+	+	+	hi		hi						Lo	hi
CD25														var
CD27					-	var	-	-			+	hi	+	var
CD34		-	-	-	hi									
CD38					hi		hi		hi	hi		hi	hI	
CD40					+									
CD138												-	+	
CD269												+	+	
ckit	lo	-	-	-	-									
CXCR4													+	
Flt3	lo	-	-	-	-	-	-	-	-	-	-	-	-	
IgA									-	-	+	+		
IgD					-		lo						-	var
IgE												+		
IgG									-	-	+	+		
IgM	-	-	-	+	+		hi		-	-	+	+	-	hi
IL3R	+	+	+	+										
IL4R		+	+	+										
IL7R	+	+	+	+	-									
MHCII												+	-	
PDL1														+
TACI											+	+	+	



B220	+	+	+	+	+	+	+	+	+	+	+	lo	lo	+	+
CD5															
CD1d						+								Hi	
CD5														+	
CD9						+								+	
CD19	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
CD21						hi	var							var	
CD22						hi									
CD23						-	var							var	lo
CD24	+	+					+							-	
CD25		var	var	var										var	
CD38													lo		
CD35						hi									
CD43	+	lo	lo	lo	-										
CD93					+	-	+							var	
CD138												+	+		
ckit	lo														
CXCR4	+											+	hi		
FAS									+	+					
Flt3	+														
GL7									+	+					
IgA												+	-		
IgD					lo	Lo							-	var	
IgE												+	-		
IgG												+	-		
IgM	-	-	-	-	+	hi	hi					+	-	hi	hi
IL7R	+	+	+												
MHCII													+		+
PDL1															
PNA									+	+					
TIM														+	

1.7 B cell development in the bone marrow

Stages of B cell development have traditionally been aligned to stages of recombination of the BCR gene and therefore it is appropriate to briefly discuss Ig before describing B cell development. Ig consist of two heavy chains and two light chains. Heavy chains are transcribed from genes independent of light chains which are transcribed from either kappa (κ) or lambda (λ) genes. The heavy chain is transcribed from three gene regions: the variable (V) region, the diversity (D) region and the joining (J) region. The nature of the heavy chain determines antibody isotype (figure 1.12). For example, a μ heavy chain is associated with IgM while a γ heavy chain is associated with IgG. The light chain consists of two gene regions: the V and J regions which can either be kappa or lambda. In humans, there are 65 different heavy chain V gene segments, 27 heavy chain D segments and 6 joining heavy chain J segments (figure 1.14). Multiple gene segments encoding for the light chain also exist.

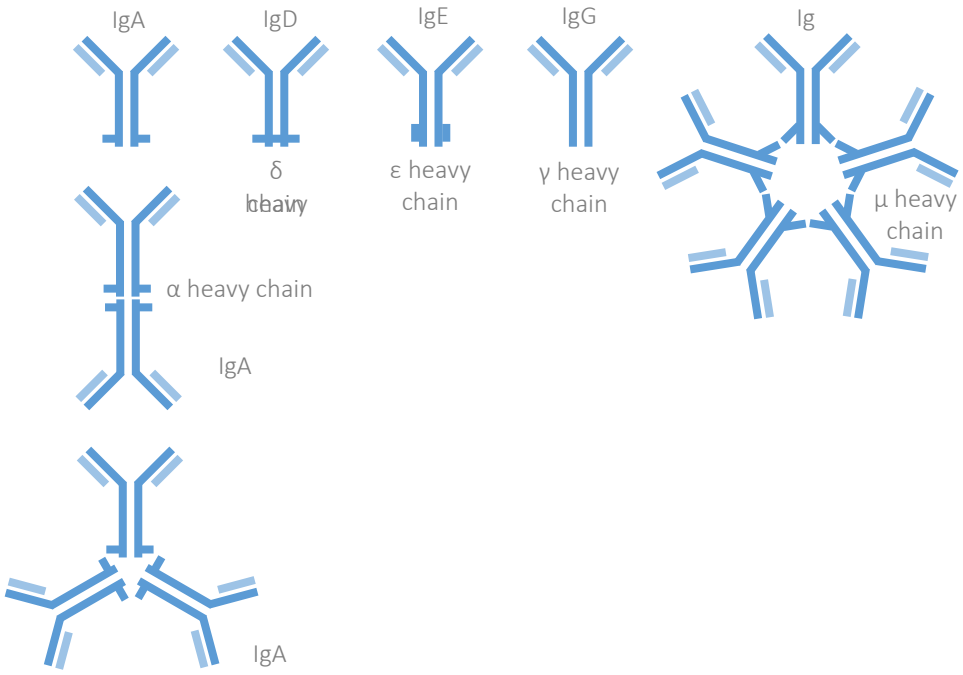


Figure 1.12: The type of heavy chain determines the antibody isotype.

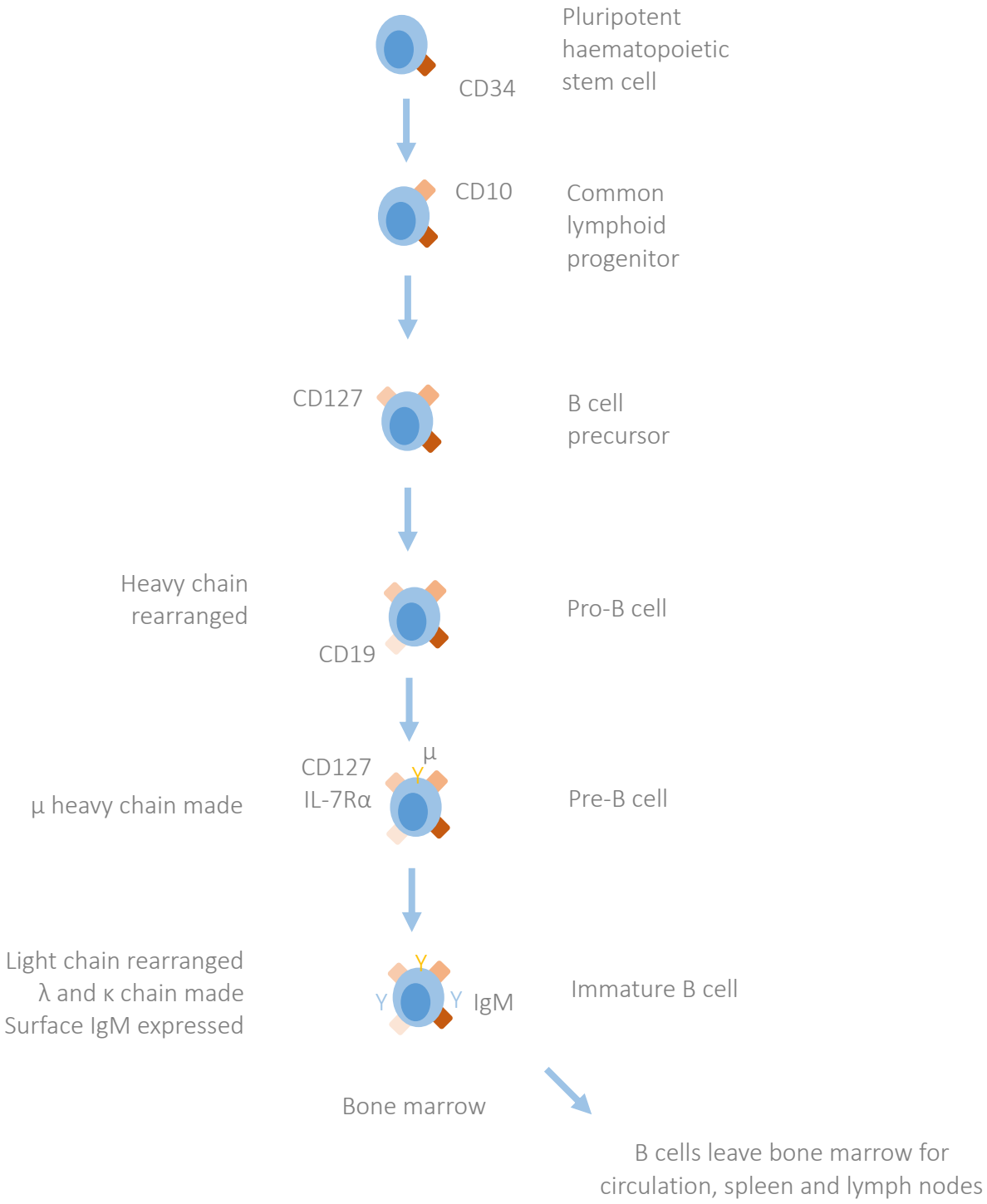


Figure 1.13. Stages of B cell development in the bone marrow. B cells arise from common lymphoid progenitors and proceed through a series of distinct developmental stages, as indicated. They finally emerge from the bone marrow

as mature naïve B cells and circulate in the blood to spleen and lymph nodes. Figure modified from (Parnham 2014).

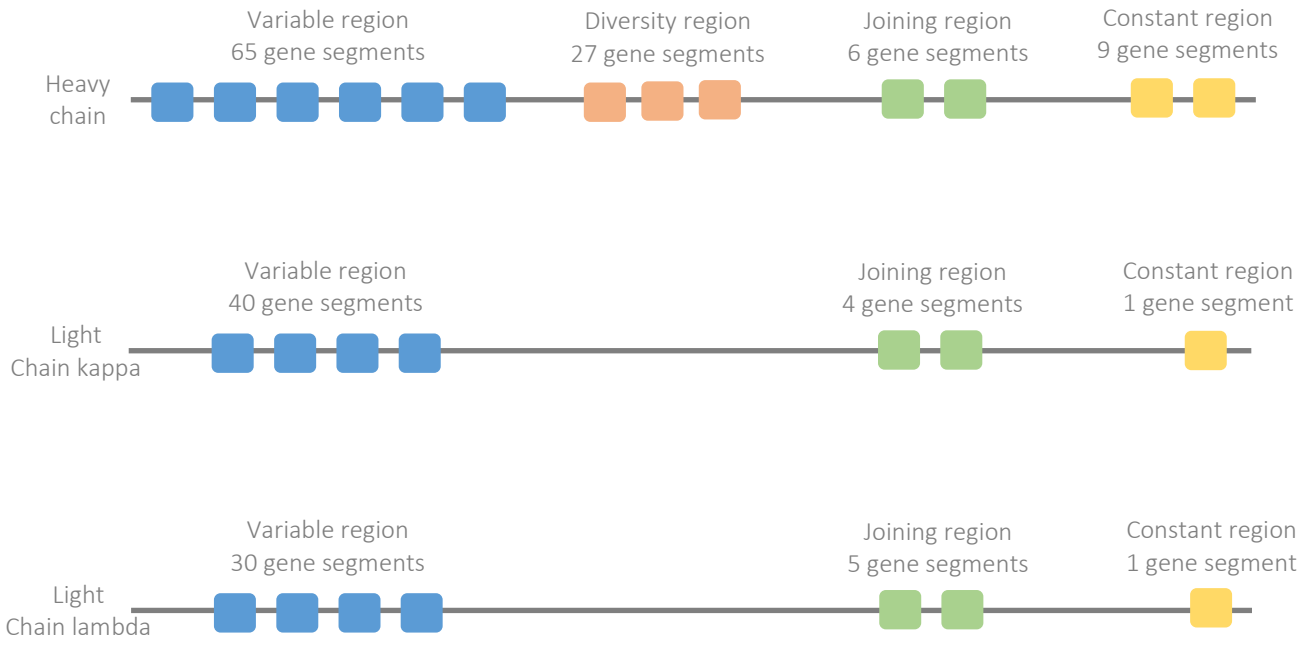


Figure 1.14: Variable, diversity, joining and constant regions of heavy and light chains. Figure modified from (Parnham 2014).

Rearrangements of genetic material encoding the V, D and J regions of the heavy chain are called VDJ recombination (figure 1.14). The first recombination to take place is between the D and J regions followed by the V region. Non-relevant V, D and J regions are deleted from the genome during this process. Subsequently, an equivalent V-J recombination of the light chain genes also takes place. The light chain does not have a D domain. The genes are transcribed and the assembly of the heavy and lights chains takes place in the cytoplasm.

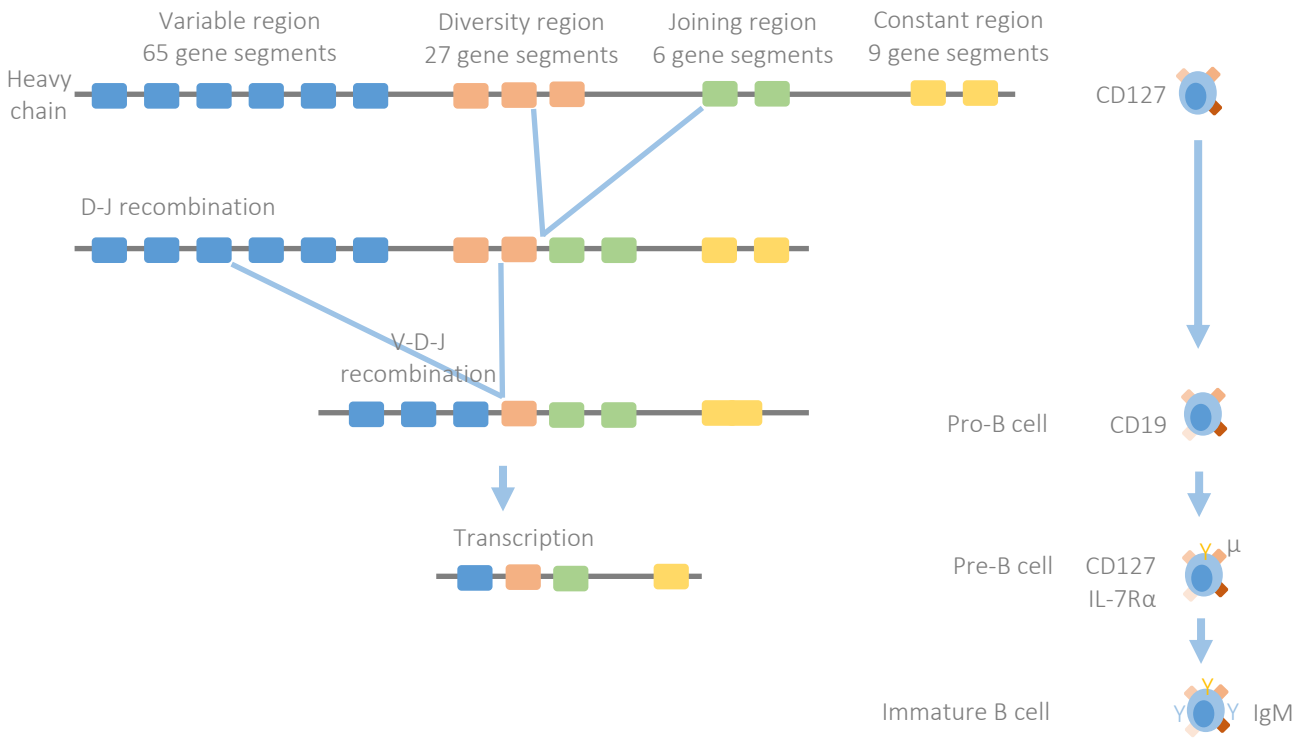


Figure 1.15: Schematic diagram of VDJ recombination when gene segment are randomly assembled during the pro-B cell phase. Figure modified from (Parnham 2014).

In most mammals, including mice and humans, B cells originate in the bone marrow. Here, pluripotent haematopoietic stem cells develop, in chronological order, into a common lymphoid progenitor, a B cell precursor and eventually a pro-B cell when rearrangements of genes encoding for the heavy chain of Ig take place (figure 1.15). Following the pro-B cell phase, the pre-B cell phase consists of the large pre-B cell phase, when μ heavy chains are first made, and the latter small pre-B cell phase when VJ rearrangement of the light chain genes first take place. The final stage of development in the bone marrow is the immature B cell phase when genes for the light chains κ and λ are rearranged and made. During this phase light and heavy chain proteins are made and it is possible for the first time to synthesize these two proteins to form a surface IgM (figure 1.15).

1.8 B cell maturation

Immature B cells leave the bone marrow via the bloodstream and arrive in the spleen for further maturation. During the transit from the marrow to the spleen, B cells are termed transitional 1 (T1) B cells and express high levels of CD24 and CD38 in humans and CD21 and 23 in mice. Engagement of the BCR induces apoptosis during this time. Once in the spleen, BAFF signalling (B cell activating factor belonging to the tumour necrosis factor family) induces B cells to develop from a T1 phenotype to a T2 phenotype. T2 cells go on to differentiate in follicles into circulating follicular B cells or into non-circulating B cells in the marginal zone (MZ). Within the follicles, follicular dendritic cells (FDC) are crucial in retaining B cells through production of B cell lymphocyte chemoattractant (BLC), also known as C-X-C motif ligand 13 (CXCL13). Upon exposure to antigen, follicular B cells can migrate to the follicular borders and present peptides to T cells in the context of MHC. In return, B cells are also stimulated through CD40 upon receipt of T cell help. B cells return to the follicle to form a germinal centre (GC) within which B cells undergo somatic hypermutation in an attempt to produce antibody with a higher affinity for antigen (Di Noia and Neuberger 2007). They also undergo class switching, so that the heavy chain present in antibody changes from μ to one of the other isotypes. During many rounds of division and hypermutation, B cells with a high affinity BCR are positively selected and differentiate into either memory B cells or plasma cells (Goodnow, et al. 2010). A subset of CD4 T cells, known as T follicular helper (Tfh) cells, are engaged by GC B cells presenting antigen to them. This Tfh-B cell interaction is essential for the progress of the GC reaction and for the development of memory B cells and plasma cells (Vinuesa, et al. 2010).

Only a small proportion of plasma cells arising from the GC become established as long-lived plasma cells in the bone marrow. They reside within a number of limited niches, do not proliferate, but act as long-term antibody factories, producing IgG (Nutt, et al. 2015). Plasma cells have also been described in inflamed tissues in autoimmunity and within allografts (Cassese et al., 2001; Kerjaschki et al., 2004; Thaunat et al., 2005; Wehner et al., 2010).

1.9 B1 cells

Conventional B cells, which we have discussed in previous paragraphs are classified as B2 cells. Another distinct category of B cells, B1 cells, was first described in leukemic cells where it was noted that some B cells expressed CD5, a protein more commonly expressed in T cells (Lanier, et al. 1981) (Wang, et al. 1980). Subsequently, CD5 expression was found on normal B cells (Hardy, et al. 1982) (Manohar, et al. 1982). While B1 cells are found to reside in the peritoneal cavity, pleural cavity, spleen and bone marrow under normal circumstances, their origin remains controversial (Hayakawa, Hardy and Herzenberg 1986) (Hayakawa, Hardy and Parks, et al. 1983). Adoptive transfer of bone marrow cells does not replenish B1 cells in the peritoneum whereas fetal liver cells do. These experiments have not convincingly answered exactly the specific fetal liver cell type which gives rise to the B1 cell population (Kantor, et al. 1992). There has also been suggestion that B1 cells arise from the yolk sac and para-aortic splanchnopleural tissues (Yoshimoto et al., 2011). Finally, complicating this question has been the suggestion that B2 cells can be induced to take on more B1 cell characteristics (Berland and Wortis 2002).

The functions of B1 cells are threefold. First, they are producers of natural Ig which are typically polyreactive IgM with modest affinity towards phosphorylcholine found on gram positive microbes such as *streptococcus pneumoniae*. These Ig were formed without antigen exposure through infection or immunisation. As much as 90% of serum IgM during health is produced by B1 cells (Sidman, et al. 1986). Not only do natural Ig recognise potential microbial threats, they can also promptly sense apoptosing cells which may be necessary to prevent an overwhelming autoimmunity during normal cell death (Chen, et al. 2009). These natural antibodies have been observed to suppress TNF-α and IL-6 by macrophages during LPS stimulation (Chen, et al. 2009). Finally, more recent literature suggests that within the B1 cell family, there is a group of GMCSF-producing cells found predominantly in the spleen that are crucial for protection against intraabdominal and respiratory microbial infections (Rauch, et al. 2012).

The presence of B1 cells in humans has also been confirmed using a “reverse engineering strategy”. Rather than identifying human B1 cells using cell surface markers common between mice and humans, B1 cells were identified by their functional profile: B

cells which produce large amounts of IgM, are efficient at T cell stimulation and have chronic intracellular signalling (Rothstein, et al. 2013). Using this criteria, a number of papers have identified human $CD20^{+}$ $CD27^{+}$ $CD43^{+}$ B cells as most resembling the murine B1 cell population (Griffin, Holodick and Rothstein 2011) (Griffin, Holodick and Rothstein 2011). The exact proportion of B cells which can be defined as B1 is debatable as CD27 and CD43 do not give discrete cell populations on cytometric analysis, however, estimates range from one to nine percent of total B cell populations (Descatoire, et al. 2011) (Verbinnen, et al. 2012). There has been suggestion that over time, in humans, total B1 cell numbers decline. Currently, their physiological relevance in humans remains uncertain, though it has been speculated that natural antibodies against antigens such as phosphorylcholine (PC), low density lipoprotein (LDL), tau and amyloid may have beneficial effect in warding off age-related illness such atherosclerosis and dementia.

B1 cells in humans have also been described to have regulatory capacity through production of IL-10. For example, Griffin *et al* describe a $CD11b^{+}$ population of B1 cells which spontaneously produce IL-10 and suppress TNFα production in activated CD4 T cells (Griffin and Rothstein 2012).

1.10 BCR signalling

The BCR has two primary functions: it recognises cognate antigen and commences a cascade of intracellular signalling; it is also able to internalise antigen which can subsequently be presented to a helper T cell. The surface Ig has no intrinsic signalling capacity but associates with a number of other proteins. The Igα and Igβ (CD79a and CD79b) form heterodimer to mediate signal transduction to activate a range of the proteins including Lyn, Blk Fyn, Syk and Btk kinases. During BCR signalling, a number of concomitant factors determine the cellular fates including survival, anergy, apoptosis, proliferation or progression to a plasma cell. These factors include the strength and duration of the BCR signal itself as well as signals from other receptors such as CD40, IL-21 receptor and BAFF-receptor. In addition, inhibitory proteins such as FcγRIIb, CD22 and SIGLEC-10 limit the strength of BCR signalling via recruitment of phosphatases that de-phosphorylate kinases.

1.11 Antibody and antibody-independent functions of B cells

The B cell's primary function was traditionally thought to be antibody production. However, the B cell mediates a number of other functions including antigen presentation to CD4 T cells and the initiation of cellular immunity. B cells can also orchestrate lymphoid tissue formation by secreting molecules such as lymphotoxin (LT). Finally, B cells produce cytokines necessary for activation and regulation of innate and adaptive immune processes (figure 1.16).

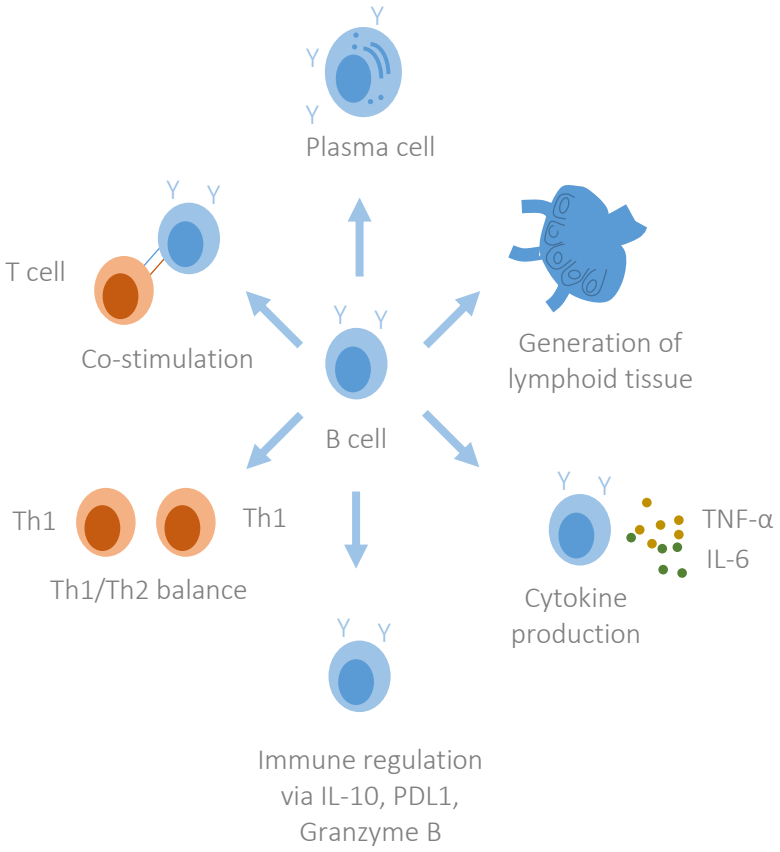


Figure 1.16. Schematic diagram of the multiple functions of B cells in addition to its primary function as an antibody producer. Figure modified from (LeBien and Tedder 2008).

1.11.1 Antibody production

Most abs are secreted into the circulation by terminally differentiated B2 cells termed plasma cells. Abs are found in the circulation, lymphatics and mucosal surfaces where they can bind

to proteins, carbohydrates and other biological macromolecules primarily found on bacteria and viruses. Abs recognise molecular shapes, or epitopes, via their variable region potentially neutralising the pathogens or toxins. Abs may also initiate an effector response by activating the complement cascade and ligating Fc receptors found on the surface of immune cells (Joller, Weber and Oxenius 2011) (Krause, Dimmock and Morens 1997).

In terms of diversity, it is possible to generate in the order of 10^{16} different antibodies specific to as many antigens. However, this antibody repertoire practically is limited to at most 10^{9} antigens, presumably as it would be physically infeasible to carry any more plasma cell clones. Antibodies directed at self-antigens, or autoantibodies, have been identified as a culprit in a number of autoimmune diseases, for example, in systemic lupus erythematosus where autoantibodies can inappropriately target a range of tissue antigens.

The precise factors that support the survival of plasma cells remain controversial. Antigen exposure however, at its simplest level, does not seem to induce survival (Manz, et al. 1998), consistent with the fact that plasma cells do not express a surface antigen receptor (BCR), unlike their B cell precursors. Previously, a number of cytokines and chemokines have been described to provide a plasma cell survival 'niche', that can be produced by a variety of cells (Moser, et al. 2006): These include A proliferation-inducing ligand (APRIL) (Belnoue, et al. 2007), IL-6 (Mohr, et al. 2009) requiring the presence of nearby stromal cells, eosinophils, macrophages and neutrophils (Chu, et al. 2014).

We currently have some, but probably incomplete knowledge of the factors which support the survival of plasma cells. Antigen exposure however at its most simple level does not seem to induce survival (Manz, et al. 1998). Previously a number of factors have been described to promote a survival 'niche' (Moser, et al. 2006). These include A proliferation-inducing ligand (APRIL) (Belnoue, et al. 2007), IL-6 (Mohr, et al. 2009) and the presence of nearby eosinophils (Chu, et al. 2014).

1.11.2 Antigen presentation to CD4 T cells

For a CD4 T cell to generate an adaptive immune response, it must be activated by antigen presented in the context of an MHC class II molecule along with co-stimulatory signals. B cells are potent antigen presenting cells (APCs) and bind antigen via the BCR which is internalised

by receptor-mediated endocytosis when the cell membrane inwardly buds to create a vesicle containing both the receptor and the antigen. The antigen is partially degraded within the acidic vesicle and other vesicles containing major histocompatibility complex (MHC) class II fuse to antigen-containing vesicles. The vesicle then travels to the cell surface where the antigen can be presented to a helper CD4 T cell. B cells are particularly effective APCs due to their high affinity antigen receptor, the BCR, and their ability to clonally proliferate. Unsurprisingly then, B cell depletion therapies have been effective in diseases which were thought to be largely mediated by T cell such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes mellitus (Claes, et al. 2015) (Edwards and Cambridge 2006) (Hinman, Smith and Cambier 2014).

1.11.3 Generation and maintenance of secondary lymphoid tissue

B cells produce LT which shape the architecture of lymph nodes and spleen (Golovkina, et al. 1999) (Gonzalez , et al. 1998) (Ngo, Cornall and Cyster 2001). The B cells' ongoing production of LTα1β2 is also required for the maintenance of subcapsular sinus macrophages that form a protective screen at the perimeter of lymph nodes (Moseman et al., 2012). B cell production of vascular endothelial growth factor A (VEGF-A) may also promote intranodal lymphangiogenesis and increase antigen and DC distribution within the lymph nodes (Angeli et al., 2006). B cells may also be involved in the generation of tertiary lymphoid structures that emerge in inflamed organs affected by autoimmunity, for example in Sjogren's disease, rheumatoid arthritis, or type 1 diabetes mellitus (Drayton , et al. 2006) (Luther, et al. 2000). These tertiary lymphoid structures appear to be a source of autoantibodies (Cassese, et al. 2001) (Espeli, et al. 2011).

1.11.4 Production of pro-inflammatory cytokines

B cells have the capacity to produce cytokines following stimulation, and can impact a variety of immune cells (Shen and Fillatreau 2015). IL-6 is a cytokine required for T cell dependent antibody responses (Kopf, et al. 1994). B cells can produce this cytokine (Barr, Brown, et al. 2010) which may contribute to autoimmune pathology, for example, via T-helper 17 (Th17) cell activation leading to exacerbation autoimmunity such as EAE (Barr, Shen, et al. 2012).

Indeed, B cells from patients with multiple sclerosis also demonstrate increased IL-6 production compared with healthy controls (Barr, Shen, et al. 2012). B cell production of tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) can also promote Th1 T cells (Menard, et al. 2007) and promote macrophage activation (Bao and Cao 2014).

More recently, innate response activator (IRA) B cells have been described. These splenic IgM-positive B cells can produce GM-CSF in response to lipopolysaccharide (LPS) and in turn mobilise neutrophils (Rauch, et al. 2012). The cell type contributes significantly to pathogen containment in mouse models of sepsis (Weber, et al. 2014) but may exacerbate atherosclerosis via Th1 cell activation (Hilgendorf , et al. 2014). These B cells are thought to originate from B1 B cells, a subset found in higher numbers in peritoneal and pleural cavities (Griffin, Holodick, & Rothstein, 2011; Martin & Kearney, 2001).

1.12 Acute kidney injury (AKI) and immune cells

In the context of clinical renal medicine, AKI remains a common condition which can have serious long-term consequences. Modulating the immune response has emerged as a potential strategy for the treatment of AKI over the past decade, however there has been relatively little research into the role of B cells in AKI. In this section, I summarise a number of disparate strands of research which are the basis of the experiments described in Chapter 2.

1.12.1 AKI definition, classification and prognostication

AKI, previously called acute renal failure, is the sudden decline in kidney function that disturbs metabolic, electrolyte and fluid volume balance (Oleska and Bagshaw 2014). The causes of AKI are varied and often multifactorial, however, intravascular volume depletion, sepsis and nephrotoxic drugs are the most common causes.

There are at least 30 different published sets of clinical criteria defining AKI, however, the RIFLE criteria (Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease), AKIN criteria (Acute Kidney Injury Network) and more recently the KDIGO criteria (Kidney Disease: Improving Global Outcomes), which amalgamates the RIFLE and AKIN, are now used by the nephrology community (Table 1.3) (Hoste 2008). These classification systems are used

while recognising that serum creatinine represents a late biomarker lagging many hours behind initial renal insult.

Stage	Serum creatinine	Urine output
1	1.5–1.9 x baseline OR ≥0.3 mg/dl (≥26.5 umol/l) increase	<0.5 ml/kg/h for 6–12 hours
2	2.0–2.9 times baseline	<0.5 ml/kg/h for ≥12 hours
3	3.0 times baseline OR Increase in serum creatinine to ≥4.0 mg/dl (≥353.6 mmol/l) OR Initiation of renal replacement therapy OR In patients <18 years, decrease in eGFR to o35 ml/min per 1.73 m2	<0.3 ml/kg/h for ≥24 hours OR Anuria for ≥12 hours

Table 1.3. KDIGO AKI criteria (KDIGO 2012)

Largely incontrovertible, however, is that severity of AKI is directly associated to mortality though this link is not necessarily causal (Chertow, Burdick, et al. 2005).

Progression of AKI to chronic kidney disease (CKD) was first described in the 1960s and there is now a wealth of data to show a strong association (Briggs, et al. 1967) (Chawla, et al. 2014) (Leung, Tonelli and James 2012) (Venkatachalam, et al. 2010) (Lo, Go and Chertow 2009) (Bucaloiu, et al. 2012) (Varrier, Forni and Ostermann 2015). The question that has never been fully answered, however, is whether a treatment for AKI can confer long-term protection from CKD. One obvious reason for the lack of such a study is that there has never been a widely available directed therapy for AKI.

1.12.2 AKI epidemiology and cost

The incidence of AKI is common among hospital in-patients. The largest known meta-study compiling data from 312 studies involving approximately 49 million patients predominantly from high-income countries estimated mean incidence of AKI among hospital in-patients to be 21.6% in adults and 33.7% in children (Susantitaphong, et al. 2013). While there is no doubt that a sizeable portion of hospital patients suffer AKI, whether the incidence has changed over time remains very difficult to ascertain. Monitoring of patients in hospital has become more intensive, therefore more likely to detect AKI. At the same time, modern treatments, such as transplantation or coronary angioplasty may themselves cause increased incidence of AKI.

In the US where cost of medical care is often arbitrarily exorbitant, one recent study places the increase in cost of having AKI as $7933 per patient (Silver, Long, et al. 2017). In the UK, in one often-quoted paper, it has been estimated that the annual hospital-related cost to the National Health Service of AKI is approximately one billion pounds or slightly more than 1% of the overall annual NHS budget (Kerr, et al. 2014). While these costs vary considerably between studies (Table 1.4), what is clear is that AKI and in particular dialysis adds on considerable cost to hospital care.

Study	Country	Cost
(Hamel, et al. 1997)	United States	Median in-hospital cost: With dialysis US $31,991
(Chertow, Burdick, et al., Acute kidney injury, mortality, length of stay, and costs in hospitalized patients 2005)	United States	Mean increase in cost: KDIGO 1: US $5,510 KDIGO 2: US $8,999
(Manns, et al. 2003)	Canada	Mean in-hospital cost: No dialysis: US $47,694 With dialysis: US $56,035
(Fischer, et al. 2005)	US	Median in-hospital cost: All AKI: US $2,600 With dialysis: $4,300
(Dasta, et al. 2008)	US	Median increase in cost:

		RIFLE R US $11,234 RIFLE I US $20,461 RIFLE F US $34,155
(Laukkanen, et al. 2013)	Finland	Mean in-hospital cost: With dialysis: US $28,527
(Hobson, et al. 2015)	US	Mean increase in cost: RIFLE R US $10,700 RIFLE I US $15,200 RIFLE F US $38,200
(Zeng, et al. 2014)	US	Mean increase in cost: KDIGO 1 US $5,400 KDIGO 2 US $15,200 KDIGO 3 US $27,300
(Kerr, et al. 2014)	UK	Total cost to UK National Health Service: UK £1,020,000,000 per annum
(Silver, Long, et al. 2017)	US	Mean increase in cost: All AKI: $1,795 With dialysis: $11,016

Table 1.4 Previously published papers on the financial cost of AKI (modified from (Silver and Chertow 2017)).

1.12.3 Immune cells and AKI

One approach to treat AKI has been to modulate the immune response seen in AKI. Virtually all types of immune cells have been implicated in the condition, often simplistically with pro- and others with anti-inflammatory and repairing effects (Linfert, Chowdhry and Rabb 2009) (Bonavia and Singbartl 2017) (Jang and Rabb 2015) (Lee, et al. 2017).

1.12.4 Neutrophils

Neutrophils are trafficked to the kidneys during AKI and the mechanism by which these cells marginate to the vascular endothelium and eventually enter the renal interstitium during sterile inflammation has been investigated in a number of studies (Awad, et al. 2009) (Bolisetty and Agarwal 2009). What actually causes neutrophils to mobilise in the first place

from neutrophil reservoirs remains unclear (Sokol and Luster 2015). In general, though there have been some exceptions, the inhibition of neutrophil endothelial adhesion and activation or the depletion of neutrophils have led to lessening of the severity of AKI in animal models.

One of the earliest studies of immune responses in AKI showed that in rabbits and rats, there was no difference in outcome following administration of monoclonal antibody clone 60.3 against CD18 which prevents neutrophil adhesion to the endothelium or an anti-neutrophil serum to reduce neutrophil numbers to negligible levels (Thornton, et al. 1989). Subsequent murine studies however show a roughly halving of serum urea and creatinine with anti-neutrophil serum treatment following renal pedicle clamping (Kelly, et al. 1996). It is not completely clear how these contradictory findings can be reconciled. One obvious difference is species differences. Looking closely at the data, perhaps another reason for this was the very severe AKI induced in Thornton's surgical clamping model. Though it is difficult to compare leporine biochemical parameters to those from other species, typical serum urea levels at a 48 hour timepoint were 63±16 mg/dl and 153± 9 mg/dl for 38 and 50 minutes of renal pedicle clamping. While these clamping times are roughly consistent with other published methods, the high urea levels suggest disease severity may have surpassed a theoretical 'point of no return' beyond which amelioration of renal function may simply not be possible due to the overwhelming amount of tissue damage from cell death.

Inhibiting activation of neutrophils is another strategy for reducing their inflammatory effects. By the use of alpha-melanocyte-stimulating hormone which inhibits neutrophil activation by an unknown mechanism, Chiao and colleagues showed it was possible to ameliorate AKI (Chiao, et al. 1997).

Cell adhesion may be inhibited on the neutrophil aspect or the endothelial aspect. On neutrophils CD11a and CD11b are abundantly found. The molecules form adhesion complexes with CD18 and inhibition of these membrane proteins, using clone TA-3 against CD11a and OX-42 against CD11b, can result in reduced neutrophil infiltration and amelioration of AKI (Rabb, Mendiola, et al. 1994) (Rouschop, et al. 2005)

On the other hand by manipulating the adhesion of neutrophils into organ tissue using both a global ICAM-1 knockout model and a neutralising anti-ICAM-1 antibody, Kelly and colleagues showed reduced neutrophil accumulation and improved biochemical and

histological outcomes (Kelly, et al. 1996). While neutrophils avidly engage with ICAM-1, as we will see later, it is possible that other cell types, namely B cells, may also employ a similar tactic for entry into renal tissue.

More recently, inhibition of vascular adhesion protein 1 with the monoclonal antibody RTU-1096 which is predominantly expressed by pericytes during AKI prevented the infiltration of neutrophils but not macrophage or T cell populations into renal tissues (Tanaka, et al. 2017). It appears that inhibiting the entry of neutrophils ameliorated AKI. Inhibition of another neutrophil chemoattractant pathway, the leukotriene B4 receptor axis either through knockout models or by chemical inhibitors, SC-57461A and U-75302, had similar effects on AKI outcomes (Deng , et al. 2017). Taken together, inhibition of neutrophil infiltration into renal tissue appears to be an effective strategy in ameliorating AKI.

Interestingly, to my knowledge, there have been no works which have looked at the effects of directly depleting neutrophils on AKI using a GR1 antibody in a murine model – perhaps one of the most obvious ways of depleting neutrophils – or using low dose anti-GR1 antibody to inhibit neutrophil egress into renal tissue (Wang, et al. 2012). More sophisiticated experiments to determine the immune mechanism by which neutrophils exert a largely negative effect during AKI have not been undertaken to date.

Moreover, the precise relationship of cellular changes observed in murine experiments to human pathophysiology of AKI is difficult to assess. However, histopathological assessment of kidney sections with acute tubular necrosis from, for example, hypoperfusion, show increased numbers of neutrophils within renal tissue consistent with the observations in murine models of AKI. Clinical trials specifically targeting neutrophils in AKI have yet to be undertaken and will be required to determine their precise role in human AKI.

1.12.5 Macrophages

Macrophages have come to be defined as having different functions during two phases of AKI. On the one hand, in the earlier stages of disease, monocytes enter or macrophages proliferate in the kidney to generate an inflammatory milieu, while in the latter stages, different phenotypes of macrophages can have divergent functions.

Infiltration of macrophages into the kidneys has been observed especially in the outer medulla, peaking at about day 5 following renal pedicle clamping in rats (Ysebaert, et al. 2000). How chemokines cause monocyte trafficking in the context of AKI is not well described, but previous studies have shown that the cytokines CCL2-5, CCL8, CCL13-16 and CX3CL1 induce mobilisation in other models (Chung and Lan 2011). Entry of monocytes into the kidneys can be prevented by blockade of the surface protein B7-1 or CD80 (clone: 3H5) (De Greef, Ysebaert and Dauwe, et al. 2001). Interestingly, De Greef's study showed improvements in serum creatinine at earlier timepoints of 24 and 48 hours but not at any later timepoints following this intervention. Blockade of CX_3CL1, which is thought to be predominantly produced by epithelial cells during AKI, has been shown to reduce infiltrating macrophages, however this has not resulted in improvement of renal parameters. Another macrophage chemotractant, osteopontin, has been shown to reduce macrophage infiltration but not affect disease course (Persy, et al. 2003).

A number of studies have presented data that depletion of macrophages using liposome-encapsulated clodronate improved multiple outcome measures after 24 hours in a surgical IRI model (Day, et al. 2005) (Jo, et al. 2006). By day 3, however, clodronate-based depletion of macrophages has shown no difference in the outcome of cisplatin-induced and IRI-induced AKI models (Lu, et al. 2008) (Jo, et al. 2006). Hints that macrophages may affect longer term outcomes came when longer fibrosis experiments were done using repeated injections of clodronate over a four to eight week period (Ko, et al. 2008). Macrophage depletion seems to have attenuated renal interstitial fibrosis.

In a key paper, which recapitulated the duality of macrophages and the reasons for at times contradictory results, Lee and colleagues showed the evolving phenotype of macrophages in the kidney over a time course during AKI (Lee, et al. 2011). M1 macrophages, which have a generally pro-inflammatory phenotype, predominated the early stages of AKI while M2 macrophages with a more anti-inflammatory reparative phenotype appeared during the later stages. Depletion, therefore, of macrophages during the onset of AKI improved outcomes while depletion three to five days later resulted in worsening of outcomes.

Thus, the literature suggests that macrophages can play both positive and negative roles in AKI pathogenesis and the subsequent development of fibrosis. One possible explanation for this is the differential effects of monocyte-derived macrophages which may be recruited during acute injury and have a more M1 profile, and tissue-resident macrophages which are prenatally seeded and have a more M2-skewed profile. *In vitro*, the factors that polarise monocytes or bone marrow pre-cursors into and M1 or M2 phenotype have been described. For example, IFN-γ, LPS, GM-CSF, oxidative, fatty acid and HMGB1 polarise macrophages to a more M1 phenotype whereas IL-4, IL-10, IL-13, TGF-β, M-CSF, AMP and GC induce more of an M2 phenotype (Zhang, et al. 2017). Differences in these local signals could modulate macrophage polarisation during AKI.

1.12.6 Dendritic cells

Dendritic cells (DCs) during AKI have been shown to be the predominant source of TNF-α (Dong, et al. 2007). Ablation of dendritic cells using CD11c-diptheria toxin receptor transgenic before or at the time of cisplatin-induced AKI resulted in worsened outcomes (Tadagavadi and Reeves 2010). However, recent work in our own laboratory and others' suggest that CD11c is not exclusively expressed on DCs and may be expressed on some macrophages, therefore, the ablated population remains unclear. Indeed all of the experimental strategies used to deplete kidney mononuclear phagocytes such as clodronate liposomes, CD11b or CD11c diphtheria toxin receptor mediated cell depletion are subject to indiscrimate depletions of multiple cell lines.

1.12.7 T cells

The evidence for the role of T cells have been difficult to interpret. Mice which are globally deficient in recombinase activating gene (RAG1) and do not have B or T cells are not protected from AKI, however, adoptive transfer of T cells into these mice confers protection (Park, et al. 2002) (Burne-Taney, Yokota-Ikeda and Rabb 2005). While these data suggest a regulatory role for T cells, other studies have suggested that the absence of CD4 and CD8 T cells actually confers protection from AKI (Rabb, Daniels , et al. 2000). Athymic mice appear to be protected from AKI and transfer of T cells exacerbates disease severity (Burne, et al.

2001). Studies which used mice deficient in CD4 T cells but not CD8 T cells were protected from AKI (Yokota, et al. 2003). *En masse* absence of multiple T cell phenotypes may be contributing to these contradictory results as well as the use of congenitally T cell deficient mice, which makes it very difficult to interpret, given that other immunological cells may not be normal in the absence of T cells.

Therapeutically, T cell modulating agents such as tacrolimus and mycophenolate mofetil as well as the blockade of the T cell CD28-B7costimulatory pathway with CTLA4/Ig-fusion protein have been effective in attenuating AKI (Sakr, et al. 1992) (Jones and Shoskes 2000) (Chandraker, et al. 1997) (Takada, et al. 1997).

1.12.8 Experimental models of AKI

Almost twenty different models of AKI have been used in previously published experiments (table 1.5) (Singh, et al. 2012). This large number of approaches reflects an attempt to model the variety of aetiologies which can induce AKI. For example, gentamicin, cisplatin, contrast media and NSAIDs mimic respective iatrogenic drug-induced AKIs. In contrast surgical clamping of the renal pedicle mimics transient renal hypoprofusion from dehydration or conditions found during transplantation. Other models reflect AKI from environmental causes or infection. Each of these models occupies a different position within a spectrum of clinical relevance, technical difficulty, scientific reproducibility and renal-specific effects.

	Technical difficulty	Clinical relevance	Reproducibility	Renal specificity
Bipyridyls	ND	Low	ND	ND
Cisplatin	Low	High	High	Low
Contrast media	Low	High	High	High
Ferric nitrilotriacetate	ND	Low	ND	ND
Folic acid	Low	Low	High	High

Gentamicin	ND	High	High	ND
Glycerol	Low	Mod (specific for myoglobinaemia)	ND	Low
Iphosphamide	ND	Low	ND	ND
Ischaemia-reperfusion	High	High	Low	High
Mercuric chloride	ND	Low	ND	ND
NSAIDs	Low	High	High	Mod
Osmotic nephrosis	ND	Low	ND	ND
Paracetamol	Low	High	High	Low
Potassium dichromate	ND	Low	ND	ND
S-(1,2-dichlorovinyl)-L-cysteine (DCVC)	ND	Low	ND	ND
Sepsis	Low	High	Mod	Low
Uranium	ND	Low	ND	ND

Table 1.5. Different methods of inducing AKI in experimental models and the author's assessment of the agent. Original list of models taken from Singh 2012. In a number of models, the characteristics of the model remained unclear. For example, the renal specificity of ferric nitrilotriacetate was unclear from the literature. Where this was the case, I have written "ND", which stands for not determined.

In our experiments, we sought a model of AKI which i) was technically uncomplicated, ii) was frequently used by other researchers, iii) was sterile in its induction of an immune

response and iv) had relatively few known off-target effects. Folic acid appeared to fulfil this criteria.

The mechanism of action is not fully understood and multiple pathways may contribute to its pathophysiology: Following administration, there is deposition of folic acid crystals along with associated apoptosis and necrosis of tubular cells. Reports suggest that following its administration in high doses, there is downregulation of B-cell lymphoma-extra large (Bcl-xL), an anti-apoptotic protein and an increase in tumor necrosis factor-a (TNF-a) (Wan, et al. 2006). Others have shown that the folic acid creates oxidative stress and consequently tissue damage: Levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were significantly decreased while lipid peroxidation (LPO) levels, a widely used manifestation of oxidative stress, increased (Gupta, et al. 2012). Mitochondrial dysfunction, as with most forms of experimental and clinical AKI, has been shown to occur following folic acid induced AKI (Stallons, Whitaker and Schnellmann 2014). Finally, also perhaps not surprisingly, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) expression is increased in the kidneys during folic acid induced AKI and the suppression of this transcription factor has been found to ameliorate disease (Kumar, et al. 2015). The extent to which these described pathways are present in all forms of AKI and how much is unique to folic acid induced AKI remains unclear.

On a practical level, folic acid provides an experimentally convenient substance. The nephrotoxin, which comes in powder form, is soluble within an alkaline medium such as sodium bicarbonate and consistent batches can be produced in 12 hours. Administration is via an intraperitoneal injection, which is technically unchallenging. The model has been used in a large number of papers. Although the whole list of references have not been included in this thesis, in 2018 alone, more than ten publications cited on the biomedical database Pubmed used a folic acid-induced model of AKI (Zhou, et al. 2018) (Honarpisheh, et al. 2018) (González-Guerrero, et al. 2018).

Although folic acid nephropathy is not directly clinically relevant (given that toxic doses in humans would require in excess of a thousand standard 5mg tablets) we felt that the drug's direct eventual effect on inducing necrosis in the tubules provided an ideal experimental model. Off-target (extrarenal) effects, as far as I know, have not been reported

whereas other more clinically relevant models have a multitude of off target effects. For example, while paracetamol-induced AKI is common, the model involves considerable liver injury or in the case of cisplatin, which is again a common cause of AKI, there are considerable effects on immune cells.

1.13 B cells and AKI

Relatively little is known about the role of B cells in AKI and what is published is often conflicting suggesting multiple roles which can both ameliorate and exacerbate disease. Initial studies showed that uMt mice which are deficient in mature B cells had less severe disease following short-term renal pedicle clamping (Burne-Taney, Ascon, et al. 2003). Following on from this work, Rabb and colleagues showed CD126 positive plasma cells trafficking into the kidney post-AKI to have a detrimental role in AKI (Jang, Gandolfo, et al. 2010). Treatment with anti-CD126 antibody have been shown to increase tubular proliferation and reduce tubular atrophy.

In contrast, IgM anti-leucocyte autoantibodies produced by B1 cells may have a protective effect in AKI (Lobo, et al. 2012). Other groups have shown that uMT mice fare worse following AKI (Renner, et al. 2010). It is not clear why there have been such contradictory results from these projects given the similar methodology of inducing AKI with renal pedicle clamping and similar timepoints. One possibility, as shown in later data, is that mice are highly variable in their susceptibility to AKI. Age-matching may be inadequate to generate comparable control groups with weight matching being potentially more useful.

Interestingly, the effect of AKI on B cell antibody production has also been investigated (Fuquay, et al. 2013). Mice immunised with foreign antigen 24 to 96 hours following AKI had increased levels of antigen specific IgG1. Such findings have implications for kidney transplantation where the donor kidney (and sometimes the recipient's native kidneys) inevitably experience ischaemia perfusion injury and AKI would potentially augment human alloimmune responses.

1.13.1 B cells and CD11b

CD11b, also known as integrin alpha M (ITGAM), forms a heterodimeric integrin complex with CD18 called alpha-M beta-2 (αMβ2). This complex is also called macrophage antigen-1 (MAC-1) or complex-receptor (CR3) and is heavily expressed monocytes, granulocytes, macrophages and NK cells. Perhaps best known of MAC-1's many ligands is intercellular adhesion molecule 1 (ICAM1) which can attach to an activated form of CD11b and can be found on endothelium of inflamed tissue (figure 1.17). In addition, other ligands of MAC-1 include LPS (Wright and Jong 1986), beta amyloid (Zhang, et al. 2011), the damage-associated molecular pattern (DAMP) high mobility group box one (HMGB1) (Gao, et al. 2011), viruses (Morrison, Simmons and Heise 2008), nucleotides such as oligodeoxynucleotide (Benimetskaya, et al. 1997) and double-stranded RNA (Zhou, et al. 2013).

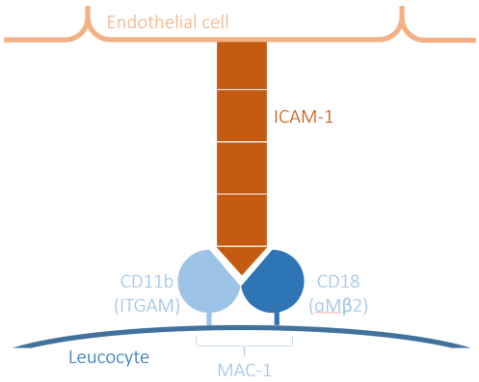


Figure 1.17. Schematic drawing of the MAC-1 consisting of CD11b and CD18 on the surface of the leucocyte (eg. myeloid cell or B cell) and its interacting protein, ICAM-1 complex on the surface of the endothelial cell.

Signalling via MAC-1 has been reported to induce expression of IL1β (Fan and Edgington 1993) (Rezzonico, et al. 2000), TNFα (Perez and Roman 1995) and tissue factor (Fan and Edgington 1998).

Relatively little work has been done on B cell expression of CD11b. Moreover, the protein's function when expressed on B cells remains unclear. CD11b expression has been previously noted on peritoneal B1 cells and may reflect a later developmental stage (Ghosn, et al. 2007). Interestingly, CD11b positive B1 cells are larger and more granular according to

early descriptions. Others have noted, somewhat controversially, that CD11b positive B1 cells in humans may have a role in expanding and stimulating T cells in lupus (Griffin and Rothstein 2011) (Griffin, Quach, et al. 2012) (Reynaud and Weill 2012). In one of the few papers known to discuss the function of CD11b on B cells, Kawai *et al* using *in vitro* experiments observed that CD11b may have an effect on its migratory potential (Kawai, et al. 2005).

1.13.2 Siglec-10 and Siglec-g

Siglecs (Sialic acid-binding immunoglobulin-type lectins) are a family of transmembrane proteins found on a wide range of immune cells which bind to sialic acids, a type of nine-carbon sugar, and mediate many different biological interactions (Chen, Brown and Zheng 2014). Siglec-g is expressed on B cells and to a lesser extent on dendritic cells and macrophages and are thought to be an inhibitor of B1a activation. The human orthologue of Siglec-g is Siglec-10. This section briefly discusses how Siglec-g regulates BCR signalling, binds to its ligands and plays a role in regulating immune responses.

There are two different SIGLECs found on the B cell surface: SIGLEC 2 (also known as CD22) and Siglec-g. Within the cytoplasmic domain of Siglec-g, there is an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain as well as an ITIM-like domain which are able to activate Src homology region 2 domain-containing phosphatase-1 (SHP-1) and inhibit B cell receptor signalling (Pfrengle, et al. 2013) (Whitney, et al. 2001). The absence of Siglec-g leads to increased calcium influx in B cells upon BCR activation (Hoffmann, et al. 2007). Although Siglec-g is expressed on both B2 and B1 cells, absence of the protein leads to increased BCR signalling in B1 cells (Hoffmann, et al. 2007) (Ding, et al. 2007).

Siglec-g can bind to both α2,6- and α2,3- linked sialic acids (Duong, et al. 2009). These sialic acids can be presented to the Siglec-g either in a *cis* fashion from surface of the same cell or in a *trans* fashion from the surface of another cell. It is unclear what the effect of non-attached sialic acids are on Siglec-g. To investigate the role of Siglec-g ligands Hutzler et al produced a knock-in mouse in which the ligand binding portion of the Siglec-g protein was altered so that both α2,6- and α2,3- linked sialic acids were unable to bind (Hutzler, et al. 2014). In doing so, they noted that this knock-in mouse called Siglec-g-R120E was phenotypically similar to global Siglec-g knockouts with increased calcium signalling and an

augmented population of B1a cells. This series of experiments suggested the chronic requirement of a Siglec-g agonist for its function. It has been suggested that one possible reason for why Siglec-g functions on B1 cells, and not B2 cells, is presence of higher concentrations of α2,3- linked sialic acids on B1 cells which may be providing basal stimulation of the protein and consequent BCR inhibition.

Phenotypical differences have been noted in Siglec-g deficient mice compared to their wild type counterparts. For example, the B1 (both B1a and B1b) population is greatly expanded in these mice (Hoffmann, et al. 2007). In addition, autoimmunity can spontaneously can occur in the Siglec-g knockout mice on a C57BL/6 background (Müller, et al. 2015). Siglec-g knockout mice have a propensity towards worse collagen-induced arthritis following immunisation or lupus-like disease when Siglec-g knockouts were crossed with a lupus-prone MRL/lpr mouse (Bökers, Urbat, et al., 7 2014). When mice are deficient in both Siglec-g and SIGLEC-2, it has been observed that they develop spontaneous lupus-like disease with age (Jellusova, et al. 2010).

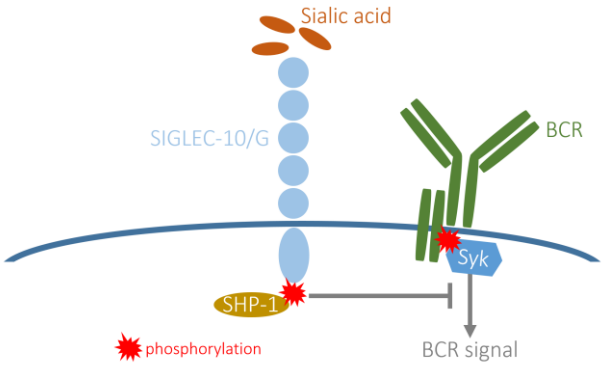


Figure 1.18. Diagram of the signalling pathway of Siglec-g/10 showing its inhibition of BCR signalling via activation of SHP-1.

Siglec-g is expressed predominantly by B cells and to a limited extent by dendritic cells (figure 1.19). Given that the Siglec-g knockout has an expanded B1a cells population and reduced threshold of activation of the B cell population, it has been used as a model to investigation excessive B cell – especially B1a cell – activity in a model of acute kidney injury (Hoffmann, et al. 2007). It should be noted that there are other inhibitory proteins which

regulate B cell activity such as paired immunoglobulin-like receptor B (PirB), B- and T-lymphocyte attenuator (BTLA, CD72), FcγRIIb (CD32), programmed cell death protein 1 (PD-1, CD279) and platelet endothelial cell adhesion molecule (CD31) (Pritchard and Smith 2003) (Odorizzi and Wherry 2012). However, the expression of these other inhibitory proteins was not limited to B cells but was present in a range of other immunological cell types (figure 1.20): For example, BTLA is also expressed on T cells. Consequently, it was felt that Siglec-g knockouts provided the most ideal model for looking at B cell exclusive disinhibition in AKI.

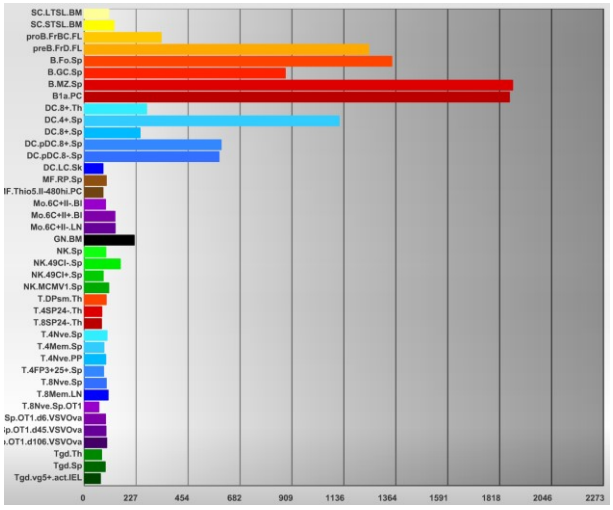
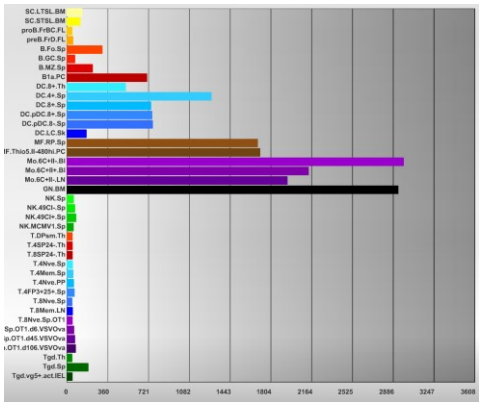
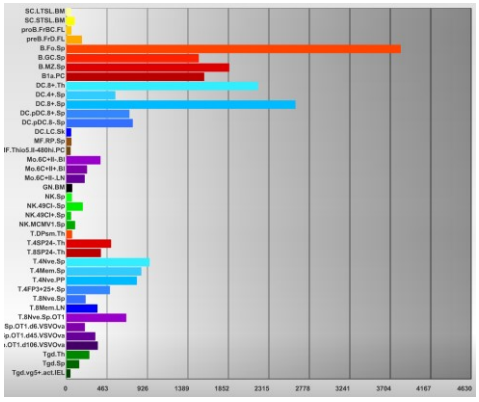


Figure 1.19 (above). Gene expression of Siglec-g in different leucocyte subsets. Data from the Immunological Genome Project Gene Skyline application (Immunological Genome Project 2019).

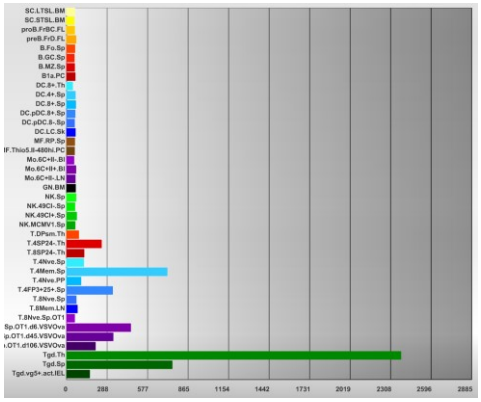
PirB



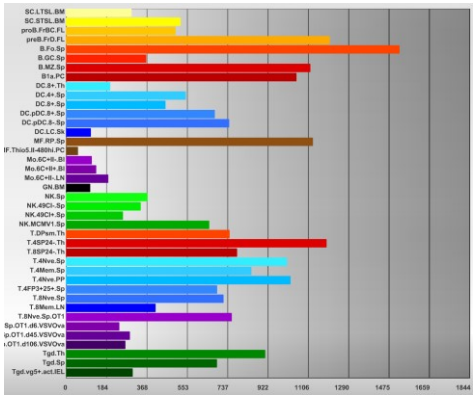
BTLA



PD-1



CD31



FcγRIIb

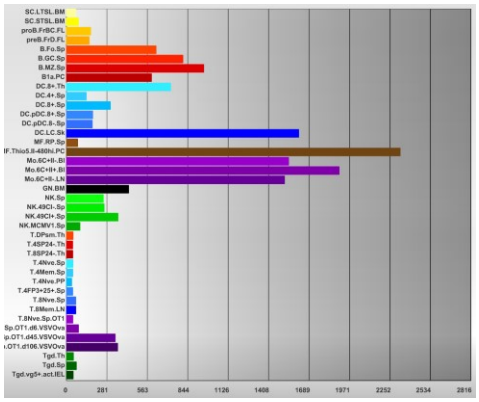


Figure 1.20 (above). Gene expression of PirB, BTLA, PD-1, CD31 and FcγRIIb in different immunological cell types. Data from the Immunological Genome Project Gene Skyline application (Immunological Genome Project 2019).

1.14: *In vivo* manipulation of regulatory B cells

There is now a wealth of research suggesting that B-regs play an important role in a range of conditions, therefore B-reg-based immunotherapy may be a potentially fruitful avenue for novel treatments. Virtually all *in vivo* studies have either been observational studies in humans or transfer experiments in mice. In clinical practice, however, cell-based therapies can be costly and are therefore less likely to be feasible for broad clinical application. Thus, the crucial question is whether B-regs can be induced *in vivo* pharmocologically. In this section, I will discuss a number of disparate fields and consider recent research into B-regs and previous clinical trials into low-dose IL-2 therapy.

As early as the late 1960s, there was evidence that a group of splenocytes from recently immunised mice, when transferred into another mouse, could suppress the production of antibodies (Morris and Möller 1968). However, there was no convincing evidence at the time that this was mediated by B cells. In the 1970s, a number of groups showed that splenocytes which were depleted of B cells failed to suppress delayed hypersensitivity reactions in passive transfer experiments (Katz, Parker and Turk 1974) (Neta and Salvin 1974) (Rosser & Mauri, 2015). Subsequently, Wolf and colleagues showed that mice deficient in mature B cells were unable to recover from experimental autoimmune encephalitis, a murine experimental model of human multiple sclerosis (Wolf, et al. 1996).

The mediator of immune regulation produced by B cells that has received most attention is IL-10, although other proteins such as IL-35, TGF-β, granzyme-B and PDL1 have been implicated. IL-10 produced by B cells has been shown to suppress inflammation in colitis, EAE and collagen-induced arthritis (Mizoguchi, et al. 2002) (Fillatreau, et al. 2002) (Mauri, Gray, et al. 2003). TGF-β from LPS-stimulated B cells has been shown to induce anergy and apoptosis in T cells. More recently, IL-35 from B cells has also been shown to be crucial in controlling EAE and experimental uveitis (Shen, Roch, et al. 2014) (Wang, et al. 2014). Contact-dependent mechanisms such as PDL1 expression (Khan, et al. 2015)and direct granzyme-B-dependent cytotoxicity have also been noted as immunomodulatory mechanisms employed by B cells (Chesneau, et al. 2015).

In mice, several groups have identified IL-10-producing B cells within a number of B cell subsets including B1, transitional and marginal zone subsets (Miles, Simpson, et al. 2018) (Evans, et al. 2007) (Gray, Miles, et al. 2007). As in mice, human IL-10-producing B cells are present within different subsets, including transitional and memory CD27 positive B cells. Several markers, such as CD5, CD1d, Tim-1, CD9 and CD80, have also been reported to be coexpressed by regulatory cells (Blair, Noreña, et al. 2010) (Ding, et al. 2011) (Iwata, et al. 2011) (J. Sun, et al. 2015) (van de Veen, et al. 2013).

The available data would suggest that there is not a separate population of regulatory B cells. When we refer to dedicated populations, this is frequently defined by the presence of one or more transcription factors that determine cell phenotype and allow the identification of a specific lineage. For example, regulatory T cells express the transcription factor Foxp3. In

the case of regulatory B cells, specific transcription factors or markers identifying a distinct population of regulators has not been described. The current evidence suggests that a regulatory B cell simply produces relatively more anti-inflammatory cytokines than inflammatory ones, and that this is determined by environmental cues, as explained later for example, by cytokines such as IL-2.

1.14.1 Diseases implicated by regulatory B cells: Multiple sclerosis / EAE

One of the earliest experiments observing the role of B cells in negatively regulating autoimmunity was in experimental autoimmune encephalomyelitis (EAE), a murine disease model thought to mimic multiple sclerosis seen in humans. Mice lacking the μ chain transmembrane region (μMt), therefore unable to generate mature B cells, failed to recover from EAE, suggesting that B cells may play a non-redundant role in limiting inflammation (Wolf, Dittel, Hardardottir, & Janeway, 1996). Subsequently, experiments using chimeric mice with B cell specific IL-10 deletion failed to suppress a type I immune response (Fillatreau, et al. 2002).

1.14.2 Arthritis / Collagen-induced arthritis

Mauri and colleagues have shown in a series of experiments that B-regs play a central role in controlling collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis (Carter, Rosser and Mauri 2012) (Evans, et al. 2007) (Mauri, Gray, et al. 2003). This suppression is mediated via IL-10 which inhibits T helper 1 differentiation. Later studies suggested that there was an enrichment of B-regs in a $CD21^{hi}$, $CD23^{hi}$, IgM^{hi} population which was labelled transitional 2-marginal zone precursor population. It is likely that this population is plastic, given that these cells, as the name suggests, are precursors of marginal zone B cells.

1.14.3 Colitis

A CD1d positive population of B-regs have been shown to be crucial immune regulators in the mesenteric lymphoid tissue during gastrointestinal inflammation via IL-10 which had effects on IL-1 and STAT3 activation (Mizoguchi, et al. 2002).

1.14.4 Systemic lupus erythematosus

In healthy subjects, $CD24^{hi}$ $CD38^{hi}$ B cells suppress differentiation of T helper 1 cells but this is impaired in patients with SLE (Blair, Noreña, et al. 2010). More recently, it has emerged that normal crosstalk between plasmacytoid dendritic cells and $CD24^{hi}$ $CD38^{hi}$ B cells induced an appropriate balance of IL-10 and TNF-α production. This balance appears to be perturbed in patients with SLE (Menon, et al. 2016).

1.14.5 Tolerance to apoptotic cells

It may not be necessary to implicate the role of regulatory B cells to particular clinical conditions, but rather to the underlying mechanism at play. Specifically, immune tolerance to apoptotic cells – the breakdown of which is thought to induce a range of autoimmune conditions – may be mediated by regulatory B cells. For example, Gray *et al* showed that B cells co-cultured with apoptotic cells take on an immunomodulatory phenotype by provision of IL-10 (Gray, Miles, et al. 2007). Subsequent work has shown exposure to apoptotic cells results in the engagement of TLR9 pathways (Miles, Heaney, et al. 2012) and that B1a cells, which are identified by the $CD43^{+}CD19^{hi}CD5^{+}IgM^{hi}IgD^{lo}$ signature, are responsible for the majority of the IL-10 produced (Miles, Simpson, et al. 2018).

1.14.6 Transplantation

Tolerance towards a transplanted organ graft with minimal immunosuppression remains the gold standard of modern transplantation. It has become apparent from a number of studies that lower rates of antibody-mediated rejection are associated with enrichment of a $CD24^{hi}$ $CD38^{hi}$ population (Shabir et al., 2015). In addition, the balance between the production of IL-10 and pro-inflammatory cytokines such as IL-6 and TNF-α may be important in regulating immune responses. For example, a reduction in the numbers of $CD24^{hi}$ $CD38^{hi}$ B cells, which produce the highest ratios of IL-10 to TNF-α, is associated with worse allograft outcome in renal transplant recipients (Cherukuri et al., 2014). In contrast, in 'operationally tolerant' patients with drug-free long-term graft function due simply to non-compliance, a significant increase in IL-10 producing $CD24^{hi}$ $CD38^{hi}$ B cells along with a corresponding decrease in

$CD20^-$ $CD38^+$ $CD138^+$ plasma cells have been observed (Chesneau et al., 2015; Chesneau et al., 2014; Nova-Lamperti et al., 2016).

Cell type	Murine surface markers	Human surface markers	Location	Mechanism of action	References
Transitional 2 marginal zone precursor	CD19, CD21, CD23, CD24		Spleen	IL-10	(Blair, Chavez-Rueda, et al. 2009) (Carter, Rosser and Mauri 2012) (Evans, et al. 2007) (Schioppa, et al. 2011)
Marginal zone	CD19, CD21, $CD23^-$		Spleen	IL-10	(Bankoti, et al. 2012) (Gray, Miles, et al. 2007) (Miles, Heaney, et al. 2012)
B1a	CD43, CD19, CD5, IgM, IgD^{lo}		Spleen, peritoneum	IL-10	(Miles, Simpson, et al. 2018)
B10	CD5, CD1d	CD24, CD27	Spleen, blood	IL-10	(Horikawa, et al. 2013) (Iwata, et al. 2011) (Matsushita, Horikawa, et al. 2010) (Yanaba, et al. 2008)
Plasma cells	B220, CD138, $MHC\text{-}11^{lo}$		Spleen	IL-10, IL-35	(Neves, et al. 2010) (Shen, Roch, et al. 2014)
TIM-1	CD19, TIM-1		Spleen	IL-10	(Wilson 2017) (Ding, et al. 2011) (Xiao, et al. 2012)
Plasmablasts	CD138, CD44	CD19, CD24, $CD27^{int}$	Draining lymph (mice) and blood (humans)	IL-10	(Matsumoto, et al. 2014)

Immature cells		CD19, CD24, CD38	Blood	IL-10	(Blair, Noreña, et al. 2010) (Bosma, et al. 2012) (Das, et al. 2012) (Flores-Borja, et al. 2013)
B regulatory 1		CD19, CD25, CD71	Blood	IL-10	(van de Veen, et al. 2013)
$PDL1^{hi}$ Found in multiple splenic compartments.	CD19, PDL1		Spleen	PDL1	Khan 2015

Table 1.6 (above) Previously identified regulatory B cell types. Modified from (Rosser and Mauri 2015).

Disease	References
Systemic lupus erythematosus	Blair et al, 2009. Blair et al, 2010.
Colitis / Inflammatory bowel disease	Mizoguchi et al, 2002
Arthritis	Mauri et al 2003. Evans et al 2007. Carter 2012.
Hypersensitivity reactions	Katz, 1974. Neta 1974. Van de veen 2013.
Multiple sclerosis / Experimental autimmune encephalitis	Wolf et al, 1996. Mann et al, 2007. Filatreau et al 2002. Matsushita et al, 2010.
Transplantation	Nova-Lamperti et al, 2016. Cherukuri et al, 2014.

Table 1.7 (above) Disease processes and conditions in which B-regs are implicated.

1.15 Previous studies in IL-2 therapy and regulatory T cells

The effect of recombinant human IL-2 therapy on T-cells illustrates the bimodal effects of the cytokine (Klatzmann and Abbas 2015). At high doses IL-2 was shown in the 1970s and 1980s

to induce survival, proliferation and most importantly differentiation of naive T cells into effector T cells (Robb 1984). For this reason, IL-2 was approved by the United States Food and Drug Administration as perhaps one of the earliest oncological 'immunotherapies' for renal cell carcinoma in 1992 and melanoma in 1998, but was not widely prescribed due to its large side-effect profile which included vascular leak syndrome and renal and liver impairment (Rosenberg 2014).

Paradoxically, in the 1990s genetically altered mice deficient in various components of the IL-2-IL-2-receptor axis developed autoimmunity, rather than immunodeficiency, which one might assume would be the result given the scientific and clinical experience described above. IL-2 deficient mice have a high infant mortality rate of about 50% and all of the survivors, without exception, develop inflammatory bowel disease (Sadlack, et al. 1993). Similarly deficiency of either the α- or β-chain of the trimeric IL-2 receptor (CD25 and CD122 respectively) led to uncontrolled autoimmunity (Suzuki, et al. 1995) (Willerford, et al. 1995). These data helped form the conclusion that IL-2 was necessary for sustaining the regulatory T cells *in vivo* (Yu, et al. 2009). It should be noted that these experiments did not use models of T cell specific deletion. The extent to which other cell types are receptive to IL-2 has never been fully addressed.

On the basis of these results, low-dose IL-2 therapy has been applied in humans trials where it was thought the therapy would lead to T-reg proliferation, in preference to effector T cells, and be useful in the treatment of autoimmune conditions.

Authors	Condition	Patient numbers treated with rIL-2	Conclusion
(Soiffer, Murray and Cochran , et al. 1992)	Bone marrow transplant	N=13	No graft versus host disease seen. Interestingly, transplant was T cell depleted
(Soiffer, Murray and Gonin , et al. 1994)	Bone marrow transplant	N=29	Reduced disease relapse. T cells were also

			depleted in this study.
(Piscitelli, et al. 1996)	HIV	N=18	Subcutaneous rIL-2 well tolerated
(Ahmadzadeh and Rosenberg 2006)	Renal cell carcinoma or metastatic melanoma	N=8	Although a relatively high dose was used (7.2 x 10^5 iu/kg *tds*), this paper is included to show that Tregs may be induced at higher doses
(Zorn, et al. 2006)	Cancer	N=12 metastatic cancer patients N=9 chronic myelogenous leukemia patients after allogeneic hematopoietic stem cell transplantation	
(Zorn, et al. 2009)	Graft versus host disease following allogeneic hematopoietic stem cell transplantation	N=10	Expansion of Treg numbers
(Koreth, et al. 2011)	Chronic graft versus host disease	N=29	Treg numbers increased. Glucocorticoid use reduced.
(Saadoun, et al. 2011)	Hepatitis-C induced vasculitis	N=10	8/10 vasculitis improvement
(Long, et al. 2012)	Diabetes mellitus	N=9	Tregs increased. No difference in clinical/metabolic parameters
(Hartemann, et al. 2013)	Diabetes		Final results to be published
(Matsuoka, et al. 2013)	Chronic graft versus host disease	N=14	

(Castela, et al. 2014)	Alopecia areata	N=5	4/5 patients had clinical improvement
(Ito, et al. 2014)	Healthy subjects	N=21	Tregs increased
(Kennedy-Nasser, et al. 2014)	Acute graft versus host disease following allogeneic hematopoietic stem cell transplantation	N=16	No grade 2-4 GVHD in treated patients
(Truman, et al. 2015)	Diabetes mellitus		Mutiple dose dose-finding trial; Final results to be published
(He, et al. 2016)	Lupus		Marked improvement
(Todd, et al. 2016)	Diabetes mellitus		Single dose dose-finding trial
(von Spee-Mayer, et al. 2016)	Lupus	N=5	Treg numbers replenished
(Medicine 2017) Induction of Regulatory t Cells by Low Dose il2 in Autoimmune and Inflammatory Diseases (TRANSREG) ClinicalTrials.gov Identifier: NCT01988506	Rheumatoid Arthritis Ankylosing Spondylitis Systemic Lupus Erythematosus Psoriasis Behcet's Disease Wegener's Granulomatosis Takayasu's Disease Crohn's Disease Ulcerative Colitis Autoimmune Hepatitis Sclerosing Cholangitis Gougerot-sjögren	N=132 (projected)	Final results to be published.

Table 1.8: Compilation of human trials using low-dose rIL-2 for autoimmune conditions

1.15.1 Low dose IL-2 therapy and B cells

Although clinical trials using low-dose IL-2 have not specifically looked at its effect on B cells, there have been at least two reports of a decrease in circulating CD19 positive B cells during treatment (Hartemann, et al. 2013) (Saadoun, et al. 2011). It is not clear what caused this reduction: It is possible that IL-2 induced a redistribution from the circulation to the tissues or

secondary lymphoid organs. Alternatively, the reduction in B cells numbers may have been an indirect results of reduction in Tfh survival. To this extent, papers have suggested reduction in levels of circulating Tfhs in IL-2 treated subjects (He, et al. 2016). It is unknown what phenotypic changes may occur as a result of IL-2 treatment.

Chapter 2: Investigating the role of B cells in acute kidney injury

Over the past decade, there has been a concerted effort to improve the medical management of AKI. In the United Kingdom, an important turning point was the publication of the National Confidential Enquiry into Patient Outcome and Death report, *Acute Kidney Injury: Adding Insult to Injury* published in 2009 (Stewart, et al. 2009). The publication highlighted the widespread prevalence and less than ideal management of AKI and suggested future improvement strategies. The impetus from this report and other similar publications resulted in clinicians' increased vigilance towards AKI as well as the generation of novel tools such as automated electronic reporting of lab results, which identify patients with worsening renal function to optimise kidney care in a timely fashion (Breighner and Kashani 2017) (Wilson 2017).

While prevention of AKI can no doubt help patients, there remains a cohort of patients for whom AKI and subsequent renal scarring resulting in chronic kidney disease are inevitable in spite of optimum treatment. For example, a review of previously published data suggests that despite almost constant monitoring, between 20 and 50 percent of patients in an intensive care setting will develop AKI (Case, et al. 2013). This situation begs the question why in spite of the high prevalence of AKI, the mainstay of treatment in most cases for the condition has remained unchanged for many years: This typically consists of the triad of optimising fluid management, withdrawing nephrotoxic medications and treating any underlying condition such as sepsis. In spite of the many publications which have shown

amelioration of AKI by manipulation of the immune response in animal models (as discussed earlier), there remains no widely available or commonly used therapy specifically directed towards AKI in humans.

Relatively little work has been done on B lymphocytes and AKI. Indeed previous work on B cells has focused on their role in adaptive immunity as antibody-producers, but there is increasing evidence that B cells have a much broader functional remit. For example, innate B1a cells located predominantly in the spleen produce granulocyte macrophage colony-stimulating factor (GM-CSF) during Gram-negative sepsis and induce distant neutrophil expansion and mobilisation from bone marrow and limit bacteraemia (Rauch, et al. 2012). Others have shown that B cells can also exert local effects attracting monocytes into myocardial tissue following acute myocardial infarction (Zouggari, et al. 2013).

Here, we show that there is re-distribution of immune cells in the early hours of AKI. B cells, and in particular $CD11b^+$ B cells move from the spleen and from bone marrow into the kidney where they produce CCL7 attracting other immune cells. Cell-intrinsic CD11b deficiency reduces the entry of B cells into the kidney while blockade of CCL7 using a monoclonal antibody ameliorates disease. On the other hand, Siglec-g knockout mice, which have proportionally higher numbers of B1 and $CD11b^+$ B cells relative to their wildtype counterparts, are more prone to AKI. Siglec-g is an inhibitory molecule found almost exclusively on B cells, and intravenous infusion of sialic acid, a Siglec-g agonist, ameliorates AKI. Finally, analysis of human kidneys from deceased donors deemed unsuitable for transplantation shows that CCL7 transcript levels are increased in kidneys which have AKI thus showing clear human relevance to our murine studies. Together our data suggest that blockade of CD11b or CCL7 or engagement of Siglec-g/10 using sialic acids represent potential novel therapeutic avenues for the treatment of AKI.

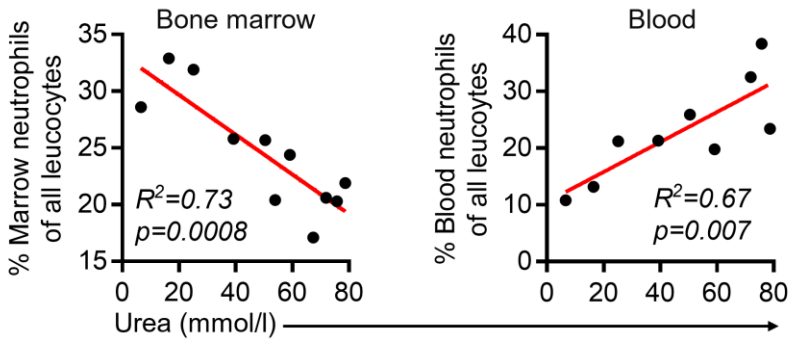
2.1 Results

To investigate the systemic immune response during AKI, there are a number of murine models of AKI including cisplatin- and folic acid-induced AKI as well as renal artery clamping that could be utilised. Each of these methods has its advantages and disadvantages, as

discussed in section 1.12.8. For the majority of experiments in this chapter, in order to keep consistency between the experiments, we used folic acid-induced acute kidney injury.

2.1.1 Bone marrow neutrophil mobilisation during AKI

To investigate the systemic immune response in AKI, we performed flow cytometric analysis of bone marrow (tibial and femoral) aspirates. Neutrophil numbers were analysed 15 hours following administration of varying amounts of folic acid in order to produce variable severity of AKI. We observed a negative linear relationship between the severity of AKI, as measured by serum urea, and the proportion of neutrophils in the bone marrow (figures 2.1 and 2.2). The reduction of bone marrow neutrophils was mirrored by an increase in circulating blood and kidney neutrophils (figures 2.2 and 2.5) supporting the view that during AKI a systemic inflammatory response is induced in proportion to the severity of AKI.



Figures 2.1 (left) and 2.2 (right). Percentage of bone marrow and blood neutrophils among CD45 positive leucocytes against serum urea. All analysis of blood was done using cardiac bleeds. Mice were a mixture of males and females between 6 to 12 weeks old. Mice were culled and analysed 16 hours after varying weight-adjusted intraperitoneal folic acid injection. Neutrophils were defined as the population of single, CD45-positive, Ly6C-high and GR1-high events on flow cytometry. Note a viability stain was not part of the gating strategy as most neutrophils die rapidly both *in* and *ex vivo*. Two experiments combined. N=11 mice in total (2 samples were excluded due to samples

clotting). Linear regression. R^2 represents goodness of fit and p value represents whether the slope is significantly non-zero.

Throughout this series of experiments, in order to distinguish intravascular and extravascular leucocyte in the kidneys, we employed a technique in which mice were injected intravenously via a tail vein with a fluorochrome-labelled anti-CD45 antibody one minute prior to culling in a carbon dioxide chamber (figure 2.3). During laboratory processing, tissues were stained again with an anti-CD45 antibody conjugated to a different fluorochrome. Flow cytometry analysis showed that leucocytes which were doubly positive for both intravenous and *ex vivo*-applied CD45 antibody were from the intravascular compartment (figure 2.4). Those cells not in the vasculature were singly positive for *ex vivo*-applied CD45 antibody. To confirm that the intravenous injection was successful, blood leucocytes from all mice were also analysed. Consistently, more than 90% of blood leucocytes were labelled with intravenous anti-CD45 antibody. Samples were excluded from any further analysis if blood leucocytes were not successfully labelled as distinction between intravascular and extravascular leucocytes was not possible. Using this approach, usually only a negligible proportion of intravascular cells were stained if, for example, there was extravasation of the tail vein injection.

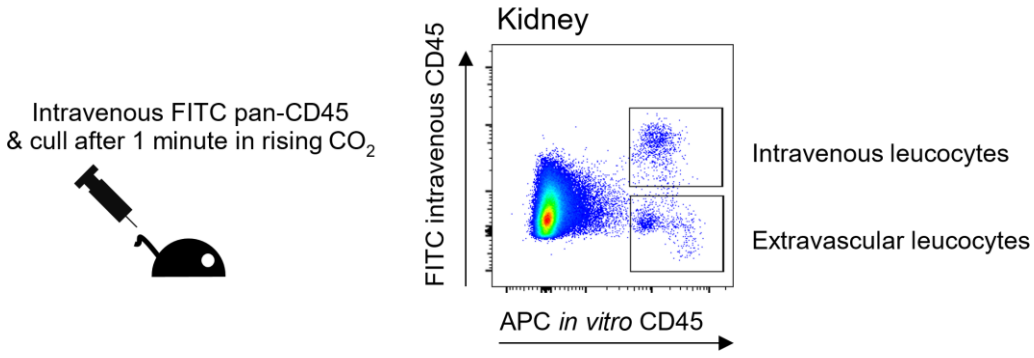


Figure 2.3 (left). Diagram showing injection of FITC anti-CD45 antibody intravenously prior to culling. Figure 2.4 (right). Representative flow cytometry

plot of CD45 with two different fluorochromes showing two different populations of intravascular and extravascular leucocytes in the kidney.

Perhaps one critique of this technique is that there may be some degree of 'leakage' of the intravascular anti-CD45 antibody into the extravascular space. However, it has been shown in a number of other works that this leakage is minimal (Anderson, et al. 2014). In future, one potential method of assessing the extent of such leakage may be to cull mice at different time points following intravenous injection of anti-CD45 antibody (for example, 1, 2, 5, 10, 60 minutes) and examine the kidneys using confocal microscopy. Intravascular and extravascular cells may then be counted manually.

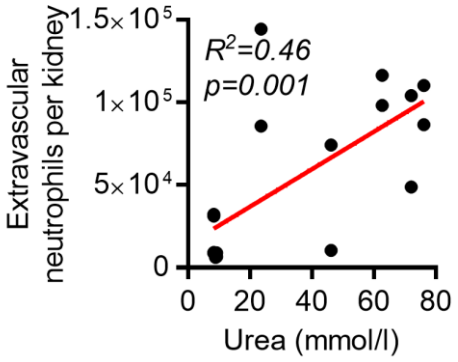


Figure 2.5: Number of extravascular kidney neutrophils against serum urea. Mice were a mixture of males and females between 6 to 12 weeks old and were culled and analysed 16 hours after varying weight-adjusted intraperitoneal folic acid injections. Neutrophils were defined as the population of single, *in vitro* stained pan CD45 positive, *in vivo* stained intravascular CD45 negative, Ly6C high and GR1 high events on flow cytometry. Two experiments combined. N=13 mice in total. Linear regression. R^2 represents goodness of fit and p value represents whether the slope is significantly non-zero. (Note: This experiment is completely independent from the experiments whose data are shown in figures 2.1 and 2.2 as the technique

for differentiating intravascular and extravascular leucocytes was not yet developed when the earlier experiments were done.)

Though these data show the dynamics of neutrophils in an AKI severity-dependent manner, there are nonetheless some caveats which may in future merit further investigation. First, in the experiment whose data are shown in figures 2.1 and 2.2, while we chose mice which were between 6 and 12 weeks old, we made no effort to balance the number of males and females. Additionally, a degree of genetic drift may be present between the individual mice as the mice used were not littermates and often were the simply unneeded wildtype siblings of genetically modified mice. For reasons of expense, we have not fully genetically sequenced these mice to assess comprehensively for genetic drift. It was felt at the time of the experiment that it would be more ethical and more economical to use these mice in experiments and derive some data from them rather than purchasing further mice from outside providers or re-deriving multiple generations of backcrossed wildtype mice. Moreover, one could argue that the variation in background of these mice in fact reinforces the strength of these data given that even with some variable genetic backgrounds, a statistically significant trend was observed.

2.1.2 Dynamics of B cells during AKI

We next investigated the global dynamics of B cells, T cells, and neutrophils in the spleen, blood and kidney in the early stages of AKI (figure 2.4). AKI was again induced by administration of folic acid. We observed that the splenic B cell count reduced by half with a corresponding increase in extravascular renal B cells.

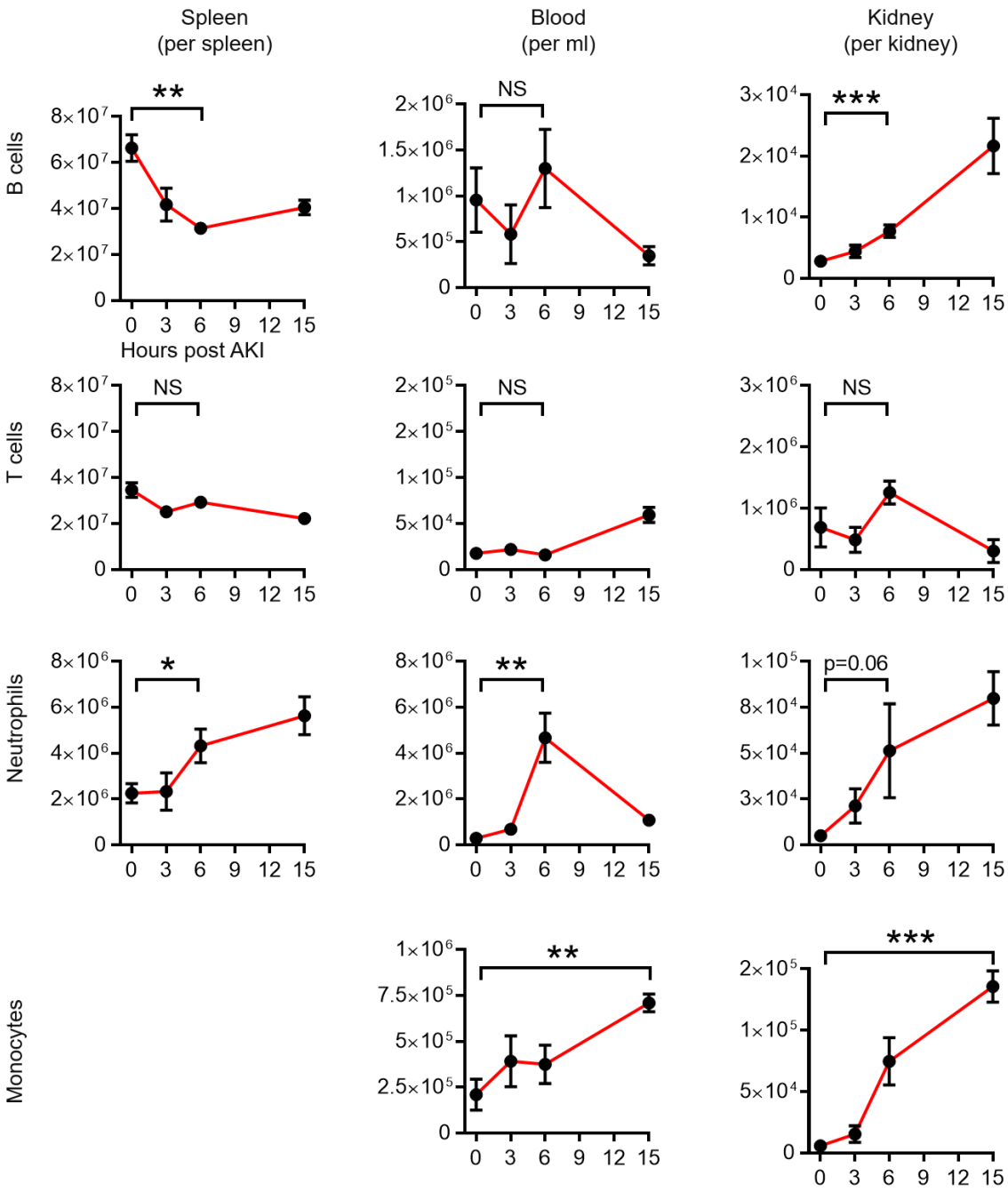


Figure 2.6. Global dynamics of B cells, T cells, neutrophils and monocytes during early stages of AKI. Following folic acid induced AKI, mice were culled at 3, 6 and 15 hours. Flow cytometry counting beads were used to estimate the total number of cells in the whole organ. Three experiments combined. N = 16 in total. Significance calculated using a non-paired parametric two-tailed T-test. As for all figures in this thesis, asterisks represent the following p values: * p <

0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, NS not significant. Error bars represent standard error of the mean (SEM).

2.1.3 CD11b and its role in B cells

We examined the blood more closely to assess the phenotype of trafficking B cells. Strikingly, whereas in the normal healthy state, about 1% of B cells were $CD19^{+}B220^{-}$, by 15 hours after AKI induction, approximately 20% of all B cells were $CD19^{+}B220^{-}$ (figures 2.7 and 2.8).

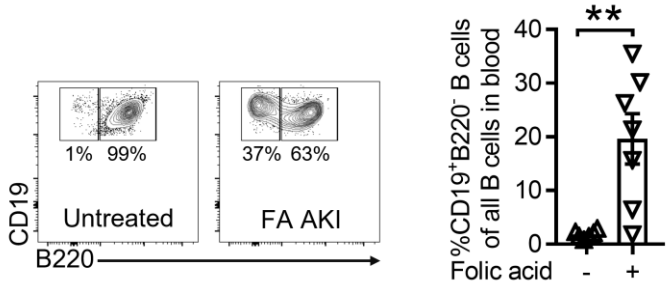


Figure 2.7 (left). Flow cytometry analysis of blood taken from untreated and folic acid treated mice. Gated on single, viable, CD19 positive events. Figure 2.8 (right). Quantification of $CD19^{+}B220^{-}$ B cells in the blood. Error bar represents standard error of the mean. N=13 in total (six given vehicle and seven given folic acid). Mice were a mixture of males and females between 6 to 12 weeks old. Mice culled and tissue processed 16 hours following insult. Statistical significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean (SEM).

To confirm that these cells were indeed B cells, we stained for CD20, an additional B cell surface marker, as well as other markers which are traditionally considered to be more myeloid cell surface markers such as CD11b and GR1. The $CD19^{+}B220^{-}$ subset expressed CD20 at levels similar to other $CD19^{+}B220^{+}$ B cells (figure 2.9). Given their high expression of CD5 and CD11b, we concluded that the $CD19^{+}B220^{-}$ blood B cells were enriched in $CD11b^{+}$ B1 B cells which have previously been described in both humans and mice (Griffin and Rothstein 2011).

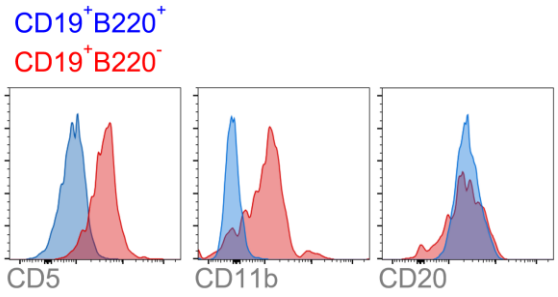


Figure 2.9. $CD19^+B220^-$ B cells express high levels of CD20, CD5 and CD11b. Gated on single, viable events. Mice culled and tissue processed 16 hours following insult. Blue histograms are representative of $CD19^+B220^-$ events and the red histograms representative of $CD19^+B220^+$ events.

We examined the distribution of these $CD11b^+CD19^+B220^-$ B cells within the kidney. In the kidneys of mice treated with vehicle, less than 10% of B cells in the extravascular space expressed CD11b (figure 2.10). During AKI there was a significant increase in $CD11b^+CD19^+B220^-$ B cells. Indeed, in the extravascular space, more than 30% of B cells expressed CD11b during AKI.

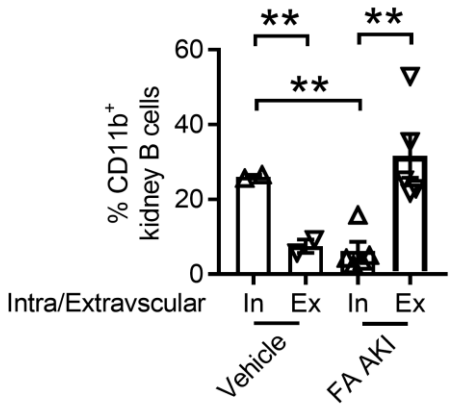


Figure 2.10. Percentage of $CD11b^+$ B cells within the intravascular and extravascular spaces during health and AKI. Gated on single, viable, CD19 positive events. Mice culled and tissue processed 16 hours following insult.

N=7 in total (2 and 5 mice in each respective condition). Significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean (SEM).

Given that CD11b along with CD18 forms part of the MAC-1 complex used by myeloid cells to tether to the vasculature during extravasation into interstitial spaces, and that one of the ligands for MAC-1 is ICAM which is known to be highly expressed in the kidneys during AKI, we hypothesized that B cells also use CD11b for a similar function (De Greef, Ysebaert and Persy, et al. 2003). To investigate this hypothesis, a competition assay was set up in which µMt mice lacking mature B cells were injected with $5x10^6$ wildtype CD45.1 B cells and an equal number of $CD11b^{-/-}$ CD45.2 B cells (figure 2.11). 24 hours after B cell transfer, AKI was induced with folic acid. The kidneys were harvested after 16 hours. In the extravascular space of the kidneys, there were significantly more wildtype B cells than knockout cells by a ratio of more than two to one (figure 2.12). In contrast, in the intravascular space, this ratio was approximately reversed.

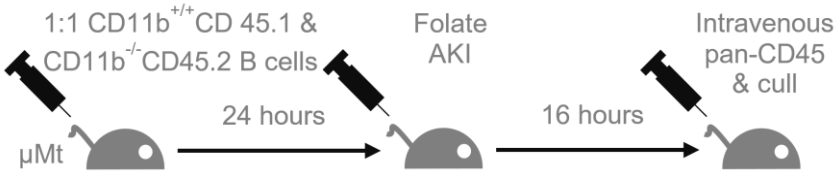


Figure 2.11. Schematic diagram of the experimental setup for injection of $CD11b^{+/+}$ and $CD11b^{-/-}$ B cells before AKI. µMt mice were injected with $5x10^6$ $CD11b^{+/+}$ CD45.1 wildtype and $5x10^6$ $CD11b^{-/-}$ CD45.2 B cells. 24 hours after injection, AKI was induced with folic acid. 16 hours later, intravenous FITC anti-pan-CD45 was injected before culling.

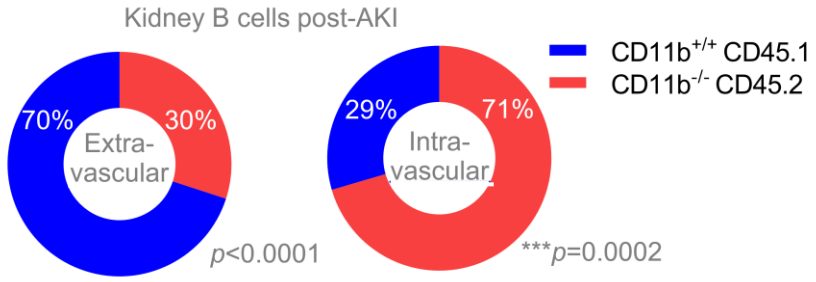


Figure 2.12. Extravascular and intravascular wildtype and CD11b$^{-/-}$ B cells following AKI. N=12. Significance calculated using a non-paired parametric two-tailed T-test.

A number of comments can be made about this set of experiments. First, there has been some controversy regarding the presence of CD11b positive B cells as illustrated in a number letters to the editor (Reynaud and Weill 2012). Specifically, it has been suggested that the initial discovery by Griffin and colleagues that this population may in fact be a sorting artefact (Griffin and Rothstein 2011). It was suggested that these events may represent 'doublets' of two attached cells, for example, between a B cell and a myeloid cell such as a monocyte. However, during our experiments we meticulously gated out doublets. Moreover, there have been other subsequent publications which have suggested the existence of CD11b positive B cells in other experimental models such as autoimmune hepatitis (Liu, et al. 2015). To ultimately answer these questions in future, single-cell RNA-seq may be useful to understand better the transcriptomic signature of this cell population.

Second, the use of µMt mice as a carrier for these B cells may not replicate an immunologically ideal model if the goal is to recapitulate a mouse which has both CD11b$^{+/+}$ and CD11b$^{-/-}$ B cells in equal numbers. For example, I am aware that after a few weeks µMt mice which have been given B cells no longer have detectable B cells, though this was not the case in our three day experiment (Scott 2018). Moreover, µMt mice do not have a completely normal myeloid population as B cells may regulate and promote their development. However, for the purposes of this experiment, which was to assess the ability of B cells to enter the kidney shortly after being injected, µMt mice provide a convenient carrier.

2.1.4 CCL7 and B cells

The role of these B cells remained to be determined. We hypothesized that B cells may be important producers of chemokines that mobilise and activate other immune cells. To screen for potential candidate cytokines, we induced AKI in both wildtype and µMT mice and analysed their whole kidney lysate by qPCR for cytokine transcripts (figure 2.13). CCL7 was significantly lower in mice lacking B cells and there was a less substantial difference in CCL5, CXCL1 and CXCL2 in µMT compared to wildtype mouse kidneys. We focussed primarily on CCL7 given that this chemokine was dramatically altered in the µMT mice.

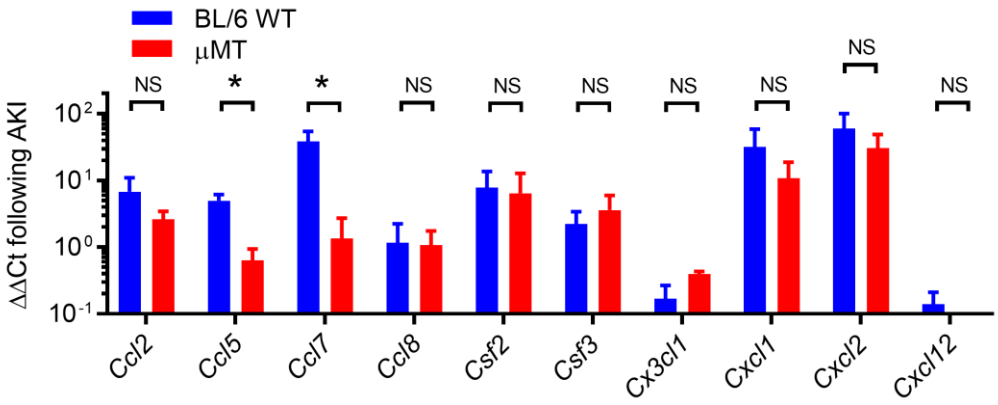


Figure 2.13. qPCR of whole kidney lysate from wildtype and µMt mice to screen for significant differences in cytokine production following folic acid-induced AKI. N=6 in total (3 per group). Mice culled and tissue processed 16 hours following insult. Significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean (SEM).

To confirm that B cells have the potential to produce CCL7, we negatively isolated splenic B cells then co-cultured them with various stimuli and measured the supernatant cytokine concentrations by ELISA (Figure 2.14). There was a significant increase in CCL7 following stimulation with DAMPs such as HSP70 and following BCR cross-linking using hen egg lysozyme (HEL) in HEL-IgM-BCR transgenic (MD4) mice. CXCL2 was also produced following CpG and heat shock protein 70 (HSP70) stimulation (data not shown).

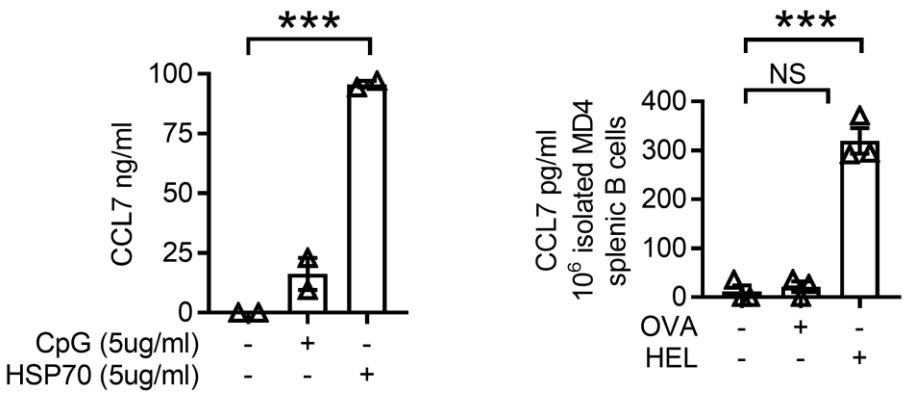


Figure 2.14. CCL7 production following stimulation with a TLR-ligand, DAMP and BCR antigen by $5x10^5$ negatively-isolated B cells over 60 hours. B cells isolated from a single murine spleen. Supernatant measured by ELISA. Both experiments performed twice with similar results. Significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean (SEM).

To assess the role of CCL7 in AKI *in vivo*, anti-CCL7 neutralising antibody was injected at the same time as folic acid. CCL7 neutralisation significantly reduced AKI severity as indicated by serum urea (2.16 and 2.17).

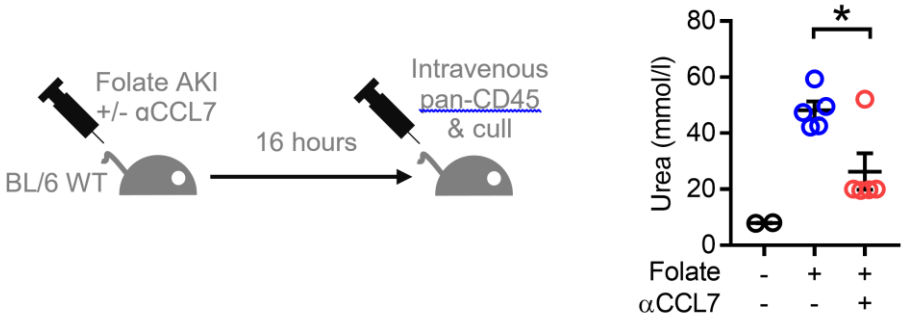


Figure 2.16 (left). Diagram of protocol for injection of anti-CCL7 neutralising antibody along with folic acid. Figure 2.17 (right) Quantification of serum urea 16 hours following AKI induction. Two experiments combined. N=12 in total (2, 5 and 5 in each respective condition). Error bars represent standard error of the mean (SEM).

As with experiments discussed in the previous section, the use of μMt mice can be problematic given their global immunological aberrations. However, in these experiments, μMt mice have been used as a screening tool to narrow down potential cytokines which might be useful to assess more closely. Another method of conducting the experiments described in figure 2.13 is to deplete B cells using a depleting antibody against CD19 or CD20, followed by folic acid administration. However, until recently, these antibodies have been very costly and difficult to access.

Our data demonstrate that CCL7 blockade does ameliorate AKI in this murine model. This is not a complete blockade of pathology, in that urea levels do not return to normal levels, but they are substantially reduced compared to animals receiving the isotype control antibody. The extent to which this blockade was pharmacologically complete, i.e. that all CCL7 in the mouse was bound by antibody, was not formally assessed.

Finally, future investigations will need to look at the signals necessary for this B cell mobilisation and activation in the context of AKI. B cells may be directly responding to DAMPs or to cytokines released by myeloid cells in response to DAMPs. Our experiments show that BCR cross-linking induces CCL7 production, and this may occur in the presence of multimeric T-independent antigen that can be found on the surface of necrotic cells.

2.1.5 Siglec-g$^{-/-}$ mice with increased B1 cells and their susceptibility to AKI

Since the blockade of CCL7 led to amelioration of AKI, we hypothesized that mice with increased numbers of B1 cells might have worse AKI. Siglec-g is a protein found almost exclusively on B cells (figure 2.18). Siglec-g deficiency results in a fourfold increase in B1 cells in mice (Hoffmann, et al. 2007). In our analysis, flow cytometric analysis of splenocytes from Siglec-g$^{-/-}$ showed an approximately seven-fold increase in CD19^{+}B220^{-} B cells (figure 2.19). Thus Siglec-g deficient mice seemed a useful model to test the effects of excess B1 cells *in vivo*. In addition to an increase in the B1 cell population, Siglec-g$^{-/-}$ B cells also have a lower threshold of activation given the protein's inhibitory function. We concluded that this feature was also useful when investigating the effects of excess B cell function in AKI.

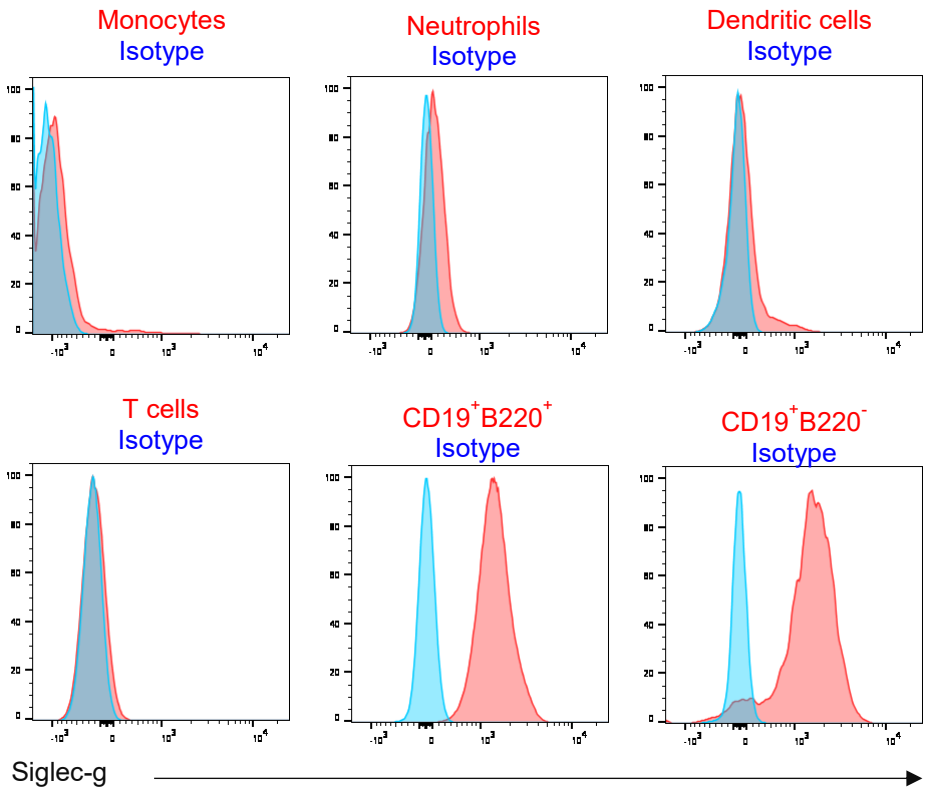


Figure 2.18. Representative flow cytometry histograms illustrating expression of Siglec-g in different immune cells. Blue histograms represent isotype control.

Low-dose intraperitoneal folic acid (150mg/kg) was given to Siglec-g$^{+/+}$ wildtype and Siglec-g$^{-/-}$ mice. We found that in younger mice which were under 25 grams, there was no difference in serum urea levels (figure 2.20). However, above 25 grams, wildtype mice showed only a small urea rise after folic acid induced AKI, while Siglec-g$^{-/-}$ mice had significantly higher urea levels. The knockout mice had higher numbers of neutrophils in the circulation and kidney as well as CD19^{+}B220^{-} B cells in the kidney (figure 2.21).

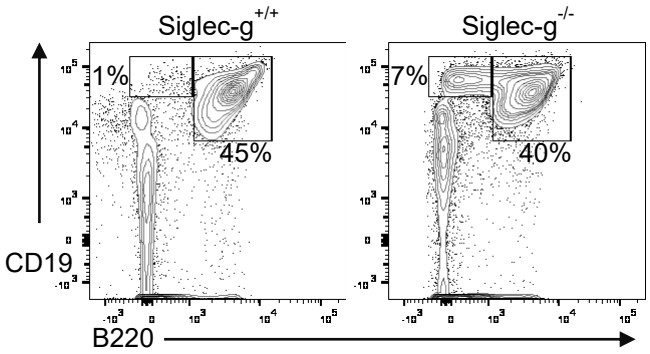


Figure 2.19 Representative flow cytometry plot of splenic CD45 positive leucocytes from Siglec-g$^{+/+}$ wildtype and Siglec-g$^{-/-}$ mice.

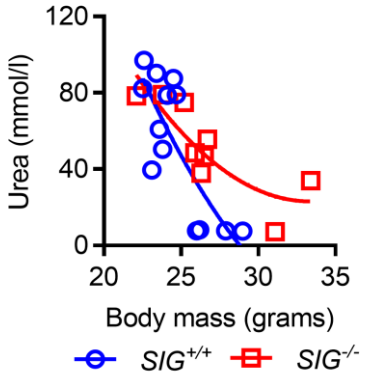


Figure 2.20. Serum urea concentrations from mice given low-dose folic acid induced AKI. Mice culled and tissue processed 16 hours following insult. N=22 in total (13 and 9 in each group).

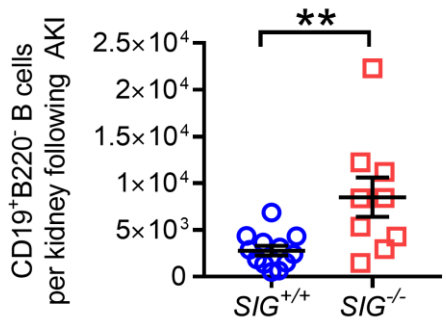
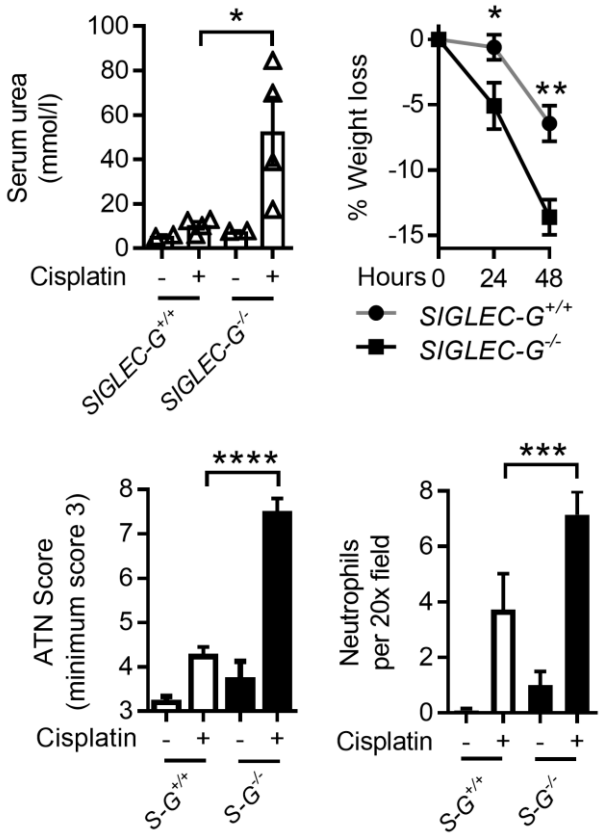


Figure 2.21. Extravascular renal $CD19^{+}B220^{-}$ B cells following low-dose folic acid induced AKI. Mice culled and tissue processed 16 hours following insult. N=22 in total (13 and 9 in each group). Significance analysed using a parametric unpaired two-tailed T test. Error bars represent standard error of the mean (SEM).

We repeated experiments in two other models of AKI: cisplatin-induced AKI and temporary surgical clamping of the renal pedicle. Following cisplatin-induced AKI, urea levels were approximately four times greater and weight loss was more than 5 per cent greater in the Siglec-$g^{-/-}$ group (figure 2.22). Histological sections of the kidney were viewed using confocal microscopy and stained with a GR1 antibody. GR1 positive cells per visual field were significantly greater in number in Siglec-$g^{-/-}$ mice following AKI. Haematoxylin and eosin staining of fixed tissue was analysed in a blinded fashion by a trainee nephrologist (figure 2.22 and 2.23). Tissue was scored on the basis of tubular necrosis, tubular dilatation and cast formation, which is a standard scoring system for experimental AKI (Clatworthy, et al. 2012).



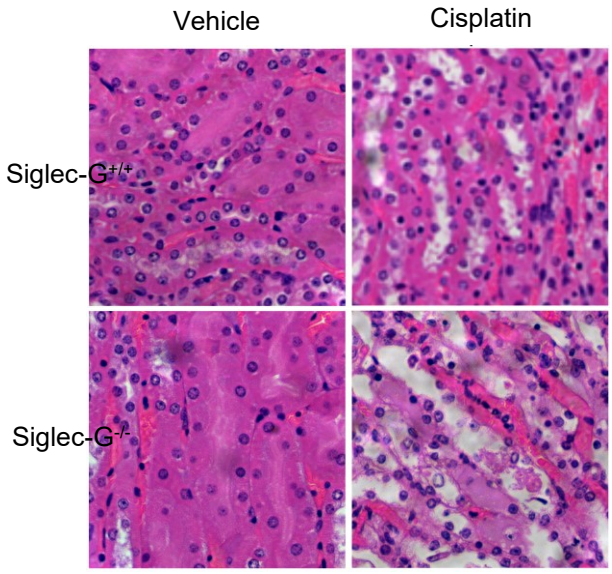


Figure 2.22 (top) Cisplatin-induced AKI in Siglec-g$^{-/-}$ mice. Quantification of serum urea, weight loss, ATN score and neutrophil infiltration in the kidney. Figure 2.23 (bottom) Representative histological sections of kidney (right). N=12 in total. Significance analysed using a parametric unpaired two-tailed T test. Error bars represent standard error of the mean (SEM).

Although cisplatin remains a commonly used experimental tool to induce AKI, the agent has a number of disadvantages. For example, given its chemotherapeutic mode of action of inhibiting DNA replication by forming adducts between the cisplatin molecule and DNA, the nephrotoxin could have immunological side-effects which may not be helpful in investigating the immune response during AKI. Therefore, another model of AKI was used. In collaboration with our surgical colleagues, Mr Kourosh Saeb-Parsy and Mr Jack Martin, laparotomised mice underwent temporary renal pedicle clamping for 45 minutes under general anaesthetic. As observed in the cisplatin model, Siglec-g$^{-/-}$ mice had similarly higher levels of urea and increased neutrophil levels in the kidney when this surgical approach was used (figure 2.24).

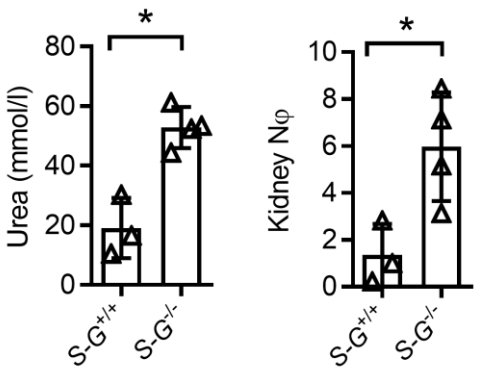


Figure 2.24: Surgical renal pedicle clamping in AKI. Quantification of serum urea and neutrophil infiltration by flow cytometry. N=7 in total (3 and 4 in respective groups). Neutrophils as a percentage of all viable events. Please note this experiment was done before the technique for labelling intravascular and extravascular leucocytes was developed. This experiment was conducted once. Mice culled and tissue processed 16 hours following insult. Significance analysed using a parametric unpaired two-tailed T test. Error bars represent standard error of the mean (SEM).

A number of caveats to this set of experiments should be mentioned. The main consideration is that the wildtype mice used in these experiments were bred in parallel but were not sibling littermates. As with other experiments discussed in this thesis, there may be some degree of genetic drift that has taken place which we have not actively analysed. In addition, as the birthdates are not identical between compared mice in such a model, AKI may be induced at slightly different timepoints in the mouse's life. We did not use littermate siblings for a number of reasons including cost and efficiency as mice heterozygous in the Siglec-g gene would need to be culled as they would not be required for these experiments and larger numbers of wildtypes or knockout mice could be generated (especially if bred as a trio) in parallel as all offspring would be of one preferred genotype or another.

2.1.6 Sialic acid and its amelioration of AKI

One of the agonist ligands for Siglec-g and other Siglec proteins is sialic acid. In humans, the normal physiological level of sialic acid is about 68mg/100ml (Carter and Martin 1962). We hypothesized that administration of intravenous sialic acid immediately prior to giving intraperitoneal cisplatin to wildtype mice during AKI may ameliorate the condition. Using a naturally occurring sialic acid, Neu5AC, during AKI, we observed reduced urea levels as well as neutrophil infiltration (figure 2.25).

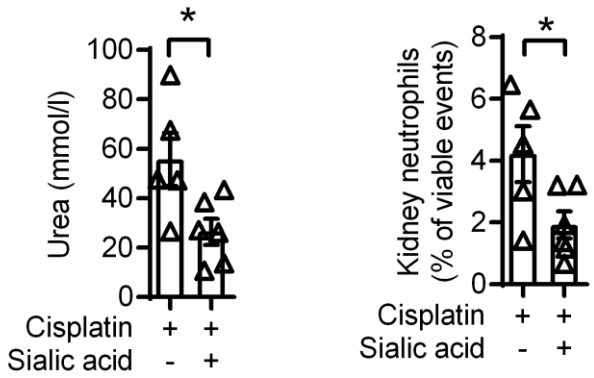


Figure 2.25: Intravenous sialic acid administration immediately prior to intraperitoneal cisplatin-induced AKI ameliorates disease severity. Quantification of serum urea and neutrophil infiltration. N=11 in total (5 and 6 mice in each respective group). Neutrophils as a percentage of all viable cells. Please note this experiment was done before the technique for labelling intravascular and extravascular leucocytes was developed. Mice culled and tissue processed 16 hours following insult. Experiment repeated twice. Significance analysed using a parametric unpaired two-tailed T test. Error bars represent standard error of the mean (SEM).

This experiment poses a number of questions which remain unanswered. For example, is it possible to know whether the effect of sialic acid is specific to Siglec-g? Even more specifically, is the effect specific to Siglec-g on B cells? The causality remains unclear. To address whether sialic acid is having a specific effect on Siglec-g, one possible experiment

would be to test its effects in Siglec-g knockout mice. To answer whether Siglec-g specifically affects B cells, it would be necessary to use a mouse with a B cell specific deficiency of Siglec-g by employing, for example, a lox-cre construct. In future, we also plan to analyse the numbers and characteristics of the infiltrating B cells when sialic acid is given.

2.1.7 Human AKI kidneys and expression of *CCL7* and *CXCL2*

To assess the relevance of these findings in human disease, we investigated kidney samples from deceased transplant donors (Table 2.1). The demographic details were collected (Table 2.1) and all samples were from the renal cortex. They included 8 patients without AKI and 7 patients with AKI. Whole kidney lysates were analysed by qPCR for *CCL7*, *CXCL2* and *CD19*. *CCL7* was undetectable in all but one non-AKI kidney sample (figure 2.26). All but two AKI kidneys had high levels of *CCL7* transcript. Significant differences were observed in *CXCL2* levels. *CD19* transcript levels, which represent B cell infiltration, were not significantly different. However, there was an overall trend towards higher levels in the AKI group.

Anonymised donor number	Age/Sex	Serum creatinine (µmol/L)	Cause of death
10	57/Male	52	Intracranial haemorrhage
14	78/Female	50	Intracranial haemorrhage
35	59/Female	48	Hypoxic brain injury from choking
37	70/Male	57	Fall and hypoxic brain injury
41	48/Male	31	Pneumonia
43	77/Male	54	Aortic dissection
65	73/Female	52	Intracranial haemorrhage
121	69/Female	44	Intracranial haemorrhage
20	55/Male	210	Cardiac arrest (cause unknown)
18	57/Female	167	Cardiac arrest (cause unknown)
52	73/Male	180	Unknown
62	61/Male	230	Cardiac arrest (cause unknown)
67	71/Male	174	Hypoxic brain injury
77	36/Male	145	Intracranial haemorrhage
94	66/Male	263	Intracranial haemorrhage

Table 2.1. Demographic details of the patients. Blue font represents donors with no AKI according to pre-terminal serum creatinine and red font represents donors with AKI. Serum creatinine was the last sample taken prior to death.

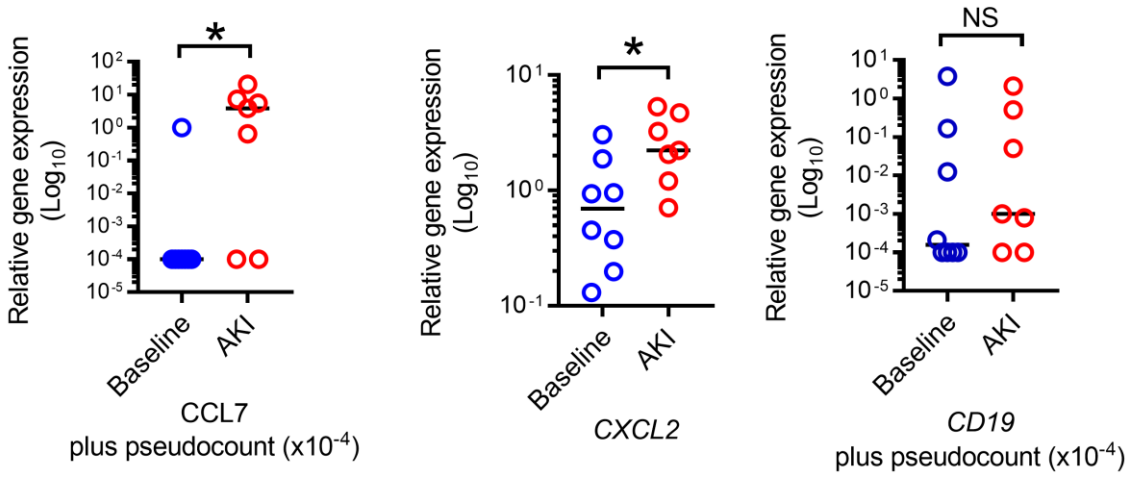


Figure 2.26 *CCL7*, *CXCL2* and *CD19* transcript levels respectively from non-AKI and AKI cadaveric donor kidneys. N=15 in total (8 and 7 donors in each respective group). Significance analysed using a parametric unpaired two-tailed T test.

The significance of these data is that they begin to recapitulate in humans murine findings described earlier. Clearly, even within the small number of subjects in this study, there are greater amounts of CCL7 and a trend towards greater amounts of CD19 transcripts indicative of B cell infiltration into human kidneys with AKI.

It should be mentioned that there are a number of small caveats to these data which do not detract from the central thrust of the argument but are nonetheless worth discussing. First, it is not clear that patients who have recently sustained a severe illness such as non-recoverable intracranial haemorrhage and subsequently died, even when serum creatinine is within the normal range, can be assumed to have normal kidneys. For example, hormonal surges just prior to death may be altering the transcriptomic landscape within renal tissue.

Ideally, it will be necessary in future to derive corroborative data from, for example, biopsies taken from normal kidneys such as during a live-donor kidney transplant.

Second, the samples were not collected wholly randomly. For example, members of the laboratory may have decided to turn down offers of kidneys for research for a whole range of reasons including long ischaemic times or severe AKI. In other words, kidneys which may have suffered from excessive ischaemic injury – and are therefore the kidneys most appropriate for this study – may have been inadvertently excluded. Indeed, of over 200 kidneys collected, only a handful had AKI. All kidneys which sustained AKI and were snap-frozen, and therefore suitable for processing, were included in this study. Control samples were chosen from the first eight samples found with serum creatinine of less than 60 µmol/L.

Third, we do not yet have direct evidence that, in human kidneys, the primary source of CCL7 is from extravascular B cells within the kidney. To ascertain this, I have started an experiment to process human kidney tissue by flow cytometry using an intracellular CCL7 antibody. Initial experiments show that B cells appear to produce the greatest amount of CCL7 of all leucocytes, however, further investigation is necessary to confirm these findings.

Finally, to investigate whether *CCL7* has the potential to be a biomarker of AKI, we obtained urine from healthy subjects and hospital in-patients who had AKI. Consistent with our findings from whole kidney transcripts, CCL7 concentrations were higher in the urine of patients with AKI (figure 2.27).

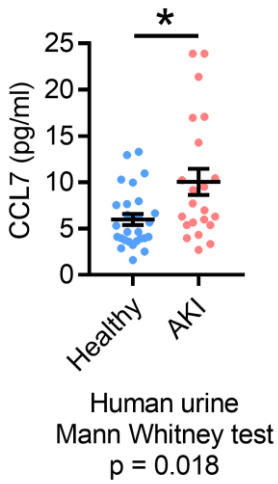


Figure 2.27. CCL7 concentration in the urine from healthy and AKI subjects measured by ELISA. Significance analysed using a parametric unpaired two-tailed T test. Error bars represent standard error of the mean (SEM).N=49 in total.

While it is tempting to infer from these data that CCL7 may represent a potential biomarker of AKI, this will require further validation due to a number of limitations of the current dataset we have generated. Firstly, we selected CCL7 for measurement in a hypothesis-driven manner based on murine data. However, there are many molecules present in urine and an unbiased assessment using, a broader, proteomic or metabolomic approach might identify additional more sensitive and specific biomarkers.

Secondly, during most phases of AKI, urine concentration can be deranged as a result of increased tubular permeability and lower osmolarity of the renal interstitial fluid. As a result, the vast majority of molecules may be more dilute. To adjust for this, other investigators have simultaneously measured urinary creatinine which can provide a surrogate marker for urinary concentration. For example in clinical practice, urinary albumin levels have historically been measured over a 24 hour period (which can be practically tedious for patients) but this has now largely been replaced by measurement of the ratio of albumin to creatinine in a spot urine sample. Thirdly, our data would require validation in an independent, preferably larger, sample collection.

2.2 Discussion

In summary, observation of B cell dynamics during the early hours of AKI suggests they have an important role in mobilising other immune cells into the kidneys. Within 16 hours following induction of AKI, a large number of $CD11b^{+}CD19^{+}B220^{-}$ B cells appear in the circulation and employ the MAC-1 complex to enter inflamed renal tissue. After entering the kidneys, B cells release CCL7 which has the potential to attract a range of myeloid cells receptive to this cytokine. Blockade of CCL7 can ameliorate disease. Conversely, deficiency of Siglec-g, an inhibitory protein found predominantly on B cells, leads to an excess of B1 cells and lower threshold of activation in B cells and an increase in the severity of AKI. A Siglec-g

agonist, sialic acid, ameliorates AKI. Finally, analysis of human kidneys with AKI shows an increase in CCL7 suggesting there is clear human relevance to these findings.

These findings are in concordance with previous studies which have shown that CCL7 from B cells is crucial in attracting myeloid cell populations during myocardial infarction (Zouggari, et al. 2013). Additionally, just as the lack of Siglec-G worsened AKI outcomes in our study, others have shown the absence of Siglec-g exacerbating B cell dependent autoimmune conditions such as arthritis (Bökers, Urbat, et al. 2014).

2.3 Future plans

A number of additional experiments would be useful to build on this work. In humans, we know there is an activated form of CD11b where a conformational change has taken place. In fact, an antibody has been developed which specifically identifies activated CD11b in human cells (Diamond and Springer 1993). In mice, however, an antibody has not yet been developed which identifies the activated form of this surface protein. It would be useful to have such a tool to assess whether, during AKI, B cells change their CD11b conformation. Indeed conformational change may explain why there are more $CD11b^+$ B cells in the renal vasculature during the healthy state.

Moreover, it may be that $CD11b^+$ is in some way involved in cytokine signalling. Previous publications have suggested MAC-1 has downstream effects on the transcription factor NF-κβ. Experiments not described here have not shown a significant increase in CCL7 levels following stimulation with either or both CpG and soluble ICAM. However, given that one of the most potent inducers of CCL7 is BCR antigen as shown in figure 2.14, perhaps concomitant stimulation with antigen and ICAM may induce greater amounts of CCL7. *In vivo*, it is possible that B cells may be reacting, for example, to apoptotic cells via BCR ligation.

Chapter 3: Induction of regulatory B cells *in vivo*

The public health burden of autoimmune diseases and organ transplantation is substantial and increasing. Though it is difficult to generate accurate statistics due to discrepancies in classification, one recent publication conservatively estimates that in the United States alone there were approximately 14.7 million people diagnosed with some form of autoimmune disease, an increase from 9 million 15 years previously (Hayter and Cook 2012) (Jacobson, et al. 1997). Organ transplants have also progressively increased over the past decade. In 2016, 33,600 organ transplants were undertaken in the United States – a 20 percent increase from five years previously (United Network for Organ Sharing 2017). The majority of patients with autoimmune disease and virtually all patients with organ transplantation require some form of immunomodulatory therapy for disease control and prevention of immunological rejection respectively.

The potential wide-ranging role of B-regs in autoimmunity and transplantation, as discussed in the introduction, begs the question whether this cell type can be effectively harnessed therapeutically in humans. The majority of studies to date have relied on murine models involving B-reg cellular transfer followed by observation of the effect on disease severity. However, directly translating these models into human treatments is difficult as cellular therapies are labour-intensive and costly (Abou-El-Enein, Bauer, Medcalf, Volk, & Reinke, 2016). Additionally, other questions such as the long-term efficacy and safety of cellular therapies remain unanswered. For example, how long do *ex vivo*-induced B-regs retain their regulatory phenotype and what is the risk of these cells undergoing malignant

transformation? At the same time, inducing B-regs *in vivo* through pharmacological means has not been adequately explored. From an economic and regulatory point of view, repurposing of previously approved drugs may be an attractive alternative to developing new therapies.

With these clinical and scientific problems in mind, two strands of research were planned. First, we noted transcriptional studies by others demonstrating high expression of CD25, the alpha component of the trimeric IL-2 receptor, on IL-10-producing B-regs (van de Veen, et al. 2013) and speculated whether IL-2 may play a role in modulating IL-10 production and how the cytokine may fit with our understanding of the signalling pathway. Second, given the recent renewed interest in low-dose recombinant human IL-2 therapy as a potent inducer of regulatory T cells in a number of human clinical trials, including at least four local trials in Cambridge, England, an analysis of patients' peripheral blood samples was undertaken to see whether low-dose IL-2 therapy augments B-reg populations.

We hypothesized the following:

1. Some B cells express the trimeric IL-2 receptor and can be receptive to IL-2.
2. Given that downstream of the IL-2 receptor is the PI3k pathway, engagement of the IL-2 receptor may skew B cells towards to a more anti-inflammatory phenotype.
3. *In-vivo* administration of low dose IL-2 may induce regulatory B cells in mice and humans with autoimmune conditions and may provide a novel treatment option for a range of conditions where regulatory B cells have been implicated.

3.1 Results

3.1.1 IL-2 and IL-10 production in B cells

Reanalysis of publicly available data suggested higher CD25 transcript levels in IL-10 producing human B cells compared with non-producing cells (Figure 3.1).

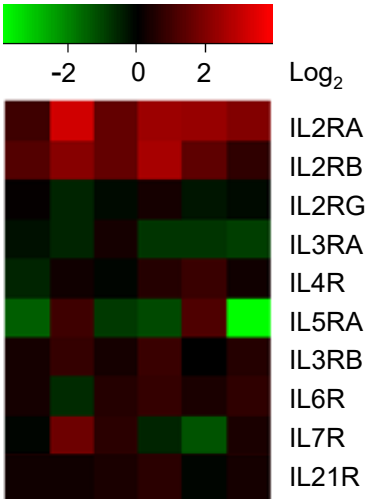
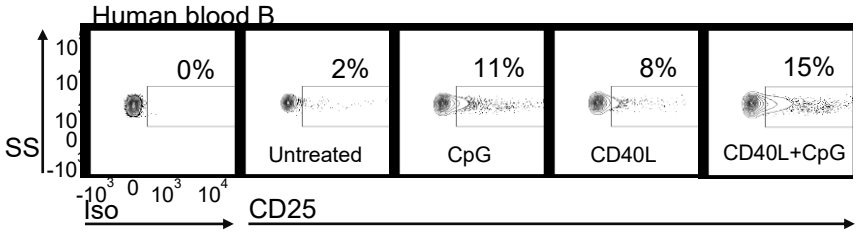


Figure 3.1. Interleukin receptors sharing the common IL2Rγ chain (CD132) on IL-10 producing regulatory B cells over non regulatory B cells. Raw gene expression data were extracted from a publically available transcriptomic dataset (GEO accession number GSE35002) generated by (van de Veen, et al. 2013) in which IL10 positive and negative B cells were transcriptionally characterised using gene mircoarrays. Each column represents an independent sample of regulatory B cells. Brightness of red represents log-fold increase (brightness of green represents log-fold decrease) in RNA over non-regulatory B cells. N = 6 healthy donors.

To assess whether these RNA data correlate to actual protein expression, we conducted a number of flow cytometry experiments on B cells. In human peripheral blood, basal surface CD25 expression was detectable in only 2% of B cells (Figures 3.2 and 3.3). However, following activation *in vitro* with the TLR9 ligand CpG alone, or with CD40 ligand, we observed a five to seven-fold increase in the proportion of CD25-positive cells ($p<0.01$; Figure 3.2).



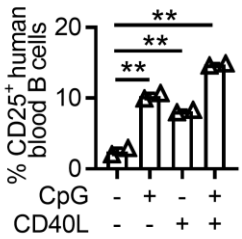
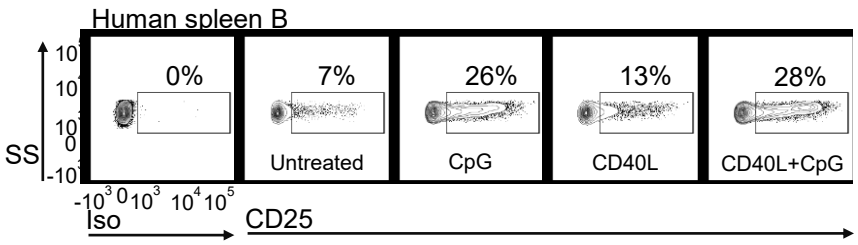


Figure 3.2 (top). Human blood B cell expression of CD25 following overnight stimulation with TLR and CD40 ligands. Leucocytes were isolated through a histopaque gradient. Figure 3.3 (bottom). Quantification of percentage of CD25 positive human blood B cells following stimulation. Single healthy blood donor. Experiment conducted three times with similar results. Significance calculated using a non-paired parametric two-tailed T-test. Asterisks represent the following p values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS not significant. Error bars represent standard error of the mean (SEM).

Since B cell activation takes place in secondary lymphoid organs, and B cells in these organs differ in their functional characteristics and their responsiveness to immunomodulation, we also investigated human splenic B cells which were donated after death. We found an increase in the proportion of B cells that expressed CD25 following stimulation with CpG and CD40L, from 7% at baseline to 28% post-stimulation ($p<0.001$, figures 3.4 and 3.5). Similarly, in murine splenic B cells, CD25 expression was markedly increased following stimulation with TLR ligands and CD40 ligation (figures 3.6 and 3.7). It should be noted that in murine *in vitro* stimulation experiments, we used LPS as well as CpG and CD40 ligand. In human experiments, we did not use LPS, as TLR4 is minimally expressed by unstimulated human B cells (Bourke, et al. 2003) (Hornung, et al. 2002) (Muzio, et al. 2000).



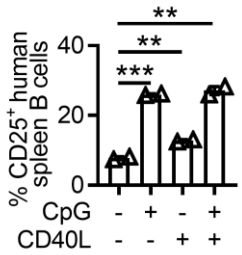
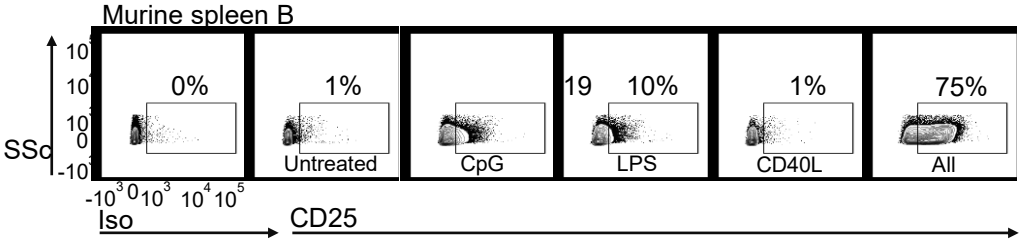


Figure 3.4 (top). Human splenic B cell expression of CD25 following overnight stimulation with TLR and CD40 ligands. Figure 3.5 (bottom). Quantification of percentage of CD25 positive human splenic B cells following stimulation. Significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean (SEM). Single human spleen. Experiment conducted three times with similar results.



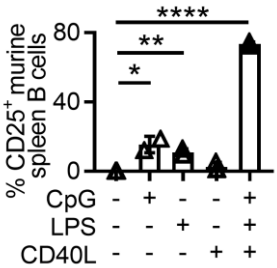


Figure 3.6 (top). Murine splenic B cell expression of CD25 following overnight stimulation with TLR and CD40 ligands. Figure 3.7 (bottom) Quantification of percentage of CD25 positive murine splenic B cells following stimulation. Significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean (SEM). Single murine spleen. Experiment conducted three times with similar results.

Together these data show that in both humans and mice at rest, a small proportion of peripheral and splenic B cells have the capacity to respond to IL-2, but that following activation following conventional stimuli, an upregulation of CD25 significantly increases this number.

On reflection, it could be argued that one weakness in these B cell stimulation experiments (figures 3.2-3.6) is that samples have been derived from a single human or mouse and that replicates are experimental and not biological, though each of these experiments have been conducted multiple times. Here, biological replicates were not undertaken for a couple of reasons: First, though the overall pattern of CD25 expression following stimulation does not differ between individuals, the actual proportional values do differ. For example, following CpG stimulation, B cells from some subjects expressed greater amounts of CD25 than other subjects at baseline without stimulation and this discrepancy was accentuated when stimulated with ligands such as CpG and CD40 ligand. Therefore, given this variability and given the cost and time involved to achieve statistical significance these larger experiments were not conducted. Moreover, given that the purpose of these experiments was simply to prove that B cell had the *potential* to express CD25 and not to quantify a population average it did not seem appropriate to pursue leucocyte samples from a larger cohort of patients. Finally as human spleens were donated at irregular and unpredictable times, it was not possible to process the samples simultaneously. As a result, further discrepancy between samples would be introduced by repeating the experiment at different times.

Another question which these experiments do not answer is which types of B cells most avidly upregulate their CD25 expression. We did not co-stain for other surface markers as these often change after stimulation. One potential future experiment would be to pre-sort different B cell subsets and to stimulate them and then stain for CD25.

3.1.2 Difficulty with assessing CD25 on human B cells

Interestingly, although we observed little CD25 on the surface of unstimulated B cells, this may not be the case *in vivo*. Suspicion that there may be more CD25 expression than previously seen on resting B cells arose while looking at data from Prof John Todd's research

group which showed significantly greater amounts of CD25 expression by unstimulated B cells. This group had analysed leucocytes in a flow cytometer after strong red cell lysis but no histopaque gradient. To assess this further, we conducted a head-to-head analysis. Human blood was taken from a subject and mixed with citrate and red cell lysis. Even if the red cell lysis was subjectively inadequate with the sample still retaining a rosé coloured appearance, the cells were stained for CD19 and CD25. What would normally be considered an excessive number of events were collected on flow cytometry on the assumption that the majority of events would be irrelevant red blood cells. In a second sample, leucocytes underwent a histopaque gradient followed by red cell lysis and antibody staining. A comparison was made between these two different preparations.

Two observations can be derived from this experiment (figure 3.7). Firstly, in samples taken from the same subject, there was considerably more CD25 in unstimulated cells than shown above (figure 3.2 to figure 3.4). Secondly, there was considerably less CD25 following the longer and more strenuous leucocyte preparation involving a histopaque gradient. These observations led us to test whether CD25 was released from stimulated splenic human B cells which had been negatively isolated (figure 3.8). The supernatant from these cells was analysed by ELISA. Indeed, large amounts of soluble CD25 were detected.

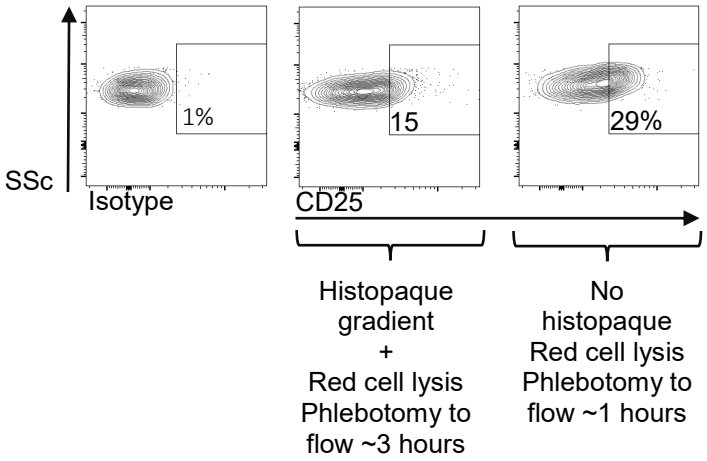


Figure 3.7. Representative flow cytometry plots of CD25 expression by human blood B cells following different preparation techniques. Single subject. Events gated on single, viable, CD19 positive events.

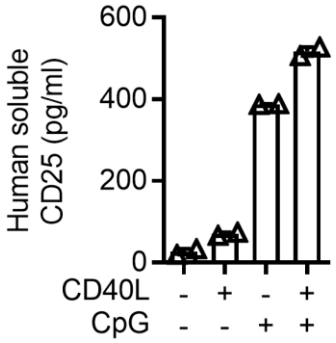


Figure 3.8. Quantification by ELISA of soluble human CD25 following stimulation. Each well contained $5x10^5$ negatively isolated B cells and was stimulated for 60 hours. Error bars represent standard error of the mean (SEM). Single human spleen. Experiment conducted twice with similar results.

These experiments, although not the main focus of investigation, open up a wide variety of questions. For example, it remains unclear whether surface CD25 is being cleaved and released or whether a splice-variant of CD25 which is soluble and does not contain the transmembrane portion of the protein is being released. Moreover, it raises the question of what the biological relevance of B cell-released soluble CD25 might be. Could the molecule be neutralising IL-2? For example, in a recent paper by Li et al, it was shown that IL-2 from T cells can be 'quenched' by soluble CD25 from dendritic cells within the germinal centre (Li, et al. 2016). The original purpose of these experiments was simply to assess the methodological rigour of our experimental techniques and not to assess the biological relevance of this phenomenon. Therefore, at least for the time being, we have not looked at these questions, but they may be topics for further research in the future.

3.1.3 IL-2 and its effect on B cell cytokine production

We next asked how IL-2 would impact B cell cytokine production following activation *in vitro*. Stimulation of human splenic B cells with IL-2 alone did not induce IL-10 (Figure 3.9), IL-6 or TNFα, (data not shown) but addition of even very low quantities of IL-2 to CpG- or CD40L-stimulated B cells led to a 6-fold augmentation of IL10 production (figure 3.9). We confirmed the production of IL-10 through intracellular staining of B cells (figures 3.10)

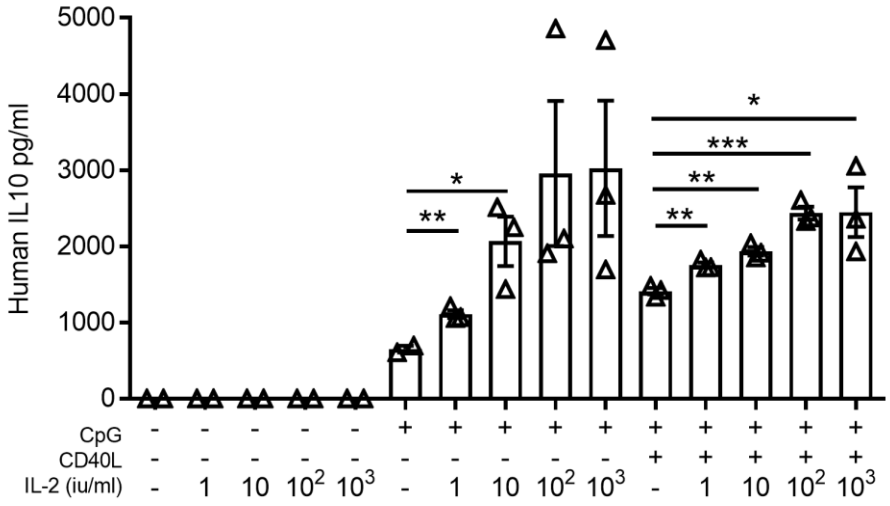


Figure 3.9. Quantification of IL-10 in culture supernatants from negatively isolated human splenic B cells stimulated with IL-2 and/or CpG and/or CD40L. Each well contained $5x10^5$ negatively isolated B cells and was stimulated for 60 hours. Significance calculated using a non-paired parametric two-tailed T-test. Error bars represent standard error of the mean. Single human spleen. Experiment conducted three times on different donated human spleen B cells with similar results.

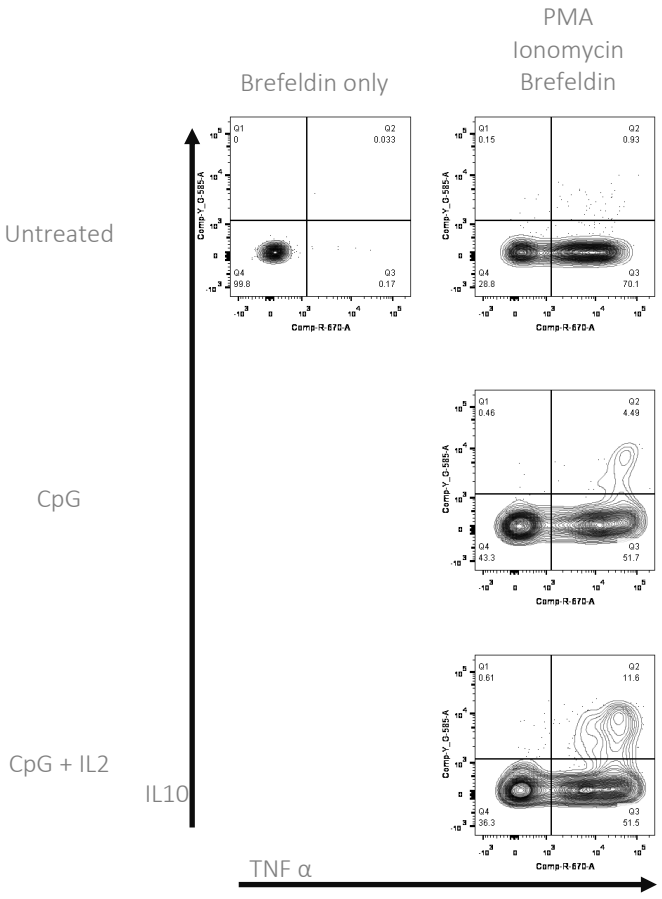
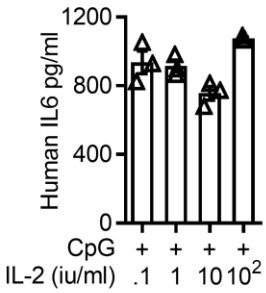
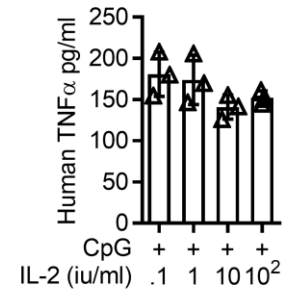


Figure 3.10: Representative flow plots of IL-10 and TNFα expression following IL-2 and CpG stimulation followed by co-culture with brefeldin, ionomycin and PMA (a standard protocol for staining for IL-10). These B cells were negatively selected from a single human spleen sample using a magnetic selection kit. Cells were gated on single, viable CD19 positive B cells. This experiment was conducted twice with an independent human spleen sample with similar results.

In contrast, IL-6 and TNFα production was not affected by IL-2 (Figure 3.12 and 3.13) demonstrating that in human B cells, IL-2 skews their cytokine profile towards immunoregulation.





Figures 3.12 (left) and 3.13 (right). Quantification of IL-6 and TNFα in culture supernatant from human splenic B cells stimulated with IL-2 and CpG. Each well contained $5x10^5$ negatively isolated B cells and was stimulated for 60 hours. Error bars represent standard error of the mean (SEM). Single human spleen. Representative of three experimental repeats.

Similarly, in murine splenic B cells, the addition of IL-2 enhanced IL-10 production by CpG stimulated B cells (Figure 3.14), confirming its capacity to augment IL-10-production by B cells *in vitro*.

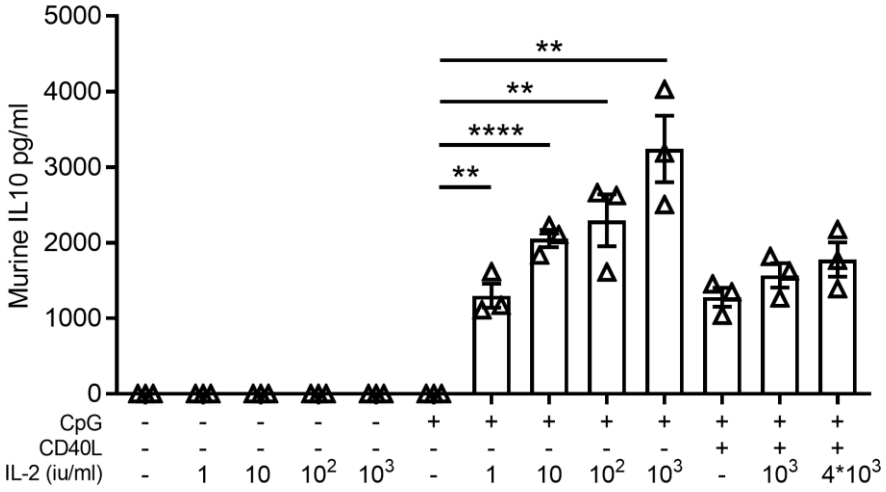


Figure 3.14. Quantification of IL-10 in culture supernatants from murine splenic B cells stimulated with IL-2+/- CpG or CD40L. Each well contained $5x10^5$ negatively isolated B cells and was stimulated for 60 hours. Significance calculated using a non-paired parametric two-tailed T-test. Single murine spleen. Error bars represent standard error of the mean. Representative of three experimental repeats.

To assess whether IL-2-mediated augmentation of IL-10-producing B cells would enhance their regulatory capacity, we activated human B cells *in vitro* in the presence or absence of IL-2, and subsequently co-cultured them with stimulated CD4 T cells. IL-2-treated B cells suppressed CD4+ T cell TNFα production (Figure 3.15), suggesting that IL-2 promotes the development of IL-10 producing B cells with the functional capacity to regulate CD4+ T cells.

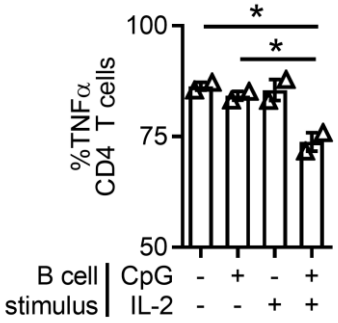
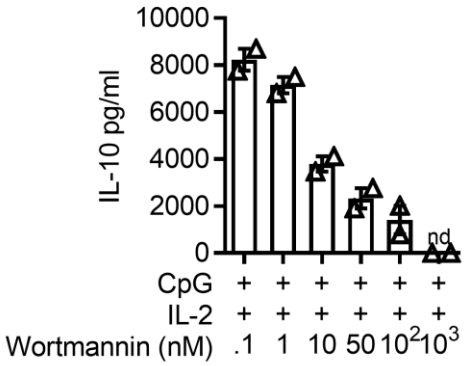


Figure 3.15. Proportion of TNFα positive human CD4 T cells, as evidenced by intracellular cytokine staining, following co-culture with activated B cells generated in the presence or absence of IL-2. Gated on single, viable, CD4 positive events. Graphs show mean and standard error of the mean (SEM) of duplicates. of duplicates. Single human spleen. Significance calculated using a non-paired parametric two-tailed T-test. Representative of three experimental repeats.

3.1.4 Pathways activated by IL-2 in B cells

IL-2 receptor ligation activates three signalling pathways: i) the PI3K/Akt pathway ii) the JAK3/STAT5 pathway and iii) the MEK/ERK pathway. It has been suggested that in some subsets of regulatory B cells, IL-10 production requires Akt activation (Matsushita, Le Huu, et al. 2016). To examine the relative contribution of these pathways in IL-2-induced IL-10 production in B cells, we used inhibitors of PI3K and MEK during stimulation of B cells and assessed supernatant protein levels (figure 3.16 and 3.17). Increasing levels of the PI3K

inhibitor wortmannin caused progressively reduced levels of IL-10. However, increasing levels of the MEK inhibitor U0126 had no or little impact on the cytokine's production.



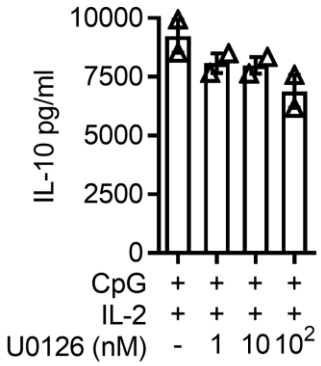
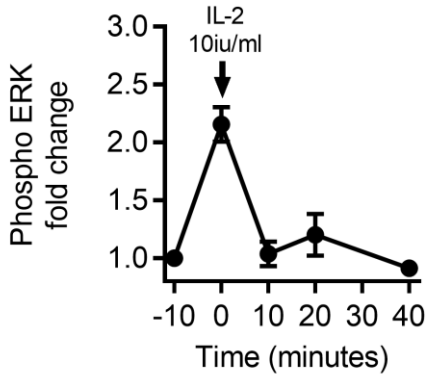
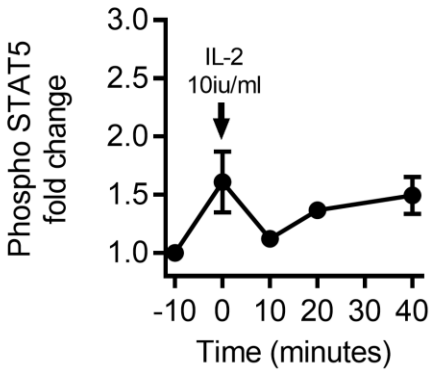


Figure 3.16 (left) B cells stimulated as indicated in the presence of wortmannin, a PI3k inhibitor. Figure 3.17 (right) B cells stimulated in the presence of U-126, a MEK inhibitor. Stimulations in both cases were for 60 hours. Graphs show mean and standard error of the mean (SEM) of duplicates of cells isolated from a single murine spleen. Data representative of two experiments.

We also examined phospho-Akt, phospho-STAT-5 and phospho-ERK levels by intracellular flow cytometry. Initial studies suggested that stimulation with just 10 iu/ml of IL-2 induced an immediate increase in phosphorylated ERK levels followed by normalisation within a few minutes (figure 3.18). It is assumed that even with near-instantaneous fixation of tissue, signalling took place rapidly enough that the ERK is already phosphorylated in the time 0 sample. In contrast, Akt phosphorylation slowly increased but was sustained over the duration of the experiment.





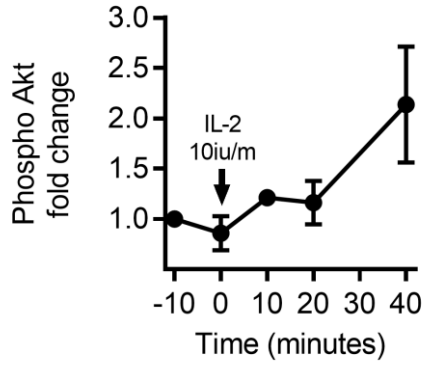


Figure 3.18, 3.19, 3.20 Quantification of phosphorylation of ERK (top), STAT5 (middle) and Akt (bottom) over time following IL-2 stimulation. NB: The data point correlating to 'Time 0' reflects samples which were IL-2-stimulated and then immediately fixed.

There are couple of potential weaknesses in this set of experiments which are worth noting. First, it is not clear whether the reduction in IL-10 production following concomitant inhibition of AKT through wortmannin actually inhibited the production of IL-10, reduced the proliferation of B cells, or even caused cell death of B cells. To understand this better, a viability stain and cell count after the stimulation would seem appropriate. Moreover, wortmannin is known to have some off target effects (Bain, et al. 2007) and other more specific inhibitors may be more appropriate for further testing in future.

3.1.5 *In vivo* effect in mice

We developed an *in vivo* murine model to look at the effects of IL-2 on antibody production. 40 million splenocytes were transferred from a BALB/c mouse into a C57BL/6 mouse intravenously to induce B cell activation and donor-reactive antibody production. In the treatment group, mice were simultaneously given IL-2 daily. This is similar to a model described by Cravedi and colleagues (Cravedi, Lessman and Heeger 2013). We hypothesized that mice given IL-2 would have a greater number of IL-10 producing regulatory B cells. We observed a greater number of CD21high, CD23 high B cells (figures 3.21 and 3.22). This population was enriched in CD9 positive B cells, which have been recently shown to provide a useful marker of regulatory B cells (figure 3.21, far right plot) (J. Sun, et al. 2015). We have not been able to analyse IL-10 production due to problems with intracellular staining. This work is on-going.

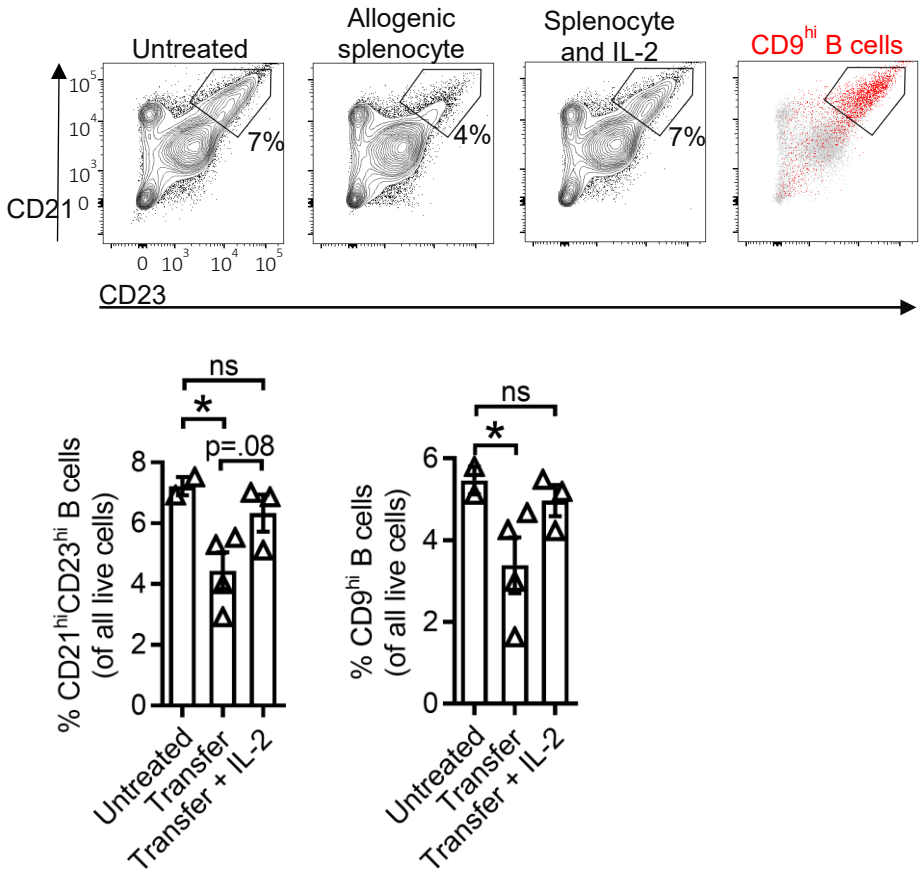


Figure 3.21 (top). Representative flow cytometry plot showing increased numbers of $CD21^{+}CD23^{+}$ transitional B cells following allogeneic splenic

transfer and IL-2 treatment. Far right flow cytometry plot previously gated on single viable CD19 positive CD9 high events. Figures 3.22 and 3.23 (bottom left and right). Quantification of $CD21^{+}CD23^{+}$ transitional B cells. N=9 in total (2-4 per group). Significance calculated using a non-paired parametric two-tailed T-test. Experiment performed twice with similar results.

A number of aspects of the experiments discussed in this section need development. However, before considering these questions it is worth asking whether, given that there are now strong data in the following section suggesting increases in the number of regulatory B cells in humans following low-dose IL-2 treatment, the need to develop this murine experiment may have lessened somewhat. After all, the vast majority of murine experiments in medical science serve as a surrogate to understand human physiology and pharmacology. This reason is in part why this experiment has not been further developed.

If, however, this experiment were to be developed, one of the key questions that still needs to be asked is, while we know that the CD21 high CD23 high population are enriched in CD9 cells, are these cells truly IL-10 producing cells?

3.1.6 *In vivo* effect in humans

To determine the relevance of these observations to the generation of B-regs in humans *in vivo*, we analysed peripheral blood mononuclear cells from patients enrolled in the Adaptive Study of IL2 Dose Frequency on Regulatory T Cells in Type 1 Diabetes (DILfrequency) study (Truman, et al. 2015). Participants enrolled in this low-dose IL-2 trial were aged between 18 and 70 years and had been diagnosed with type I diabetes within 60 months of enrolment. Patient were given a dose (0.09 to 0.47 x 10^6 international units (IU) per m^2) of the recombinant IL-2 (rIL-2) aldesleukin every two to five days. To put these doses into context, a typical dose used for high dose IL-2 for the treatment of renal cell carcinoma or malignant melanoma is in the order of 720,000 iu/kg three times per day for up to 15 doses. In a 75kg individual, this equates to 162x10^6 IU per day. In contrast low-dose therapy is about 0.172 x10^6 units per day for average patient with a surface area of 1.8m^2. Therefore, the low dose IL-2 treatment was approximately one thousand-fold lower than the high dose therapy.

We chose to analyse samples taken from subjects given the lowest aldesleukin dose of 0.09 x 10^6 IU/m^2 as a control as this had no effect on T cells, and from subjects given the high*er* but still generally low-dose ranges of 0.2-0.47 x 10^6 IU/m^2 (Figure 3.24). See above reference for full details of the study.

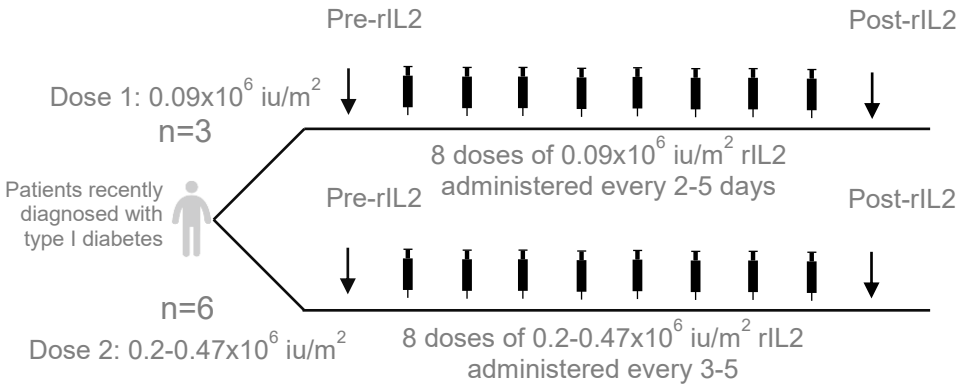


Figure 3.24 Diagrammatic schema summarizing the two treatment arms of the rIL-2 study. Patients with recently diagnosed type 1 diabetes mellitus received negligible dose (0.09x10^6 IU/m^2) or low dose (0.2-0.47x10^6 IU/m^2) aldesleukin every two to five days.

Overall, there was no change in peripheral B cell counts between samples taken prior to rIL-2 administration and those taken at visit 10 (after 8 doses), 20-50 days after commencing treatment, a timepoint when aldesleukin was completely washed out (figures 3.25 and 3.26).

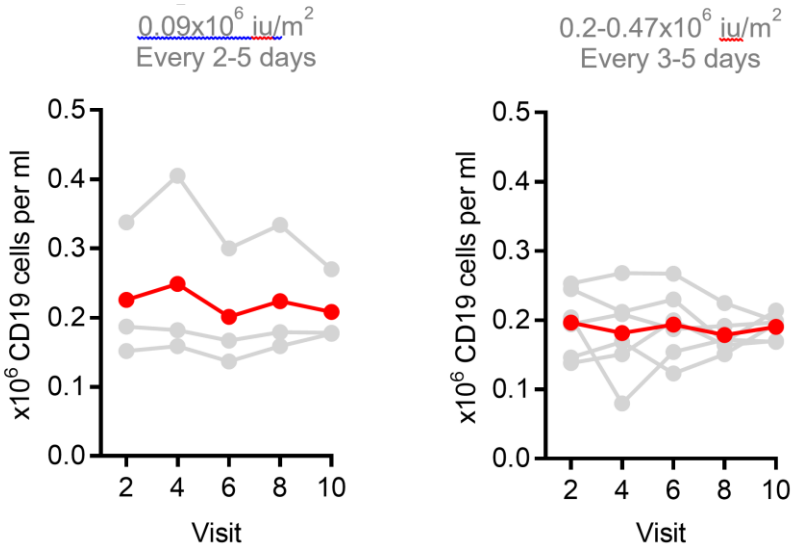
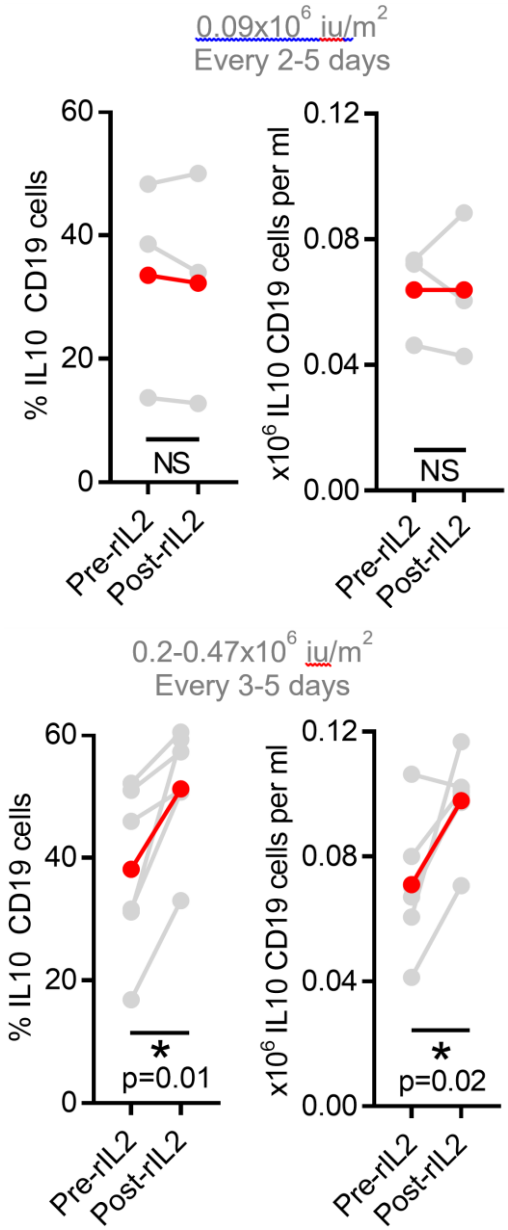


Figure 3.25 (left) Total peripheral blood B cell numbers remain unchanged during rIL2 treatment B cell count taken during visits 2, 4, 6, 8 and 10 of DILfrequency trial of patient given 0.09x10^6 IU/m^2 aldesleukin. Figure 3.26 (right) Total peripheral blood B cell numbers in patients given 0.2-0.47x10^6 IU/m^2 aldesleukin. Red line indicates mean B cell number. There was no significant difference at any time point in comparison to the original timepoint. Significance calculated using a paired parametric two-tailed T-test.

However, in participants who received 0.2-0.47 x 10^6 IU/m^2 rIL-2 we observed a significant increase in the number and proportion of IL-10 producing B cells in contrast to those receiving a 0.09 x 10^6 IU/ml^2 dose of rIL-2 ($p<0.05$; Figures 3.27 to 3.30).



Figures 3.27 and 3.28 (top left and right respectively). Proportion or absolute number of IL-10-positive B cells in patients prior to and following the administration of $0.09x10^6$ IU/m^2 aldesleukin. Figures 3.29 and 3.30 (bottom left and right respectively). Proportion or absolute number of IL-10-positive B cells in patients prior to and following the administration of $0.2\text{-}0.47x10^6$ IU/m^2 (bottom) aldesleukin.

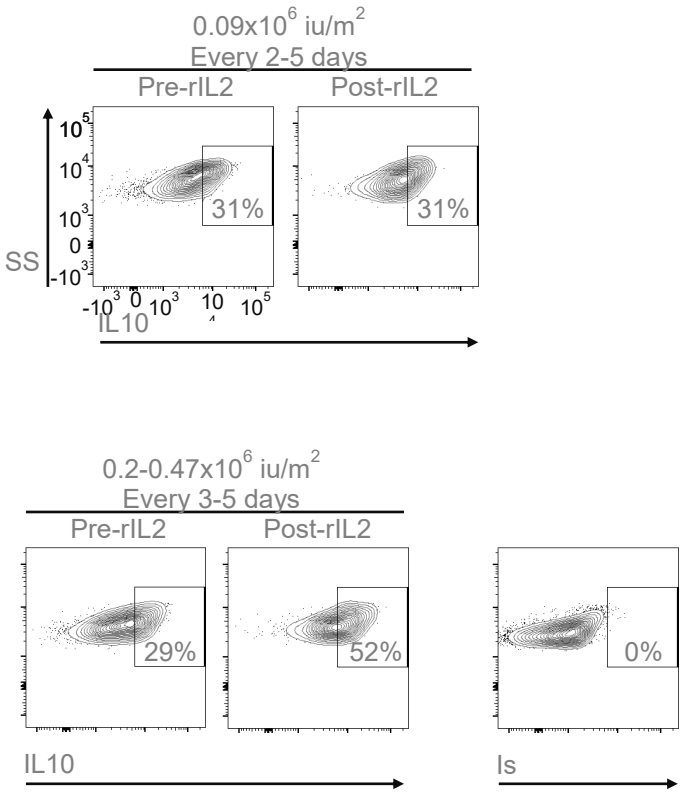


Figure 3.31 (top) Representative flow cytometry plot of IL-10 expressing B cells pre- and post-administration of $0.09x10^6$ IU/m^2 aldesleukin. Figure 3.32 (bottom). Representative flow cytometry plot pre- and post $0.2\text{-}0.47x10^6$ IU/m^2 aldesleukin.

A number of methodological and practical considerations are worth mentioning at this point. Without doubt, a prospective double blinded study would be an ideal methodological framework within which to assess *in vivo* the effects of IL-2 in B cells. However, in a doctoral program which from the outset was not based around a clinical trial, such an undertaking would not be practically possible because of both time and financial commitments required. In addition, although the nine participants were able to generate statistical significance, a greater number of patients in the study would be useful in future studies. Finally, while we are aware that the 0.09 x 10^6 IU/m^2 dose does not have significant effect on T cells and therefore assumed likewise of B cells, a better control population may be use either no IL-2 or a placebo. While these data provide solid provisional results which confirm the increase in

IL-10-producing B cells following low-dose IL-2 administration, these methodological shortcomings show the need for a future study tailored to studying B cells specifically.

3.2 Discussion

In summary, the activation of human peripheral blood and splenic B cells in the presence of IL-2 promotes B cell production of the immunoregulatory cytokine IL-10, augmenting functional CD4 T cell suppression *in vitro*. This appears to be mediated via a PI3k pathway in agreement with earlier experiments. *In vivo*, the administration of low-dose IL-2 to murine and human subjects increased the number of circulating IL-10-producing B cells, with therapeutic potential to modulate deleterious immune responses.

Though B cells may not necessarily encounter both IL-2 and CpG *in vivo*, as discussed earlier, CpG was used as surrogate stimulus in our experiments for TLR ligation and such stimulation may occur in autoimmune conditions where self-nucleic acid is present. In secondary lymphoid organs, adjacent T cells may potentially act as a source of IL2. Therefore, given the proximity of T cells and B cells in secondary lymphoid tissues, it would not be surprising if B cells came in contact with IL-2 *in vivo* in a paracrine fashion along with TLR stimulation.

3.3 Future plans

Many questions remain unanswered and experiments are currently being devised to address these:

1. In a number of disease processes such as systemic lupus erythematosus, it is possible to induce disease remission through the use of IL-2. However, it remains unclear whether this is wholly T-reg-mediated or partially – or even wholly – B-reg mediated given the pluripotent effects of IL-2.
2. How long can B-regs be maintained? For example, what is the maximum effective period between doses? Does the efficacy of IL-2 drop after multiple doses? What is the washout period of IL-2?
3. How do pegylated versions of IL-2 treatment affect drug availability?

4. What is the effect on other types of leucocytes? With the advent of single-cell RNAseq, it may become easier to answer these questions.
5. Given the effects IL-2 has on T follicular helper cells, will immunisation against antigens become less effective in these patients (Ballesteros-Tato, et al. 2012)?
6. Can IL-2 treatment be given to patients with solid-organ transplants? Already, we are aware of one study at Guy's and St Thomas' Hospital, London in which liver transplant recipients are receiving rIL-2 treatment. Can this be expanded to kidney, pancreas and multi-visceral organ transplant recipients? It is our hope that such a trial may be possible soon.
7. Are there other off-the-shelf drugs which might improve outcomes? A screening of currently approved and available drugs may be helpful.

Chapter 4: Conclusion and general future directions

The experiments described in the previous two chapters explore two sides of B cell behaviour independent of their capacity as antibody producers: In the first half of the thesis, I have explored the more pro-inflammatory aspects of B cell behaviour in the context of acute kidney injury (figure 4.1). In the early stages of AKI, $CD11b^{+}CD19^{+}B220^{-}$ B cells become prominent in the blood and these lymphocytes employ the MAC-1 complex to migrate into damaged renal tissue. Upon exiting the vasculature and entering the renal interstitium, B cells release CCL7 which attract a wide range of inflammatory myeloid cells. By blocking CCL7 it is possible to ameliorate disease. Conversely, deficiency of Siglec-g, an inhibitory protein found predominantly on B cells, leads to an excess of B cell activity and B1 cell numbers, and worsens the severity of AKI. A Siglec-g agonist, sialic acid, ameliorates AKI. Analysis of human kidneys with AKI showed an increase in CCL7 levels, suggesting human relevance to these findings.

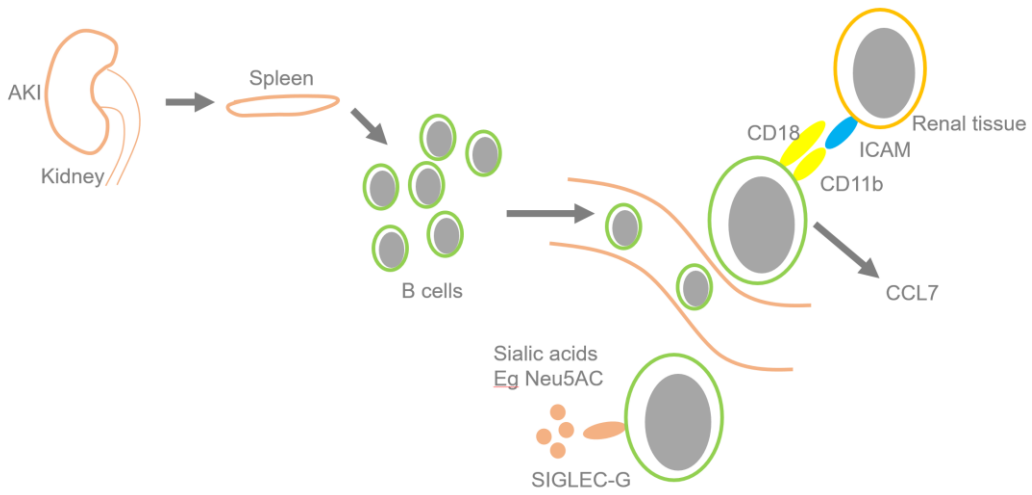


Figure 4.1: Schematic drawing of the behaviour of B cells during AKI.

In the second half of this thesis, I looked at how B cells are able to regulate inflammation. Specifically, I looked how such B cells might be induced *in vivo*. Following activation, B cells upregulate expression of CD25, a key component of the IL-2 receptor, and thus make themselves receptive to IL-2 signalling. The IL-2 signal is crucial in turning B cells from an inflammatory phenotype, defined by the production of IL-6 and TNFα, to a regulatory one defined more by IL-10. *In vivo*, the administration of low-dose IL-2 to both mice and human subjects increased circulating levels of IL-10-producing B cells, suggesting this may be a method of inducing regulatory B cells.

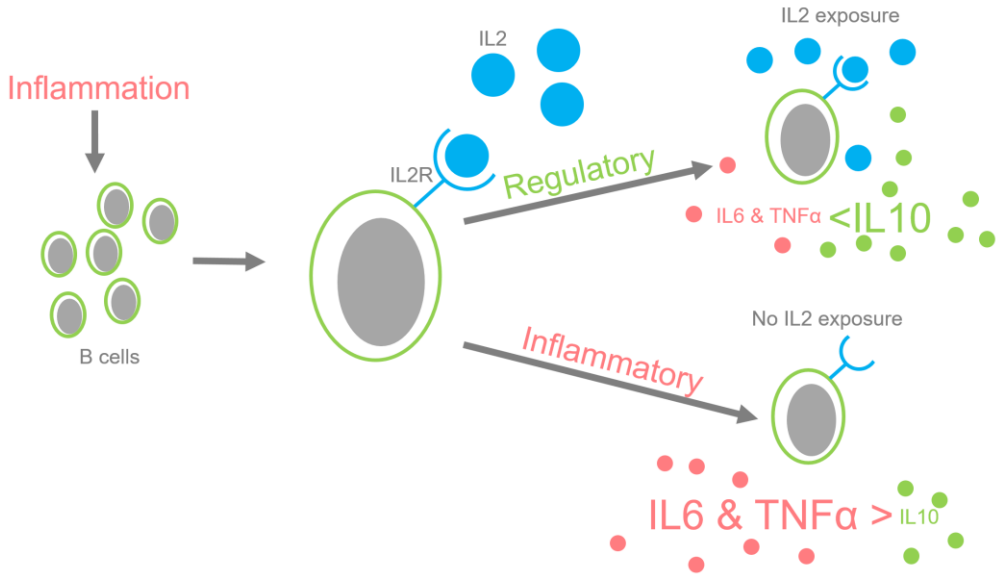


Figure 4.2: Schematic drawing of behaviour of stimulated B cells following IL-2 exposure.

A number questions – and perhaps potential conflicts – arise from these two sections and are discussed in this conclusion:

1. The most obvious question which brings together both of these chapters, is how it is possible to reconcile these almost diametrically opposing functions of proinflammatory B cells in AKI on the one hand and anti-inflammatory B cells induced by IL-2 on the other. This raises the question, more specifically,

of whether the same B cell can have the dual pro-inflammatory and anti-inflammatory roles, and if so, is the B cell's functional remit temporally and environmentally dictated? For example, in AKI, are infiltrating B cells initially pro-inflammatory but later anti-inflammatory during the resolution phase? Or are these dual roles of B cells the result of distinct B cell populations, one pro-inflammatory and the other anti-inflammatory?

2. How do CD11b-expressing and CCL7-producing B cells fit into our current understanding of inflammatory B cells?
3. How does the role of IL-2 fit into our current understanding of regulatory B cells?

4.1 The duality of B cells

Our experiments show that B cells can have both pro- and anti- inflammatory effects. Such dual functionality is a phenomenon observed in many immune cell subsets; for example, there are CD4 and CD8 T cells with both effector and regulatory functions (Dias, et al. 2017). In the germinal centre, there are T follicular helper cells and T follicular regulatory cells (Linterman, et al. 2011). Similarly in innate immunity, macrophages may produce pro-inflammatory cytokines and chemokines, but in other contexts produce negative regulatory cytokines including IL-10 (Cohen and Mosser 2013).

Our data show that B cells may also have diverse functions in immunity beyond antibody production. In the context of AKI, B cells produce the monocyte-recruiting chemokine CCL7, promoting inflammation. This is consistent with previous descriptions of B cell function following myocardial infarction (Zouggari, et al. 2013).

We also show that B cells can produce the regulatory cytokine IL-10, and this is augmented by exogenous IL-2. This raises the possibility that the current use of low dose IL-2 clinically might induce B cells with a regulatory function. This may also be relevant to AKI. Our experiments addressed the role of B cells at early time points, but over time there may be transformation towards a more regulatory B cell phenotype. This concept is supported by data showing that during dehydration and AKI, IL-2 levels within the kidney rise (Goes, et al. 1995) (Sugama, et al. 2013). More recently, there has been a suggestion that administration of either an IL-2/anti-IL-2 complex or IL-233, a hybrid cytokine of IL-2 and IL-33, ameliorates

AKI by their effects on regulatory T cells (Kim, et al. 2013). However this effect could be mediated via effects on B cells rather than T cells alone. Such observations provide the impetus for future experiments.

4.2 CD11b-expressing and CCL7-producing B cells and how these ideas may fit into the current literature

The notion of CD11b-expressing B cells, while controversial, has not been without a body of literature as previously discussed. Most recently, Liu and colleagues have described CD11b positive B cells in the liver having an immunoregulatory effect on CD4 T cells in a murine experimental model of autoimmune hepatitis (Liu, et al. 2015). Moreover, there is extensive literature on how myeloid cells take advantage of CD11b and CD18 to enter endothelium. Thus, it is not particularly surprising that CD11b might be the mechanism by which B cells enter renal tissue.

Broadly speaking, one of the main questions that remains unanswered is whether CD11b expression on B cells regulate the severity of AKI. To answer this, two potential approaches are possible. One is to give either CD11b wildtype or knockout B cells to a B cell deficient mouse such as a µMt mouse, then give a renal insult and analyse the outcome by serum and tissue analysis (figure 4.3). Alternatively, another method may be to generate a tissue-specific knockout through a flox-cre construct. A $CD11b^{flox/flox}$ CD19-cre mouse would be generated such that there would be reduced or no expression of CD11b on B cells while other cell lines would express CD11b as normal. Subsequently, again, these mice, along with their appropriate controls, would undergo folic acid-induced AKI and a comparison of the two groups could be made.

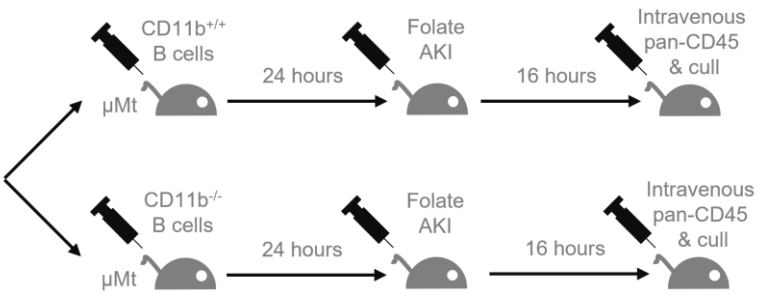


Figure 4.3: Schematic of a proposed model of exploring the role of CD11b on B cells in regulating the severity of AKI

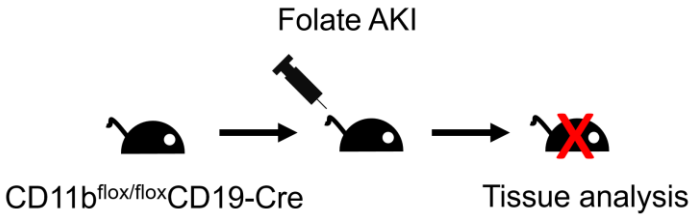


Figure 4.4: An alternative schematic proposed model of exploring how important CD11b on B cells may be for regulating the severity of AKI.

Other questions include, is CD11b newly expressed following the renal insult? If so, what are the signals that drive this expression on B cells? Is the expression of CD11b transient? If so, does the lack of expression of CD11b cause B cells to leave the kidney? Or is CD11b expression present even before the insult? If so, is the source of these B cells exclusively from the spleen or could there be mobilisation of B cells from sources such as the peritoneal cavity where there are known to be far greater numbers of CD11b positive B cells, albeit with overall smaller total numbers of B cells? As with all other questions raised in this chapter, future experiments will need to address these points.

The expression of CCL7 by B cells is broadly in concordance with a recent publication by Zouggarri and colleagues who reported this phenomenon in the context of myocardial infarction, another form of sterile inflammation (Zouggari, et al. 2013). As with CD11b, the main questions that remain relate to what drives the expression of this protein. For example, while I have shown earlier how B cells have the potential to produce this cytokine following TLR and BCR stimulation *in vitro*, what are the stimuli that may induce its expression *in vivo*?

Thus, whilst the roles of CD11b for extravasation and CCL7 expression by B cells have not previously been described in AKI, hints that this may be important arise from other experimental models of sterile inflammation such as autoimmune hepatitis and myocardial infarction.

4.3 IL-2-induced B cells and how these ideas may fit into the current literature

It has been known for some time that B cells may have the potential to respond to IL-2. The work of Korsmeyer et al and Muraguchi et al demonstrated that the IL-2 receptor was present on B cells (Korsmeyer, et al. 1983) (Muraguchi, et al. 1985). In addition, at about the same time, there was a suggestion that IL-2 may stimulate B cell proliferation (Mingari, et al. 1984) (Gearing, et al. 1985). However, there has been little consideration of how IL-2 might impact the function of B cells. Our work demonstrates that IL-2 promotes IL-10 production, adding to the cytokines known to augment IL-10 production in B cells, with BAFF being one of the most important to date (Yang, et al. 2010).

As noted previously, regulatory B cells are not a distinct subset identified by the expression of one specific transcription factor or marker. Rather, a number of B cell subsets have the capacity to produce IL-10, for example, B1 cells and transitional B cells. Therefore, which subset of B cells are most enriched with CD25 positive cells? And which subset is most responsive to IL-2? In terms of the capacity of these subsets to respond to IL-2, we have not determined the proportion of each subset that expresses CD25 at rest or after stimulation; rather we have measured this in total B cells. Complicating this question is that in humans, the markers identifying B1 cells are contentious and the population is not discrete, as discussed in the introduction. Given this limitation, it is not clear if one could address whether human B1 cells are enriched in CD25 positive cells. Investigating the upregulation of CD25 in B cell subsets post-stimulation would require subset sorting prior to stimulation, due to the changes in surface markers that occur with stimulation. The limited number of samples available from the clinical trial precluded such an approach in the current study.

Perhaps most importantly, what requires further inquiry is whether these IL-2 induced B cells are clinically relevant for ameliorating autoimmunity or inducing transplant tolerance. Strictly speaking, the clinical trial has seen an augmentation of the IL-10 population but further work is necessary to ascertain whether this population is having an effect on disease. On one end of the spectrum of relevance, IL-2 stimulated B cells may have no effect at all on autoimmune diseases or transplantation, though this is unlikely. At the other extreme, IL-2 stimulated B cells may be sufficient – independent of the aid of IL-2 induced regulatory T cells – to ameliorate disease. To answer these questions, further studies are required.

Chapter 5: Methods

All temperatures are in Celsius.

5.1 Washing of cells

1. Unless otherwise stated washing of cells involved filling the container with 2 to 3 millilitres of ice-cold PBS.
2. The container was centrifuged at 1300 RPM for 5 minutes at 4 degrees without a lid.
3. The excess fluid was tipped out.
4. The container was ‘racked’ on a rough surface such as the top of a tube rack to disperse the cell pellet.

5.2 Enzyme-linked immunosorbent assay

1. In the interests of expenditure, most ELISA assays were carried out with a number of changes from the manufacturer's instructions. Most of the ELISAs done in this thesis were purchased from R&D
2. 50ul of capture antibody was used to coat the bottom of an ELISA plate and stored overnight in the dark at 4 degrees with a lid.
3. The capture antibody was 'flicked off' into a sink.
4. To wash, the whole plate was submerged in PBS with tween and then the fluid was again flicked off. This was repeated three times. The plate was subsequently submerged in PBS alone and the fluid was flicked off. This was also repeated three times.

   The plate was patted dry at the end of washing.
5. 100ul of blocking solution was added to each well and stored with 1 hour at room temperature.
6. The plate was washed as above.
7. 50ul of antigen-containing solution was added to each well and stored for minimum 2 hours at room temperature or overnight at 4 degrees.
8. Plates were washed as above.
9. 50ul of detection antibody was added to each well and stored for 2 hours at room temperature.
10. Plates were washed as above.
11. 50ul of streptavidin horseradish peroxidase was added to each well and stored for 20 minutes at room temperature.
12. Plates were washed as above.
13. 50ul of 3,3',5,5'-Tetramethylbenzidine substrate solution was added to each well and stored for 20 minutes at room temperature.
14. 25ul of sulfuric acid stop solution was added to each well.
15. The plates were read immediately using a BMG LABTECH Clariostar Monochromator Microplate Reader.

5.3 Preparation of tissue for confocal microscopy

1. Portions of splenic tissue were cut into sections approximately 8 millimeters long.
2. The tissue was fixed in 1% paraformaldehyde for 24 hours followed by 30% sucrose solution for a further 24 hours
3. Tissues were set in OCT moulds and frozen down to minus 80 degrees
4. 25 micrometer sections of tissues were cut at -25 degrees using a cryostat and placed on poly-L-lysine slides.
5. A border was drawn around the tissue section using a wax pen.
6. Sections were air-dried for approximately 15 minutes and subsequently frozen to -20 degrees until the tissue was ready for staining.
7. Sections were defrosted and rehydrated using PBS for 15 minutes
8. PBS was aspirated off and tissue was blocked for non-specific staining using 1% murine serum and 1% Fc block in PBS for 1 hour at room temperature
9. Above blocking solution was aspirated off and 75 ul of antibody solutions were added to each sample. Antibody solution was made up in either PBS or permeabilisation solution depending on whether intracellular stains were included.
10. Samples were incubated for 24 hours at 4 degrees in a damp chamber to prevent evaporation of antibody solution.
11. Slides were washed in PBS with 1% tween for 15 minutes three times, while ensuring that excess light was not applied to the slides.
12. The slides were dried as much as possible without disrupting the tissue.
13. A cover slip was applied with 15 microlitres of mounting solution per tissue sample.
14. The slides were dried at room temperature with ambient humidity.
15. Slides were analysed within 48 hours on a confocal microscope.

5.4 Genome-wide RNA analysis

For analysis of data shown in Figures 3.1 and 3.9, see (van de Veen, et al. 2013)

5.5 Human peripheral blood B cell CD25 and CD122 expression following stimulation

1. Following ethical approval, discarded human leucocyte cones from healthy blood donors were obtained from the National Health Service Blood and Transplant, Cambridge.
2. Until the cells were fixed, all of the following steps were carried out in a Category II biosafety cabinet.
3. Histopaque was brought out of the refrigerator to warm up to room temperature.
4. Leucocytes were diluted in ice-cold running buffer (produced in-house: 0.5% bovine serum albumin (Sigma-Aldrich), 0.4% 0.5M pH 8.0 ethylenediaminetetraacetic acid in sterile phosphate buffered solution) and isolated using a Histopaque-1077 (Sigma) density gradient.
5. Cells were washed twice at 4 degrees and resuspended in ice-cold Roswell Park Memorial Institute Solution 1640 (RPMI) supplemented with 10% fetal calf serum, 1% penicillin and streptomycin (all Sigma) in concentrations no greater than 10^6 cells per 200 µl of supernatant. Cell counting was carried out manually using a haemocytometer.
6. Cells were stimulated overnight with CpG ODN 2395 (5ug/ml, InvivoGen) and/or CD40 ligand (1ug/ml, Peprotech).
7. Cells were washed with ice-chilled PBS and blocked for non-specific staining using 1% normal rat serum (ThermoFisher) and 1% human FcR block (Miltenyi Biotec) for 30 minutes.
8. Without rewashing, cells were stained with primary surface antibodies for 30 minutes at room temperature.
9. Cells were washed at room temperature and viability stain was applied for 15 minutes at room temperature. Details of all human antibodies and staining buffers used are listed below. Two antibodies were used for both CD25 and CD122.
10. Cells were washed and preserved in fixation fluid (produced in-house).
11. Prepared samples were analysed using a BD LSRFortessa flow cytometer.
12. Flow cytometry files were analysed on FlowJo Version 10 software.

Antigen	*Clone*	*Chromophore*	*Manufacturer*	*Catalogue number*
CD19	HIB19	BV650	BioLegend	302238
CD25	M-A251	APC	BD Bioscience	555434
Mouse IgG1, κ Isotype Control	MOPC-21	APC	BD Pharmingen	555751
CD25	2a3	APC	BD Bioscience	340907
CD122	TU27	PE	BioLegend	339006
Mouse IgG1, κ istotype control	MOPC-21	PE	BioLegend	400112
CD122	Mik-β3	PE	BD Pharmingen	554525
Mouse IgG1, κ Isotype Control	MOPC-21	PE	BD Pharmingen	554680
Viability	Live/Dead	Aqua	ThermoFisher	L34966

5.6 Human spleen B cell CD25 and CD122 expression

1. Following ethical approval, human spleen was retrieved from cadaveric organ transplant donors whose organs were turned down for transplantation.
2. Until the cells were fixed, all of the following steps were carried out in a Category II biosafety cabinet.
3. Histopaque was brought out of the refrigerator to warm up to room temperature.
4. Splenic tissue typically about 1 centimeter diameter was cut into small pieces using conventional surgical scissors.
5. The tissue was mechanically pushed through a 100 micron metal filter using the plunger of a 2.5 ml syringe with approximately 50ml of ice-cold cRPMI.
6. The above step was repeated.
7. The cell suspension was centrifuged through a histopaque gradient at room temperature for 25 minutes at 2300 RPM with the gentlest acceleration and deceleration settings.
8. Cells were carefully removed from the leucocyte strata using a disposable stripette.
9. Cells were washed twice in ice-cold cRPMI at 4 degrees Celsius at 2500 RPM for 10 minutes per cycle.
10. For experiments involving cell surface expression of the IL2 receptor, cells were stimulated, stained, and analysed as described above for peripheral blood leucocytes.

5.7 Human spleen B cell IL-10 production

1. Cell suspension was generated from whole spleen as described above.
2. The splenocytes were resuspended in running buffer.
3. B cells were magnetically negatively isolated as per the manufacturer's instruction.
4. B cells were placed in a round-bottomed, 96 well plate in 200ul of supernatant at $5x10^5$ cells per well.
5. Cells were stimulated with a combination of CD40 ligand, CpG, or rIL2 (aldesleukin) for 60 hours.
6. Supernatants were removed and analysed for cytokine concentration by ELISA.

5.8 Human spleen B cell co-culture

1. For co-culture experiments, B cells and CD4 T cells were negatively isolated (Miltenyi) as per the manufacturer's instructions.
2. B cells were stimulated overnight with CpG, IL-2 or a combination of ligands.
3. Simultaneously, T cells were stimulated overnight on an anti-CD3 antibody (OKT3, Abcam)-coated 96-well flat-bottomed plate.
4. Cells were subsequently washed three times to eliminate any residual IL-2 (estimated dilution: $2.7x10^4$-fold).
5. Cells were co-cultured overnight at a ratio of 1:5 B cells to CD4 T cells.
6. For the last five hours of co-culture, cells were exposed to PMA (Sigma), ionomycin (Sigma) and brefeldin A (Biolegend).
7. Cells were stained, fixed, permeabilised and intracellularly stained as per human blood leucocyte samples described below.

5.9 Human protein phosphorylation staining

Cells from recent phlebotomy:

1. If leucocytes were derived from fresh blood, 300 ul of citrate was poured in a 50ml conical centrifuge tube.
2. Approximately 3 milliliters of blood was drawn from a healthy member of the laboratory.
3. Blood was immediately emptied into the tube containing citrate.
4. Blood was gently swivelled but not aggressively shaken as this was likely to blood stain the bottle cap unnecessarily
5. 200 ul of blood was aliquoted into FACS tubes.
6. Cells were treated with 1 ml of red cell lysis buffer for 10 minutes or until fluid changed to a paler colour.
7. Cells were washed with cRPMI 8. Excess fluid was tipped off.
8. Cells were stimulated overnight with 5ug/ml of CpG to upregulate IL-2 receptor expression.

Cells from leucocyte cones:

9. Cone leucocytes were prepared in the manner described above.
10. Cells were aliquoted into 96 well-round-bottomed plates with no more than 4 million cells per well.
11. Initially cells were suspended in 200 microlitres of cRPMI.
12. Stimulus such as CpG was suspended in a further 50 microlitres of cRPMI.
13. Initial stimulation was usually 24 hours.

After above steps, all samples were stained as follows:

14. Samples were centrifuged and supernatant pipetted off.
15. Primary surface antibodies compatible with permeabilisation kits were applied. Block for preventing non-specific staining was not done in the interests of processing tissue as quickly as possible.
16. Samples were transferred to FACS tubes if not already done previously.
17. Fixation solution was warmed to 36 degrees Celsius.
18. Cells were stimulated with IL-2 or positive control at staggered time points.

19. Samples were fixed with equal amount as the sample supernatant.
20. Samples were incubated at 36 degrees for ten minutes
21. Permeabilisation solution was chilled on dry ice.
22. 1 ml of permeabilisation solution was applied to each sample while being vortexed to ensure sample did not freeze and solidify.
23. This procedure was done in the hood to ensure minimal inhalation of methanol.
24. Samples were left to permeabilise at room temperature for 20 minutes.
25. Samples were centrifuged and excess fluid pipetted off.
26. Samples were stained with relevant phosphorylation antibody.
27. After 1 hour of staining at room temperature, a further 1 ml of distilled water was added to the samples and analysed on a flow cytometer.

5.10 rIL2-treated human blood leucocytes intracellular staining

1. Details of the methods of the DILFrequency trial have been described at length elsewhere. In brief, the trial was a mechanistic, non-randomised, repeat dose, open-label, response-adaptive study of 36 participants with type I diabetes mellitus using varying doses and frequencies of low-dose rIL-2 intended to augment Treg populations. In our study, we chose a look at a selection of patients from the study with rIL2 doses varying between 0.09 x 10^6 iu/m^2 to 0.47 x 10^6 iu/m^2 administered every 2 to 5 days. These doses reflect the full range of doses given. Analysed blood samples were taken before rIL2 treatment (visit 2) and at the beginning of every other visit thereafter (visits 4, 6, 8 and 10). Administration of the original clinical trial was conducted by Prof John Todd and Dr Frank Waldron-Lynch, along with members of their scientific and medical team. Samples were retrieved from liquid nitrogen preservation by Ms Jane Kennet, research nurse.
2. Retrieved leucocyte samples were defrosted in a water bath at 36 degrees Celsius for one minute.
3. Cells were immediately transferred to 15 millilitres conical centrifuge tubes filled with ice-cold cRPMI.
4. Cells were washed twice.
5. Cells were rested for 24 hours at a concentration of approximately 8x10^5 cells per 200 µl well. All resting and subsequent stimulation of cells took place in a 96-well round-bottomed plate in an incubator at 36ºC and 5% CO_2.
6. Cells were stimulated with CpG ODN 2395 (10ug/ml) for 48 hours.
7. During the last 5 hours of this stimulation, cells were additionally exposed to phorbol myristate acetate, ionomycin, and brefeldin.
8. Prior to primary antibody staining, cells were blocked for non-specific binding with normal rat serum and FcR block as described above.
9. Extracellullar antibodies were as described above.
10. Cells were fixed and stored overnight at 4ºC and permeabilised the following day using an cytosolic intracellular staining kit as per the manufacturer's instructions in the 96-well plates (eBioscience).

11. Intracellular stains were applied for 1 hour at room temperature in darkened conditions.
12. Cells were washed.
13. Cells were analysed immediately on a flow cytometer.
14. All samples were processed in duplicate along with an unstimulated isotype control. Stained leucocytes were analysed on a BD LSRFortessa flow cytometer.
15. Flow cytometry files were analysed on FlowJo Version 10 software.

	Clone	Chromophore	Manufacturer
CD19	HIB19	BV650	BioLegend
IL-10	JES3-9D7	PE	BioLegend
TNFα	MAb11	APC	BioLegend
Viability		Aqua	ThermoFisher

5.11 Murine splenic cell preparation for as cell suspension

1. Mice were euthanized with a rising concentration of CO_2. Murine spleen was dissected using a sterile technique immediately following confirmation of death. The spleen was suspended in sterile PBS in a 15 ml conical centrifuge tube and transported on ice from the animal facilities to the laboratory.
2. cRPMI and MACS running buffer were placed on ice. The centrifuge was pre-cooled to a temperature of 4 degrees Celsius.
3. All tissue preparation was subsequently done in a sterile biosafety cabinet using sterile techniques. As much as possible, cells were kept on ice.
4. Fat was dissected from the spleen and disposed.
5. The spleen was compacted and pushed through a 40 micrometer disposable sieve along with 4 millilitres of cold cRPMI using the rubber end of a plunger from a 1 ml syringe.
6. The cell suspension was passed through a new 40 um sieve again to reduce fat content.
7. Splenocytes were resuspended in 50ml of cRPMI in a 50 ml conical centrifuge tube.
8. Cells were centrifuged at 1300 RPM for 5 minutes at 4 degrees.
9. Excess fluid tipped off and 1 ml of red cell lysis was added to the cells for 1-2 minutes or until the colour of the suspension turned from a bright red to a colour similar to cRPMI.
10. Cells were resuspended again in 50 mls of cRPMI and centrifuged at 1300 RPM for 5 minutes at 4 degrees.
11. Excess fluid was tipped out and cells counted using a haemocytometer. Cells were ready for use.

5.12 Murine spleen B cell CD25 expression

1. Mouse spleens were collected from 6-12 week-old BL/6 wildtype mice bred within the University of Cambridge mouse facilities and processed as described above.
2. Methods for *en masse* stimulation experiments and isolated B cell experiments were as per human splenic experiments except murine CD40L (1ug/ml, Peprotech) and LPS (10ug/ml, Sigma).

	Clone	Chromophore	Manufacturer
B220	RA3-6B2	APC/Cy7	Biolegend
CD25	PC61	PerCP/Cy5.5	Biolegend
Isotype for above	G0114F7	PerCP/Cy5.5	Biolegend

5.13 Murine IL-2-treated B cell transfer

1. B cells were isolated from a wildtype BL/6 mouse as previously described
2. B cells were stimulated in 96-well round-bottomed plates.
3. Cell concentration was 1 million B cells per 250ul well
4. Cells were stimulated either with either CpG (5ug/ml) or IL-2 (100 iu/ml). Vehicle stimulated was cRPMI
5. Purity was confirmed to be >95%
6. Cells were stimulated in at 36 degrees and 5%CO_2 for 48 hours
7. Splenocytes were BALB/c mice were isolated as previously described.
8. BL/6 B cells and BALB/c splenocytes were mixed to create a final concentration of 5 million B cells and 14 million splenocytes per 200 ul injection.
9. Mice were placed in a heater for at least 10 minutes before tail vein injection of B cell and allogeneic splenocytes.
10. Daily weights of the mice were observed.
11. Mice were culled on day 7.
12. Serum and spleen were removed at the time of culling.

5.14 Alloantibody detection assay

1. Serum collected from mice were co-cultured with splenocytes or thymocytes from allogeneic mouse for 10 minutes at multiple concentrations.
2. Cell were washed.
3. Cells were stained with primary antibodies and rat fluorescently labelled anti-mouse anti-IgG antibody
4. Cells were prepared for flow cytometry as described previously.

5.15 Preparation of folic acid

1. Folic acid was weighed within a 50 millilitre conical centrifuge tube.
2. Sodium bicarbonate solution was added to the solution
3. The tube was covered in aluminium foil.
4. The solution was mixed in a shaker at 36 degrees overnight
5. Each experiment was conducted using a single batch of FA.

   NB: How well the folic acid is dissolved in solution appears to have a significant bearing on its potency to induce AKI. In order to ensure minimal batch to batch variation, FA shaken at least eight hours.

5.16 Murine intravascular labelling of cells

1. Appropriate approval from the animal facilities as well the Home Office was gained before commencing this experiment.
2. Mice were placed in a warming chamber at 36 degrees for at least ten minutes.
3. Mice were transferred to a mouse restrainer.
4. Mice were injected pan-CD45 antibody via the tail vein
5. The restrainer was released so that the mice could move within the restrainer tube for 2 minutes
6. Mice were culled using rising carbon dioxide levels.
7. Processing of mouse tissue was done as described elsewhere.

5.17 Surgical renal pedicle clamping

1. Appropriate approval from the animal facilities as well the Home Office was gained before commencing this experiment.
2. Mice were anaesthetised using inhaled vaporised isofluorane.
3. Mice were placed on a warmed surface throughout the operation.
4. A midline laparotomy was undertaken and the bowels moved to expose the renal pedicles.
5. Clamps were placed on the renal pedicle for 45 minutes.
6. All surgical procedures were done by either Mr Kourosh Saeb-Parsy or Mr Jack Martin (Department of Surgery, University of Cambridge) using an operating microscope.
7. Following the end of clamping, the mice were injected subcutaneously with 100 ul of PBS.
8. Laparotomy was closed and the mice were observed while coming out of anaesthesia.
9. Mice were inspected on the evening of the operation.
10. Mice were culled and tissue analysed in the same manner as previously described for cisplatin or folate induced acute kidney injury.

5.18 Acute tubular necrosis scoring of haematoxylin and eosin stained kidney samples

Scoring was conducted as previously described (Clatworthy, et al. 2012).

1. Kidneys were H&E stained and prepared as described elsewhere.
2. Ten random non-overlapping fields were photographed using a light microscope.
3. A trainee nephrologist assessed the histology images in a blinded fashion on a personal computer.
4. Scoring of tubulointerstitial damage involved three parameters: tubular necrosis, tubular dilatation, and cast formation. Scores were as follows: involvement of 0–25% of tubules within each cortical or medullary high-powered field (HPF), 1; 25–50%, 2; 50–75%, 3; and 75–100%, 4. Therefore, minimum score was three.
5. Data analysis was conducted on Microscoft Excel 2013 and Graphpad Prism version 7.

5.19 Competition assay of wildtype of $CD11b^{-/-}$ B cells during acute kidney injury

1. Appropriate approval from the animal facilities as well the Home Office was gained before commencing this experiment.
2. $CD11b^{-/-}$ mice were acquired from the Jackson Laboratory and transferred from the US. These mice were on a $CD45.2^{+/+}$ background.
3. $CD11b^{-/-}$ and $CD45.1^{+/+}$ mice were culled in rising CO2 as per local standard operating practices.
4. The spleen was removed in a sterile fashion and transported to the laboratory on ice in sterile PBS.
5. B cells were negatively isolated as described elsewhere.
6. B cells were counted and an equal number of each type of B cell was mixed.
7. A small sample of the cells were kept to test for cellular purity on a flow cytometer.
8. Cells were transferred to the mouse facilities on ice.
9. Mice placed in a heating apparatus set to 36 degrees to attain vasodilation.
10. Cells were injected into the mice in volumes no greater than 200ul.
11. Mice were observed for 20 minutes following the injection to ensure there were no unexpected consequences.
12. 24 hours later, mice were injected intraperitoneally with 300mg/kg folic acid.
13. 16 hours later, mice underwent intravenous CD45 labelling and culling as described elsewhere.
14. Tissues were harvested and analysed as described elsewhere.

5.20 qPCR analysis of human renal tissue following acute kidney injury

1. Following ethical approval, human kidney was retrieved from cadaveric organ transplant donors whose organs were turned down for transplantation.
2. Sample preparation was undertaken by various members of the Clatworthy laboratory including Drs Miriam Berry, Kevin Loudoun, Rebeccah Mathews and Alexandra Riding.
3. A section of kidney cortex typically measuring 8 x 8 x 8 mm was snap frozen in liquid nitrogen or in a -80 degree freezer.
4. Samples were retrieved from the freezer were placed on a petri dish on top of dry ice.
5. A small sample typically measuring approximately 2 to 3 millimeters was cut using a disposable razor blade.
6. Samples were never fully defrosted during removal of a small section.
7. Samples were placed in a Precellys tube with 800ul cell lysis buffer and shaken in a Precellys 24 tissue homogeniser.
8. To reduce air bubble, the Precellys tubes were centrifuged in a desktop centrifuge at maximum speed for 5 minutes.
9. Samples were transferred to an RNA-free container and either frozen at -80 degrees or immediately processed for RNA extraction.
10. RNA extraction was undertaken using an Ambion PureLink RNA Mini Kit as per the manufacturer's instruction.
11. RNA yield was quantified using a Thermo Scientific NanoDrop spectrophotometer.
12. RNA to cDNA conversion was done using an Applied Biosystems High Capacity RNA-to-cDNA kit.
13. All qPCR measurements were done in triplicate using Taqman reagents and TaqMan Gene Expresssion Assay primers and probes. A Life Technologies Viia 7 was used to conduct qPCR.

5.21 Human urine CCL7 measurements

1. Ethical permission was granted locally to collect urine from in-patients with acute kidney injury as well as from healthy control subjects.
2. All samples were collected between June and October 2014.
3. In-patients with AKI were located by specialist registrars in renal medicine at Addenbrooke's Hospital, Cambridge, England. Acute kidney injury was defined as a serum creatinine above 120.
4. Both a written and verbal explanation of the study was given to the patient and written consent was gained from each patient who partook in the study. Patients who were unable to fully consent, for example as a result of a recent cerebral incident, were not enlisted in the study.
5. Demographic and clinical data was collected from each patient. This information included age, sex, ethnicity, baseline serum creatinine and urea (if available), serum urea and creatinine on the date of sample collection, source of urine (catheter or fresh sample), urine dipstick analysis, and co-morbidities.
6. Up to 50 milliliters of urine was collected from each patient.
7. Multiple 1-2 milliliter samples of urine were aliquoted into plastic containers and frozen in liquid nitrogen contains at temperatures less than -196 degrees.
8. Samples were chosen and defrosted on the benchtop at room temperature.
9. Samples were not diluted as there was adequate volumes for 'neat' analysis and dilution could potentially increase error in measurement.
10. Concentrations of CCL7 were measured using a commercially available ELISA kit using a modified protocol as described elsewhere.
11. The ELISA was done by Dr Alexandra Riding as these experiments were done after my period of funding. All other portions of this experiment including analysis were done by the author.
12. Data analysis was conducted on Microscoft Excel 2013 and Graphpad Prism version 7.

5.22 Statistical analysis

1. Error bars

   In most graphs shown in this thesis, errors bars represent standard error of the mean. In a large number of experiments, because precious human samples from diseased donors are processed one-by-one, it was necessary to show an error bar which would calculated the predicted variation of the mean from current value. Thus it seemed appropriate to represent this form of error.

2. Sample size

   The calculation of sample size is based on a number of factors including level of significance sought, expected effect size, and standard deviation of the effect. As with many experiments, we sought a significance as indicated by a 5% chance of the null hypothesis being incorrectly rejected (a p value of less than 0.05), and from previous description of the use of FA-induced AKI anticipated that at least 6 mice per group would be required. However, our initial experiments demonstrated that substantially variability in the severity of AKI, particularly in mice which were between 20 and 25 grams. We therefore subsequently sought to use mice of >25g so that the variability within groups was reduced, allowing the use of groups of 5-6 mice. In some cases, the exact effect size of interventions was unknown, for example, the impact of the anti-CCL7 antibody on AKI severity. This therefore required an initial smaller pilot experiment to determine potential effect size.

   In addition to these statistical considerations, some of the experimental cohort sizes were influenced by practical considerations, and supporting the philosophy of "The 3Rs" as promoted by the National Centre for Replacement, Refinement & Reduction of Animals in Research. In particular, "Reduction" refers to "minimising the number of animals used per experiment" (National Centre for Replacement 2019). If some WT or Siglec-G-/- litters contained less than 5 mice of one sex, we opted to utilize them for an experiment, as waiting for additional mice from subsequent litters would have meant the older mice were considerably heaviesr, with the differential susceptibility to FA-induced AKI discussed earlier. The alternative

would be to schedule 1 these mice because of inadequate numbers, which we did not feel was in keeping with the 3R ethos. Where possible, we tried to establish breeding trios to increase litter number. In experiments utilizing mice on a C57BL/6 background, eg, uMTs, WT controls were utilized from other breeding lines, eg, cre-negative mice. These mice would have otherwise been culled. We accept that these controls may not be genetically completely identical to uMT and may lead to variability in the data."

All data was analyzed on either Microsoft Excel or Graphpad Prism except generation of heatmaps which were done on R.

Chapter 6: Materials[1]

6.1 General materials

Materials listed below consist of specialized apparatuses necessary for the experiments described previously. Generic materials, for example petri dishes, have not been included in the interests of space.

Description	*Manufacturer and model*	*Catalogue number*	*Notes*
Human negative B cell isolation kit	MACS Miltenyi Biotec B cell Isolation Kit II (human)	130-091-151	
Murine negative B cell isolation kit	MACS Miltenyi Biotec B cell isolation kit (murine)	130-090-862	
Blood collection tube (mouse, for cell analysis)	Sarstedt Microvette system for capillary blood collection	20.1278	
Blood collection tubes (mouse, serum)	BD Microtainer SST amber tubes	364979	Other models also used.
Cell counter	Life Technologies Countess II		Manual counting also done with haemocytometer and light microscope

[1] The materials sections contains all materials used during the PhD. A number of listed items may not have been used in the experiments described above. For example, materials used in experiments which were deemed technical failures have been included in this section.

Cell strainer	Falcon 100 µm nylon cell strainer	352360	
Citric acid	Sigma citrate concentrated soluion	S5770-50ML	
Clamps for murine renal pedicles	Micro Serrefine straight clamps (8 mm length)	18055-02	These clamps were replaced after every 2 to 3 experiments
Counting beads	Invitrogen 123 count eBeads Counting Beads	01-1234-42	
Dimethyl sulfoxide	Sigma-Aldrich	276855	
Folic acid	Sigma folic acid	F7876-10G	
Gloves	Microflex Supreno (predominantly)	SU INT M	
Heat plate for RNA to cDNA	BIO-RAD T100 thermocycler	T100	
Needle (for post-terminal bleeding)	BD Microlance 3 26 G 5/8”	304300	
Syringe (for post-terminal bleeding)	BD Plastipak 1ml	303172	
Needle and syringe (for tailbleed)	Braun Omnican 100	9151141	
Tissue homogenizer	Precellys 24 tissue homogeniser		
Tissue homogenizer tubes	Precellys tissue homogenizing tubes		

qPCR machine	Applied Biosystems ViiA7		
Red cell lysis solution	BD FACS Lysing Solution	349202	Reconstituted as per manufacturer's recommendation
Sodium bicarbonate solution	Sigma sodium bicarbonate solution (7.5%)	S8761-100ML	
Roswell Park Memorial Institute-1640 medium	Sigma-Aldrich RPMI-1640 Medium With L-glutamine and sodium bicarbonate, liquid, sterile-filtered	R8758	
'Complete' Roswell Park Memorial Institute-1640 medium			Produced in-house with 10% FCS, 1% penicillin and stretomycin
L-glutamine-penicillin-streptomycin solution	Sigma-Aldrich	G1146	As used in 'complete' RPMI
Flow cytometry fixation solution			Produced in-house: 12.5ml 40% formaldehyde, 10g glucose, 5ml 2% sodium azide in 500ml PBS
Intracellular Fixation & Permeabilisation	Affymetrix eBioscience	88-8824-00	Not intranuclear "Foxp3 Fix/Perm kit"

Buffer Set			
Foetal bovine serum	Sigma-Aldrich	F9665	
Histopaque	Histopaque®-1077 sterile-filtered, density: 1.077 g/mL	10771	
'MACS' Running Buffer			Produced in-house: 4ml -0.5M EDTA pH 8, 5g BSA, in 1l PBS
Phosphate-buffered saline			Produced in-house by the Laboratory of Molecular Biology
Wortmannin	Cayman Chemicals	10010591	
Normal mouse serum			Produced in-house
96 well round bottom plates (Nunc MicroWell Plates with Nunclon Delta surface)	Nunc	163320	

6.2 Antibodies (anti-human)

Antigen	Clone	Fluorochrome(s)	Manufacturer	Catalogue numbers
CCL7		APC	Miltenyi Biotec	130-105-358
CD1d	51.1	PerCP/Cy5.5	BioLegend	350312
CD4	OKT4	eFluor 450	eBioscience	48-0048-41
CD5	UCHT2	APC	BioLegend	300612
CD8	RPA-T8	Brilliant violet 650	BioLegend	301041
CD9	HI9a	PE	BioLegend	312106
CD19	SJ25C1	BV786	BD Horizon	563325
CD19	SJ25C1	BUV737	BD Horizon	564303
CD19	HIB19	APC eFluor 780	eBioscience	47-0199-42
CD19	HIB19	BV650	BioLegend	302238
CD24	ML5	BV605	BD Horizon	562788
CD24	ML5	ML5	BioLegend	311104
CD25	BC96	PE	eBioscience	12-0259-41
CD25	BC96	APC	BioLegend	302610
CD27	L128	BUV395	BD Horizon	563816
CD27	O323	APC/Cy7	BioLegend	302816
CD38	HIT2	APC	eBioscience	17-0389-42
CD38	HIT2	PerCP-Cy5.5	BioLegend	303522
CD45	2D1	APC/Cy7	BioLegend	368515
CD45RA	HI100	PerCP-Cy5.5	Invitrogen	45-0458-41
CD122	Mik-β3	BV421	BD Horizon	562887
CD122		PE	BD Pharmigen	554525
CD122	TU27	BV 421	BioLegend	339010
CD122	TU27	PE	BioLegend	339006
CD127	A019D5	Alexa Fluor 647	BioLegend	351317
CD138	MI15	V450	BD Horizon	562098

CD138	DL101	PE-Cy7	eBioscience	25-1389-42
CD197	G043H7	Brilliant violet 785	BioLegend	353229
CXCR5	MU5UBEE	FITC	eBioscience	11-9185-41
IFNγ	4S.B3	eFluor 450	eBioscience	48-7319-42
IgD	IA6-2	APC-H7	BD Pharmigen	561305
IgM	II/41	APC	eBioscience	17-5790-82
IL-10	JES5-16E3	PE	BioLegend	501404
IL-10	JES5-16E3	APC	eBioscience	17-7101-82
TIM-1	1D12	APC	BioLegend	353906
TNFα	Mab11	APC	BioLegend	502912

6.3 Antibodies (anti-mouse)

Antigen	Clone	Fluorochrome(s)	Manufacturer	Catalogue numbers
B220	RA3-6B2	eFluor450	eBioscience	48-0452-82
B220	RA3-6B2	PerCP/Cy5.5	BioLegend	103236
B220	RA3-6B2	PE-Cyanine7	eBioscience	25-0452-82
B220	RA3-6B2	Alexa Fluor 647	BD Pharmigen	557683
B220	RA3-6B2	APC-eFluor 780	eBioscience	47-0452-82
CD3	17A2	Brilliant Violet 510	BioLegend	100233
CD3	17A2	Brilliant Violet 605	BioLegend	100237
CD3	17A2	Brilliant Violet 785	BioLegend	100231
CD3e	145-2C11	eFluor450	eBioscience	48-0031-82
CD3e	145-2C11	Brilliant Violet 650	BD Horizon	564378
CD3e	145-2C11	Alexa Fluor 488	BioLegend	100321
CD4	GK1.5	eFluor 450	eBioscience	48-0041-82
CD4	GK1.5	FITC	eBioscience	11-0041-85
CD4	RM4-5	Brilliant Violet 605	BioLegend	100548
CD4	GK1.5	PE	eBioscience	12-0041-82
CD4	GK1.5	PE-Cyanine7	eBioscience	25-0041-82
CD4	GK1.5	APC-eFluor 780	eBioscience	47-0041-82
CD5	53-7.3	APC	eBioscience	17-0051-82
CD5	53-7.3	Brilliant Violet 510	BioLegend	100627
CD5	53-7.3	PE	Invitrogen	12-0051-82
CD8	53-6.7	eFluor 450	eBioscience	48-0081-82
CD8a	53-6.7	FITC	eBioscience	11-0081-85
CD9	eBioKMC8	eFluor 450	eBioscience	48-0091-80
CD9	eBioKMC8	FITC	eBioscience	11-0091-82
CD9	eBioKMC8	APC	eBioscience	17-0091-82
CD11b	M1/70	Alexa Fluor 488	BioLegend	101217

CD11b	M1/70	eFluor 450	eBioscience	48-0112-82
CD11b	M1/70	Brilliant Violet 605	BioLegend	101237
CD11b	M1/70	Brilliant Violet 650	BioLegend	101239
CD11b	M1/70	FITC	eBioscience	11-0112-85
CD11b	M1/70	PE	BioLegend	101208
CD11b	M1/70	APC-eFluor 780	eBioscience	47-0112-82
CD11c	N418	eFluor 450	eBioscience	48-0114-82
CD11c	N418	FITC	Tonbo	35-0114-U025
CD11c	N418	PE	BioLegend	117308
CD11c	N418	PE-Cyanine7	eBioscience	25-0114-82
CD11c	N418	APC-eFluor 780	eBioscience	47-0114-82
CD19	eBio1D3	eFluor 450	eBioscience	48-0193-82
CD19	6D5	Brilliant violet 605	BioLegend	115540
CD19	6D5	Brilliant violet 785	BioLegend	115543
CD19	1D3	PerCP/Cy5.5	Tonbo	65-0193-U100
CD19	eBio1D3	PE	eBioscience	12-0193-83
CD19	6D5	Alexa Fluor 647	BioLegend	115522
CD19	eBio1D3	APC-eFluor 780	eBioscience	47-0193-82
CD21/35	eBio4E3	PE	eBioscience	12-0212-82
CD23	B3B4	PE/Cy7	BioLegend	101613
CD25	CD61.5	PerCP/Cy5.5	eBioscience	45-0251-82
CD25	CD61.5	PE	eBioscience	12-0251-83
CD16/32	2.4G2	None	BD Pharmigen	553140
CD45	30-F11	FITC	eBioscience	17-0451-85
CD45	30-F11	APC	eBioscience	17-0451-82
CD45.1	A20	APC-eFluor 780	eBioscience	47-0453-82
CD45.2	104	FITC	BioLegend	109805
CD45.2	104	PE-Cyanine7	eBioscience	25-0454-82
CD45.2	104	APC-eFluor 780	eBioscience	47-0454-82

CD122	TM-b1	FITC	eBioscience	11-1222-85
CD138	281-2	Brilliant Violet 650	BioLegend	142517
CD138	281-2	PE/Cy7	BioLegend	142514
CD182	SA044G4	PerCP/Cy5.5	BioLegend	149308
CD268	eBIO7H22-E16	APC	eBioscience	17-5943-80
CD274	MIH5	PE	eBioscience	12-5982-82
CXCR5	L128D7	PE	Biolegend	145504
CXCR5	2G8	APC	BD Pharmingen	560615
GR-1	BM8	FITC	eBioscience	11-4801-85
GR-1	RB6-8C5	PE-Cyanine7	eBioscience	25-5931-82
F4/80	BM8	PE-Cyanine7	eBioscience	25-4801-82
F4/80	BM8	APC	BioLegend	123116
GM-CSF		PE	BD Biosciences	554406
GR-1	RB6-8C5	eFluor 450	eBioscience	48-5931-82
GR-1	RB6-8C5	BV786	BD OptiBuild	740850
GR-1	RB6-8C5	APC	BioLegend	1008412
GR-1	RB6-8C5	APC-eFluor 780	eBioscience	47-5931-82
Granzyme-B	NGZB	PE	eBioscience	12-8898-80
IgA	11-44-2	PE	Invitrogen	12-5994-81
IgD	11-26c.2a	AlexaFluor 488	BioLegend	405718
IgD	11-26c	FITC	eBioscience	11-5993-85
IgD	11-26c.2a	PE	BioLegend	405705
IgD	11-26	APC	eBioscience	17-5993-82
IgG	Polyclonal	PE	eBioscience	12-4010-87
IL-6	MP5-20F3	PE	eBioscience	12-7061-81
IL-10	JES6-5H4	PE	eBioscience	12-7021-81
IL-10	JES5-16E3	Alexa Fluor 647	BioLegend	505014
Ly6C	HK1.4	PE	eBioscience	12-5932-82

Ly6C	HK1.4	APC	eBioscience	17-5932-82
Siglec-g	SH2.1	APC	eBioscience	17-5833-80

6.3 Antibodies (phosphorylation)

Antigen	Clone	Fluorochrome(s)	Manufacturer	Catalogue numbers
Akt	M89-61	Alexa Fluor 647	BD Phosflow	560343
Stat5	47/Stat5(pY694)	PE	BD Phosflow	612567

6.4 Antibodies (isotypes)

Mouse IgG1, κ isotype	X40	BUV737	BD Horizon	564299
Mouse IgG1, κ isotype	X40	BV421	BD Horizon	562438
Mouse IgG1, κ isotype	P3.6.2.8.1	eFluor 450	eBioscience	48-4714-82
Mouse IgG1, κ isotype	P3.6.2.8.1	PE	eBioscience	12-4714-42
Mouse IgG1, κ isotype	P3.6.2.8.1	PE-Cyanine7	eBioscience	25-4714-42
Mouse IgG1, κ isotype	P3.6.2.8.1	APC	eBioscience	17-4714-82
Mouse IgG1, κ isotype	P3.6.2.8.1	APC-eFluor 780	eBioscience	47-4714-82
Mouse IgG1, κ isotype		FITC	BD Pharmingen	550616
Mouse IgG1, κ isotype	MOPC-21	PE	BioLegend	400112
Mouse IgG1, κ isotype	MOPC-21	APC	BioLegend	400120
Rat IgG2a, κ isotype	RTK2758	Alexa Fluor 488	BioLegend	400525
Rat IgG2b, κ isotype	eB149/10H5	eFluor 450	eBioscience	48-4031-80
Rat IgG2b, κ isotype	eB149/10H5	FITC	eBioscience	17-4031-81
Rat IgG2b, κ isotype	eB149/10H5	APC	eBioscience	17-4031-82
Rat IgG2a, κ	eBR2a	FITC	eBioscience	11-4321-85

isotype				
Rat IgG2a, κ isotype	eBR2a	APC	eBioscience	17-4321-81
Rat IgG2b, κ isotype	eBRG1	PerCP-Cyanine 5.5	eBioscience	45-4301-80
Rat IgG2b, κ isotype	eBRG1	APC	eBioscience	17-4301-81
Rat IgG2a, κ isotype	eBR2a	APC-eFluor 780	eBioscience	47-4321-82

6.5 Stimuli for *in vitro* experiments

Stimulus	*Manufacturer*	*Catalogue number*	*Notes*
Recombinant IL-2 (human)			Kindly provided by Prof John Todd and Dr Frank Waldron-Lynch
Recombinant IL-21 (human)	Peptrotech	200-21	
Recombinant IL-21 (murine)	Peptrotech	210-21	
Goat Anti-Mouse IgM, µ chain specific	Jackson ImmunoResearch Laboratories, Inc.	115-005-020	
F(ab')2 fragment goat anti-mouse IgM, µ chain specific	Jackson ImmunoResearch Laboratories, Inc.	115-006-020	
Lipopolysaccharide from Escherichia coli 0111:B4	Sigma-Aldrich	L3024	
ODN 2395 Class C CpG oligonucleotide	InvivoGen	TLRL-2395	
ODN 2395 control	InvivoGen	TLRL-2395c	
Recombinant soluble CD40 ligand (human)	Peprotech	310-02	
Recombinant soluble CD40 ligand (murine)	Peprotech	315-15	

6.6 *In vitro* inhibitors

Description	*Manufacturer and model*	*Catalogue number*	*Notes*
U0126	Sigma	19-147	MEK-1 and MEK-2 inhibitor
Wortmannin	Selleckchem	S2758	PI3K inhibitor

6.7 ELISA kits

Description	*Manufacturer and model*	*Catalogue number*	*Notes*
ELISA plates	Nunc-Immuno™ MicroWell™ 96 well solid plates	439454	
CCL7 ELISA (human)	R&D Systems	DY282	
IL-6 ELISA (human)	R&D Systems	DY206	
IL-6 ELISA (murine)	R&D Systems	DY406	
IL-10 ELISA (human)	R&D Systems	DY217B	
IL-10 ELISA (murine)	R&D Systems	DY008	
Soluble CD25 (human)	R&D Systems	DY2438	
TNF-α ELISA (human)	R&D Systems	DY210	
TNF-α ELISA (murine)	R&D Systems	DY410	

6.8 Polymerase chain reaction

Description	*Manufacturer and model*	*Catalogue number*	*Notes*
Water	Ambion nuclease-free water	AM9937	
RNA to cDNA conversion	Applied Biosystems High Capacity RNA-to-cDNA Kit	4387406	
Mastermix	Applied Biosystems TaqMan Fast Advanced Master Mix	4444557	
CCL7	Thermo Scientific	Hs00171147_m1	
CD19	Thermo Scientific	Hs01047413_g1	
CXCL2	Thermo Scientific	Hs00601975_m1	
GAPDH	Thermo Scientific	Hs02786624_g1	
HPRT1	Thermo Scientific	Hs02800695_m1	
Ccl2	Thermo Scientific	Mm00441242_m1	
Ccl5	Thermo Scientific	Mm01302427_m1	
Ccl7	Thermo Scientific	Mm00443113_m1	
Ccl8	Thermo Scientific	Mm01297183_m1	

Csf2	Thermo Scientific	Mm01290062_m1	
Csf3	Thermo Scientific	Mm00438334_m1	
Cx3cl1	Thermo Scientific	Mm00436454_m1	
Cxcl1	Thermo Scientific	Mm04207460_m1	
Cxcl2	Thermo Scientific	Mm00436450_m1	
Cxcl12	Thermo Scientific	Mm00445553_m1	
Gapdh	Thermo Scientific	Mm99999915_g1	
Hprt1	Thermo Scientific	Mm03024075_m1	

References

Ahmadzadeh , M, and S A Rosenberg. 2006. “IL-2 administration increases CD4+ CD25(hi) Foxp3+ regulatory T cells in cancer patients.” *Blood* 107: 2409–14.

2018. *American Association of Avian Pathologists.* https://www.aaap.info/index.php?option=com_content&view=article&id=82.

2013. *American Poultry Historical Society, Inc.* http://poultryhistory.org/class.html.

Anderson, Kristin G, Katrin Mayer-Barber, Heungsup Sung, Lalit Beura, Britnie R James, Justin J Taylor, Lindor Qunaj, et al. 2014. “Intravascular staining for discrimination of vascular and tissue leukocytes.” *Nat Protoc*, 209–222.

Artist and. date of artwork unknown. https://www.google.co.uk/search?q=edward+jenner+cow&rlz=1C1CHBF_en-GBGB714GB714&source=lnms&tbm=isch&sa=X&ved=0ahUKEwiNi7KvmKrZAhUJB8AKHc-MCYQQ_AUICigB&biw=1280&bih=654#imgdii=x3qyZoxWG63j5M:&imgrc=R-EOwfnJ-Y9IZM:.

—. date of artwork unknown. *Bust of Thucydides. 3rd c. B.C.* https://www.college.columbia.edu/core/content/bust-thucydides-3rd-c-bc.

—. date of artwork unknown. *Fabricius ab Aquapendente (Hieronymus Fabricius) 1537-1619. Italian anatomist.* http://www.alamy.com/stock-photo-fabricius-ab-aquapendente-hieronymus-fabricius-1537-1619-italian-anatomist-57311257.html.

Artist unknown. 1894. *Reproduction of image originally published in Scientific American.* https://www.pinterest.co.uk/pin/147000375314577745/.

Ascon, D B, S Lopez-Briones, M Liu, M Ascon, V Savransky, R B Colvin, M J Soloski, and H Rabb. 2006. “Phenotypic and functional characterization of kidney-infiltrating lymphocytes in renal ischemia reperfusion injury.” *Journal of Immunology* 177 (5): 3380-7.

Awad, A S, M Rouse, L Huang, A L Vergis, J Reutershan, H P Cathro, J Linden, and M D Okusa. 2009. “Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury.” *Kidney International* 75 (7): 689-98.

Bain, J, L Plater, M Elliott, N Shpiro, C J Hastie, H Mclauchlan, I Klevernic, J Simon, D R Alessi, and P Philip Cohen. 2007. “The selectivity of protein kinase inhibitors: a further update.” *Biochem J.*, 297–315.

Ballesteros-Tato, A, B León, B A Graf , A Moquin, P S Adams , F E Lund, and T D Randall. 2012. "Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation." *Immunity*, 847-56.

Bankoti, R, K Gupta, A Levchenko, and S Stäger. 2012. "Marginal zone B cells regulate antigen-specific T cell responses during infection." *J. Immunol* 188: 3961–3971.

Bao, Y, and X Cao. 2014. "The immune potential and immunopathology of cytokine-producing B cell subsets: a comprehensive review." *J Autoimmun*, 10-23.

Barr, T A, P Shen, S Brown, V Lampropoulou, T Roch, S Lawrie, B Fan, et al. 2012. "B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells." *J Exp Med*, 1001-10.

Barr, T A, S Brown, P Mastroeni, and D Gray. 2010. "TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to Salmonella enterica." *J Immunol*, 2783-9.

Bellomo, R. 2006. "The epidemiology of acute renal failure: 1975 versus 2005." *Curr. Opin. Crit. Care* 12: 557–560.

Belnoue, E, M Pihlgren, T L McGaha, C Tougne, A F Rochat, C Bossen, P Schneider , B Huard, P H Lambert, and C A Siegrist. 2007. "APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells." *Blood*, 2755-2764.

Benimetskaya, L, J D Loike , Z Khaled, G Loike, S C Silverstein, L Cao, J el Khoury, T Q Cai, and C A Stein. 1997. "Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein." *Nat Med* 3 (4): 414-20.

Berland, R, and H H Wortis. 2002. "Origins and functions of B-1 cells with notes on the role of CD5." *Annu Rev Immunol.*

Black, C Allen. 1997. "A brief history of the discovery of the immunoglobulins and the origin of the modern immunoglobulin nomenclature." *Immunology and cell biology* 75: 65-68.

Blair, P A, K A Chavez-Rueda, J G Evans, M J Shlomchik, A Eddaoudi, D A Isenberg, M R Ehrenstein, and C Mauri. 2009. "Selective targeting of B cells with agonistic anti-CD40

is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice." *J Immunol* 182: 3492–3502.

Blair, P A, L Y Noreña, F Flores-Borja, D J Rawlings, D A Isenberg , M R Ehrenstein, and C Mauri. 2010. "CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients." *Immunity* 32: 129–140.

Bökers, S, A Urbat, C Daniel , K Amann, K G Smith, M Espéli, and L Nitschke. 2014. "Siglec-G deficiency leads to more severe collagen-induced arthritis and earlier onset of lupus-like symptoms in MRL/lpr mice." *J Immunol* (192): 2994-3002.

Bökers, S, A Urbat, C Daniel, K Amann, K G Smith, M Espéli, and L Nitschke. 2014. "Siglec-G deficiency leads to more severe collagen-induced arthritis and earlier onset of lupus-like symptoms in MRL/lpr mice." *J Immunol* 192 (7): 2994-3002.

Bolisetty, S, and A Agarwal . 2009. "Neutrophils in acute kidney injury: not neutral any more." *Kidney International* 75: 674–676.

Bolisetty, S, and A Agarwal. 2009. "Neutrophils in acute kidney injury: not neutral any more." *Kidney International* 75: 674–676.

Bonavia, Anthony , and Kai Singbartl. 2017. "A review of the role of immune cells in acute kidney injury." *Pediatr Nephrol.*

Bosma, A, A Abdel-Gadir, D A Isenberg, E C Jury, and Claudia Mauri. 2012. "Lipid-antigen presentation by CD1d(+) B cells is essential for the maintenance of invariant natural killer T cells." *Immunity* 36: 477–490.

Bourke, E, D Bosisio , J Golay, N Polentarutti, and A Mantovani. 2003. "The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells." *Blood*, 956-63.

Breighner, C M, and K B Kashani. 2017. "Impact of e-alert systems on the care of patients with acute kidney injury." *Best Pract Res Clin Anaesthesiol.* 31 (3): 353-359.

Briggs, J D, A C Kennedy, L N Young, R G Luke, and M Gray. 1967. "Renal function after acute tubular necrosis." *British Medical Journal* 3: 513–516.

Bucaloiu, I D, H L Kirchner, E R Norfolk, J E Hartle, and R M Perkins. 2012. “Increased risk of death and de novo chronic kidney disease following reversible acute kidney injury.” *Kidney Int* 81: 477–85.

Burne, M J, F Daniels, A El Ghandour, S Mauiyyedi, R B Colvin , M P O'Donnell, and H Rabb. 2001. “Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure.” *J Clin Invest* 108 (9): 1283-90.

Burne-Taney, M J, D B Ascon, F Daniels, L Racusen, W Baldwin, and H Rabb. 2003. “B cell deficiency confers protection from renal ischemia reperfusion injury.” *Journal of Immunology* 171 (6): 3210-3215.

Burne-Taney, M J, N Yokota-Ikeda, and H Rabb. 2005. “Effects of combined T- and B-cell deficiency on murine ischemia reperfusion injury.” *Am J Transplant* 5 (6): 1186-93.

Burrows, Personal communication with Dr Natalie. 2017.

Carter, A, and N H Martin. 1962. “Serum sialic acid levels in health and disease.” *J Clin Pathol*, 69–72.

Carter, N A, E C Rosser, and C Mauri. 2012. “Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis.” *Arthritis Res. Ther.* 14.

Case, J, S Khan , R Khalid, and A Khan. 2013. “Epidemiology of acute kidney injury in the intensive care unit.” *Crit Care Res Pract.*

Cassese, G, S Lindenau, B de Boer, A Arce, A Hauser, G Riemekasten, C Berek, et al. 2001. “Inflamed kidneys of NZB / W mice are a major site for the homeostasis of plasma cells.” *Eur J Immunol*, 9 ed.: 2726-32.

Castela, E, F Le Duff , C Butori , M Ticchioni , P Hofman , P Bahadoran , J P Lacour , and T Passeron. 2014. “Effects of low-dose recombinant interleukin 2 to promote T-regulatory cells in alopecia areata.” *JAMA Dermatol* 150 (7): 748-51.

Chandraker, A, M Takada, K C Nadeau, R Peach, N L Tilney, and M H Sayegh. 1997. “CD28-b7 blockade in organ dysfunction secondary to cold ischemia/reperfusion injury.” *Kidney Int* 52 (6): 1678-84.

Chaudhry, M S, and A Karadimitris. 2014. “Role and regulation of CD1d in normal and pathological B cells.” *J Immunol*, 4761–4768.

Chawla, L S, P W Eggers, R A Star, and P L Kimmel. 2014. “Acute kidney injury and chronic kidney disease as interconnected syndromes.” *N Engl J Med* 371: 58–66.

Chen, Guo-Yun, Nicholas K Brown, and Pan Zheng. 2014. “Siglec-G/10 in self–nonself discrimination of innate and adaptive immunity.” *Glycobiology* 24 (9): 800–806.

Chen, Y, S Khanna, C S Goodyear, Y B Park, E Raz, S Thiel, C Grönwall, et al. 2009. “Regulation of dendritic cells and macrophages by an anti-apoptotic-cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis.” *J Immunol*, 1346–1359.

Chertow, G M, E Burdick, M Honour, and J V Bonventre. 2005. “Acute kidney injury, mortality, length of stay, and costs in hospitalized patients.” *Journal of the American Society of Nephrology* 16: 3364-3370.

Chertow, G M, E Burdick, M Honour, J V Bonventre, and D W Bates. 2005. “Acute kidney injury, mortality, length of stay, and costs in hospitalized patients.” *J Am Soc Nephrol* 16: 3365-3370.

Chesneau, M, L Michel, E Dugast, A Chenouard, D Baron, A Palli, J Durand, et al. 2015. “Tolerant Kidney Transplant Patients Produce B Cells with Regulatory Properties.” *J Am Soc Nephrol*, 2588–2598.

Chiao, H, Y Kohda, P McLeroy, L Craig, I Housini, and R A Star. 1997. “Alpha-melanocyte-stimulating hormone protects against renal injury after ischemia in mice and rats.” *J Clin Invest* 99 (6): 1165-72.

Chu, V T, A Beller, S Rausch, J Strandmark, M Zänker, O Arbach, A Kruglov, and C Berek. 2014. “Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis.” *Immunity*, 582-593.

Chung, A C K , and H Y Lan. 2011. “Chemokines in Renal Injury.” *JASN*, 802-809.

Claes, N, J Fraussen, P Stinissen, R Hupperts, and V Somers. 2015. “B Cells Are Multifunctional Players in Multiple Sclerosis Pathogenesis: Insights from Therapeutic Interventions.” *Front Immunol*, 642.

Clatworthy, M R, M I Kettunen, D E Hu, R J Mathews, T H Witney, B W Kennedy, S E Bohndiek , et al. 2012. “Magnetic resonance imaging with hyperpolarized [1,4-(13)C2]fumarate allows detection of early renal acute tubular necrosis.” *Proceedings of the National Academy of Sciences* 109 (33): 13374-9.

Cohen, Heather B, and David M Mosser. 2013. “Extrinsic and intrinsic control of macrophage inflammatory responses.” *J Leukoc Biol* 94 (5): 913–919.

Coons, A H, E H Leduc, and J M Connolly. 1955. “Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit.” *Journal of Experimental Medicine* 102: 49–60.

Cravedi, P, D A Lessman, and P S Heeger. 2013. “Eosinophils are not required for the induction and maintenance of an alloantibody response.” *Am J Transplant* 13 (10): 2696–2702.

Crocker, P R, J C Paulson, and A Varki. 2007. “Siglecs and their roles in the immune system.” *Nat Rev Immunol* 7 (4): 255-66.

Das, A, G Ellis, C Pallant, A R Lopes, P Khanna, D Peppa, A Chen, P Blair, G Dusheiko, and U Gill . 2012. “IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection.” *J. Immunol* 189: 3925–3935.

Dasta, J F, S L Kane-Gill, A J Durtschi, D S Pathak, and J A Kellum. 2008. “Costs and outcomes of acute kidney injury (AKI) following cardiac surgery.” *Nephrol Dial Transplant* 23: 1970-1974.

Davison, Fred. 2012. “The Importance of the Avian Immune System and its Unique Features.” In *Avian Immunology*, edited by Karel A Schat, Bernd Kaspers and Pete Kaiser, 3. Academic Press.

Day, Y J, L Huang, H Ye, J Linden, and M D Okusa. 2005. “Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages.” *Am. J. Physiol. Renal Physiol.* 288: F722-F731.

De Greef, K E, D K Ysebaert, S Dauwe, V Persy, S R Vercauteren, D Mey, and M E De Broe. 2001. “Anti-B7-1 blocks mononuclear cell adherence in vasa recta after ischemia.” *Kidney International* 60 (4): 1415-1427.

De Greef, K E, D K Ysebaert, V Persy, S R Vercauteren, and M E De Broe. 2003. “ICAM-1 expression and leukocyte accumulation in inner stripe of outer medulla in early phase of ischemic compared to HgCl2-induced ARF.” *Kidney International* 63 (5): 1697-707.

Deng , B, Y Lin, S Ma, Y Zheng, X Yang, and B Li. 2017. “The leukotriene B4-leukotriene B4 receptor axis promotes cisplatin-induced acute kidney injury by modulating neutrophil recruitment.” *Kidney International* 30036.

Descatoire, M, J C Weill, C A Reynaud , and S Weller . 2011. “A human equivalent of mouse B-1 cells?” *J Exp Med* 208: 2563–2564.

Di Noia, J M, and M S Neuberger. 2007. “Molecular mechanisms of antibody somatic hypermutation.” *Annu Rev Biochem*, 1-22.

Diamond, M S, and T A Springer. 1993. “A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen.” *J Cell Biol* 120 (2): 545-56.

Dias, S, A D'Amico, E Cretney, Y Liao, J Tellier, C Bruggeman, F F Almeida, et al. 2017. “Effector Regulatory T Cell Differentiation and Immune Homeostasis Depend on the Transcription Factor Myb.” *Immunity* 17 (46): 78-91.

2011. *Dignity Memorial.* December. Accessed May 2018. http://obits.dignitymemorial.com/dignity-memorial/obituary.aspx?n=Timothy-Chang&lc=4620&pid=155255223&mid=4939050.

DiLillo, D J, T Matsushita, and T F Tedder. 2010. “B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer.” *Ann N Y Acad Sci*, 38-57.

Ding, C, Y Liu, Y Wang, B K Park, C-Y Wang, P Zheng, and Y Liu. 2007. “Siglecg Limits the Size of B1a B Cell Lineage by Down-Regulating NFκB Activation.” *PLoS One*, e997.

Ding, Q, M Yeung, G Camirand, Q Zeng, H Akiba, H Yagita, G Chalasani, M H Sayegh, N Najafian, and D M Rothstein. 2011. “Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice.” *J. Clin. Invest* 121 : 3645–3656.

Dong, X, S Swaminathan , L A Bachman, A J Croatt, K A Nath, and M D Griffin. 2007. “Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia-reperfusion injury.” *Kidney International* 71: 619–628.

Drayton , D L, S Liao, R H Mounzer, and N H Ruddle. 2006. “Lymphoid organ development: from ontogeny to neogenesis.” *Nat Immunol*, 344-53.

Duong, B H, H Tian, T Ota, G Completo, S Han, J L Vela, O Miyo, et al. 2009. “Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo.” *J Ex Med*, 173-187.

Edwards, J C, and G Cambridge. 2006. “B-cell targeting in rheumatoid arthritis and other autoimmune diseases.” *Nat Rev Immunol*, 394-403.

Ehrlich, Paul. 1967. “Paul Ehrlich - Nobel Lecture.” In *Nobel Lectures, Physiology or Medicine 1901-1921*, 306. Amsterdam: Elsevier Publishing Company.

Espeli, M, S Bökers, G Giannico, H A Dickinson, V Bardsley, A B Fogo, and K G Smith. 2011. “Local renal autoantibody production in lupus nephritis.” *J Am Soc Nephrol*, 296-305.

Esposito, J J, S A Sammons, A M Frace, J D Osborne, M Olsen-Rasmussen, M Zhang, D Govil, et al. 2006. “Genome sequence diversity and clues to the evolution of variola (smallpox) virus.” *Science* 313 (5788): 807-12.

Evans, J G, K A Chavez-Rueda, A Eddaoudi, A Meyer-Bahlburg, D J Rawlings , M Ehrenstein, and C Mauri. 2007. “Novel suppressive function of transitional 2 B cells in experimental arthritis.” *J. Immunol* 178: 7868–7878.

Fabricius, Hieronymus . 1967. “The Embryological Treatises of Hieronymus Fabricius.” Edited by H B Adelman.

Fagerholm, S C, M MacPherson, M J James, C Sevier-Guy, and C S Lau. 2013. “The CD11b-integrin (ITGAM) and systemic lupus erythematosus.” *Lupus* 22 (7): 657-63.

Fagraeus, A. 1948. “The plasma cellular reaction and its relation to the formation of antibodies in vitro.” *Journal of Immunology* 58: 1-13.

Fan, S T, and T S Edgington. 1993. “Integrin regulation of leukocyte inflammatory functions. CD11b/CD18 enhancement of the tumor necrosis factor-α responses of monocytes.” *J Immunol* 150: 2972-2980.

Fan, S-T, and T S Edgington. 1998. “Coupling of the adhesive receptor CD11b/CD18 to functional enhancement of effector macrophage tissue factor response.” *J Immunol* 161: 1462–1470.

Feng, Nan, Haiying Chen, Sengwang Fu, Zhaolian Bian, Xiaolu Lin, Li Yang, Yunjie Gao, Jingyuan Fang, and Zhizheng Ge. 2016. “HIF-1α and HIF-2α induced angiogenesis in

gastrointestinal vascular malformation and reversed by thalidomide." *Scientific Reports* 6.

Fillatreau, S, C H Sweenie, M J McGeachy, D Gray, and S M Anderton. 2002. "B cells regulate autoimmunity by provision of IL-10." *Nat Immunol* 3 (10): 944-50.

Fischer, M J, B B Brimhall , D C Lezotte, J E Glazner, and C R Parikh. 2005. "Uncomplicated acute renal failure and hospital resource utilization: a retrospective multicenter analysis." *Am J Kidney Dis* 5 (46): 1049-1057.

Fleming, S D, T Shea-Donohue, J M Guthridge, L Kulik, T J Waldschmidt, M G Gipson, G C Tsokos, and V M Holers. 2002. "Mice deficient in complement receptors 1 and 2 lack a tissue injury-inducing subset of the natural antibody repertoire." *J Immunol* 169 (4): 2126-33.

Flores-Borja, F, A Bosma, D Ng, V Reddy, M R Ehrenstein , D A Isenberg , and C Mauri. 2013. "CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation." *Sci. Transl. Med.* 5.

Fuquay, R, B Renner, L Kulik, J W McCullough , C Amura, D Strassheim, R Pelanda , R Torres, and J M Thurman. 2013. "Renal ischemia-reperfusion injury amplifies the humoral immune response." *Journal of the American Society of Nephrology* 24 (7): 1063-72.

Gao, H M, H Zhou, F Zhang , B C Wilson, W Kam, and J S Hong. 2011. "HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration." *J Neurosci* 31 (3): 1081-92.

Gearing, A, R Thorpe, C Bird, and M Spitz. 1985. "Human B cell proliferation is stimulated by interleukin 2." *Immunology Letters*, 105-108.

Ghosn, Eliver Eid Bou , Yang Yang, James Tung, Leonard A Herzenberg, and Leonore A Herzenberg. 2007. "CD11b expression distinguishes sequential stages of peritoneal B-1 development." 105 (13): 5195–5200.

Glick, Bruce. 1987. "How It All Began: The Continuing Story of the Bursa of Fabricius." In *Avian Immuology: Basis and Practice*, edited by A Toivanen and P Toivanen , 1-7. CRC Press.

Glick, Bruce, Timothy S Chang, and R George Jaap. 1956. "The Bursa of Fabricius and Antibody Production." *Poultry Science*, 1 January: 224–225.

Goes, N, J Urmson, V Ramassar, and P F Halloran. 1995. “Ischemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-gamma, transforming growth factor-beta 1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10.” *Transplantation*, 565-72.

Golovkina, T V, M Shlomchik, L Hannum, and A Chervonsky. 1999. “Organogenic role of B lymphocytes in mucosal immunity.” *Science*, 1965-8.

Gonzalez , M, F Mackay, J L Browning, M H Kosco-Vilbois , and R J Noelle. 1998. “The sequential role of lymphotoxin and B cells in the development of splenic follicles.” *J Exp Med*, 997-1007.

González-Guerrero, C, J L Morgado-Pascual, P Cannata-Ortiz, M A Ramos-Barron, C Gómez-Alamillo , M Arias, S Mezzano, et al. 2018. “CCL20 blockade increases the severity of nephrotoxic folic acid-induced acute kidney injury.” *J Pathol* 246 (2): 191-204.

Goodnow, C C, C G Vinuesa, K L Randall, F Mackay , and R Brink. 2010. “Control systems and decision making for antibody production.” *Nat Immunol*, 681-8.

Gray, M, K Miles, D Salter, D Gray, and J Savill. 2007. “Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells.” *Proc. Natl. Acad. Sci. USA* 104: 14080–14085.

Gray, M, K Miles, D Salter, D Gray, and J Savill. 2007. “Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells.” *Proc Natl Acad Sci U S A* 104 (35): 14080-5.

Griffin, D O, and T L Rothstein. 2012. “Human CD11b+B1 cells spontaneously secrete IL-10 and regulate T cell activity.” *Mol Med* 18: 1003–1008.

Griffin, D O, N E Holodick , and T L Rothstein. 2011. “Human B1 cells are CD3-: A reply to “A human equivalent of mouse B-1 cells?” and “The nature of circulating CD27+CD43+ B cells”.” *J Exp Med* 208: 2566–2569.

Griffin, D O, N E Holodick, and T L Rothstein. 2011. “Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+CD27+CD43+CD70.” *J Exp Med* 208: 67–80.

Griffin, Daniel O, and Thomas L Rothstein. 2011. “A small CD11b+ human B1 cell subpopulation stimulates T cells and is expanded in lupus.” 208 (13): 2591.

Griffin, Daniel O, Tam Quach, Franak Batliwalla, Dennis Andreopoulos, Nichol E Holodick, and Thomas L Rothstein. 2012. “Human CD11b+ B1 cells are not monocytes: A reply to “Gene profiling of CD11b+ and CD11b− B1 cell subsets reveals potential cell sorting artifacts”.” *Journal of Experimental Medicine* 209 (3): 434.

Gupta, A, V Puri, R Sharma, and S Puri. 2012. “Folic acid induces acute renal failure (ARF) by enhancing renal prooxidant state.” *Exp Toxicol Pathol*, 225-32.

Hamel, M B, R S Phillips, R B Davis, N Desbiens, A F Connors, J M Teno, N Wenger, J Lynn, and A W Wu. 1997. “Outcomes and cost-effectiveness of initiating dialysis and continuing aggressive care in seriously ill hospitalized adults. SUPPORT Investigators. Study to Understand Prognoses and Preferences for Outcomes and Risks of Treatments.” *Ann Intern Med* 127: 195-202.

Hao, J, D Campagnolo , R Liu , W Piao , S Shi , B Hu , R Xiang , et al. 2011. “Interleukin-2/interleukin-2 antibody therapy induces target organ natural killer cells that inhibit central nervous system inflammation.” *Ann Neurol* 69 (4): 721-34.

Hardy, R R, K Hayakawa, J Haaijman, and L A Herzenberg. 1982. “B-cell subpopulations identified by two-colour fluorescence analysis.” *Nature*, 589-91.

Hartemann, Agnès , Gilbert Bensimon, Christine A Payan, Sophie Jacqueminet, Olivier Bourron, Nathalie Nicolas, Michèle Fonfrede, Michelle Rosenzwajg, Claude Bernard, and David Klatzmann. 2013. “Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial.” *The Lancet Diabetes & Endocrinology* 1 (4): 295–305.

Hayakawa, K, R R Hardy, and L A Herzenberg. 1986. “Peritoneal Ly-1 B cells: genetic control, autoantibody production, increased lambda light chain expression.” *Eur J Immunol*, 450-6.

Hayakawa, K, R R Hardy, D R Parks, and L A Herzenberg. 1983. “The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice.” *J Exp Med*, 202-18.

Hays, J N. 2005. *Epidemics and Pandemics: Their Impacts on Human History.* Santa Barbara: ABC Clio.

Hayter, S M, and M C Cook. 2012. “Updated assessment of the prevalence, spectrum and case definition of autoimmune disease.” *Autoimmun Rev* 11 (10): 754-65.

He, J, X Zhang , Y Wei, X Sun, Y Chen, J Deng, Y Jin, et al. 2016. “Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus.” *Nat Med* 22 (9): 991-3.

Hilgendorf , I, I Theurl , L M Gerhardt, C S Robbins, G F Weber, A Gonen, Y Iwamoto, et al. 2014. “Innate response activator B cells aggravate atherosclerosis by stimulating T helper-1 adaptive immunity.” *Circulation*, 1677-87.

Hinman, R M, M J Smith, and J C Cambier. 2014. “B cells and type 1 diabetes ...in mice and men.” *Immunol Lett*, 128-32.

Hobson, C, T Ozrazgat-Baslanti, A Kuxhausen, P Thottakkara, P A Efron, F A Moore, L L Moldawer, M S Segal , and A Bihorac . 2015. “Cost and mortality associated with postoperative acute kidney injury.” *Ann Surg* 261: 1207-1214.

Hoffmann, A, S Kerr , J Jellusova, J Zhang, F Weisel , U Wellmann, T H Winkler, B Kneitz, P R Crocker, and L Nitschke. 2007. “Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population.” *Nat Immunol* 8 (7): 695-704.

Honarpisheh, M, O Foresto-Neto, S Steiger, F Kraft, P Koehler, E von Rauchhaupt, J Potempa, K Adamowicz, J Koziel, and M Lech. 2018. “Aristolochic acid I determine the phenotype and activation of macrophages in acute and chronic kidney disease.” *Sci Rep* 8 (1): 12169.

Horikawa, M, E T Weimer, D J DiLillo, G M Venturi , R Spolski , W J Leonard, M T Heise , and T F Tedder. 2013. “Regulatory B cell (B10 Cell) expansion during Listeria infection governs innate and cellular immune responses in mice.” *J. Immunol* 190: 1158–1168.

Hornung, V, S Rothenfusser, S Britsch, A Krug, B Jahrsdörfer, T Giese, S Endres, and G Hartmann. 2002. “Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.” *J Immunol*, 4531-7.

Hoste, E A. 2008. “The epidemiology of cardiac surgery-associated acute kidney injury.” *Int. J. Artif. Organs* 31: 158–165.

Hutzler, S, L Özgör, Y Naito-Matsui, K Kläsener, T H Winkler, M Reth , and L Nitschke. 2014. "The ligand-binding domain of Siglec-G is crucial for its selective inhibitory function on B1 cells." *Journal of Immunology*, 5406-5414.

Immunological Genome Project. 2019. *Immunological Genome Project Immgen Gene Skyline.* Accessed March 2, 2019. http://www.immgen.org/databrowser/index.html.

Ito, S, C M Bollard, M Carlsten, J J Melenhorst , A Biancotto , E Wang, J Chen, et al. 2014. "Ultra-low dose interleukin-2 promotes immune-modulating function of regulatory T cells and natural killer cells in healthy volunteers." *Mol Ther* 22 (7): 1388-1395.

Iwata, Y, T Matsushita, M Horikawa, D J Dilillo, K Yanaba, G M Venturi, P M Szabolcs, et al. 2011. "Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells." *Blood* 117: 530–541.

Jacobson, D L, S J Gange, N R Rose, and N M Graham. 1997. "Epidemiology and estimated population burden of selected autoimmune diseases in the United States." *Clin Immunol Immunopathol* 84 (3): 223-43.

Jang , H R, and H Rabb . 2015. "Immune cells in experimental acute kidney injury." *Nat Rev Nephrol* 11 (2): 88-101.

Jang, H R, M T Gandolfo, G J Ko, S R Satpute, L Racusen, and H Rabb. 2010. "B cells limit repair after ischemic acute kidney injury." *Journal of the American Society of Nephrology* 21 (4): 654-665.

Jellusova, J, U Wellmann, K Amann, T H Winkler, and L Nitschke. 2010. "CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity." *J Immunol* 184 (7): 3618-27.

Jo, S K, S A Sung, W Y Cho, K J Go, and H K Kim. 2006. "Macrophages contribute to the initiation of ischaemic acute renal failure in rats." *Nephrol Dial Transplant* 21 (5): 1231-9.

Joller, N, S S Weber, and A Oxenius. 2011. "Antibody-Fc receptor interactions in protection against intracellular pathogens." *Eur J Immunol*, 889-97.

Jones, E A, and D A Shoskes. 2000. "The effect of mycophenolate mofetil and polyphenolic bioflavonoids on renal ischemia reperfusion injury and repair." *J Urol* 163 (3): 999-1004.

Kantor, A, A M Stall, S Adams, L A Herzenberg, and L A Herzenber. 1992. “Differential development of progenitor activity for three B-cell lineages.” *Proc Natl Acad Sci U S A*, 3320–3324.

Katz, S I, D Parker, and J L Turk. 1974. “B-cell suppression of delayed hypersensitivity reactions.” *Nature* 251 (5475): 550-1.

Kawai, K, N H Tsuno, M Matsuhashi, J Kitayama, T Osada, J Yamada , T Tsuchiya, et al. 2005. “CD11b-mediated migratory property of peripheral blood B cells.” *J Allergy Clin Immunol* 116 (1): 192-7.

KDIGO. 2012. “Summary of Recommendation Statements.” *Kidney International Supplements* 2: 8. doi:10.1038/kisup.2012.7.

Kelly, K J, W W Williams, R B Colvin, S M Meehan, T A Springer, J C Gutierrez-Ramos, and J V Bonventre. 1996. “Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury.” *J Clin Invest* 97 (4): 1056-63.

Kennedy-Nasser, A A, S Ku, P Castillo-Caro, Y Hazrat, M F Wu , H Liu, J Melenhorst, et al. 2014. “Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity.” *Clin Cancer Res* 20 (8): 2215-25.

Kerr, M, M Bedford, B Matthews , and D O'Donoghue. 2014. “The economic impact of acute kidney injury in England.” *Nephrol Dial Transplant* 29 (7): 1362-8.

Khan, A R, E Hams, A Floudas, T Sparwasser, C T Weaver, and P G Fall. 2015. “PD-L1hi B cells are critical regulators of humoral immunity.” *Nature communications*, 5997 .

Kim, M G, T Y Koo, J J Yan, E Lee, K H Han, J C Jeong, H Ro, et al. 2013. “IL-2/anti-IL-2 complex attenuates renal ischemia-reperfusion injury through expansion of regulatory T cells.” *J Am Soc Nephrol*, 1529-36.

Kincade, Paul W. 2003. “The Importance of Environment in the Production of Lymphocytes and Immunologists.” *J Immunol* 171 (10): 4953-4956.

Klatzmann, David, and Abdul K Abbas. 2015. “The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases.” *Nature Reviews Immunology* 15: 283-294.

Ko, G J, C S Boo, S K Jo, W Y Cho, and H K Kim . 2008. “Macrophages contribute to the development of renal fibrosis following ischaemia/reperfusion-induced acute kidney injury.” *Nephrol Dial Transplant* 23 (3): 842-52.

Kojima, Hidefumi , Hua Gu, Saeko Nomura, Charles C Caldwell, Tetsuji Kobata, Peter Carmeliet, Gregg L Semenza, and Michail V Sitkovsky. 2002. “Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1α-deficient chimeric mice.” *Proceedings of the National Academy of Science* 2170-2174.

Kopf, M, H Baumann, G Freer, M Freudenberg, M Lamers, T Kishimoto, R Zinkernagel, H Bluethmann, and G Köhler. 1994. “Impaired immune and acute-phase responses in interleukin-6-deficient mice.” *Nature*, 339-42.

Koreth, J, K Matsuoka, H T Kim, S M McDonough, B Bindra, E P Alyea, P Armand, et al. 2011. “Interleukin-2 and regulatory T cells in graft-versus-host disease.” *N Engl J Med* 365 (22): 2055-66.

Korsmeyer, S J, W C Greene, J Cossman, S M Hsu , J P Jensen, L M Neckers, S L Marshall, et al. 1983. “Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in hairy cell leukemia.” *Proc Natl Acad Sci U S A.*, 4522-6.

Krause, R M, N J Dimmock, and D M Morens. 1997. “Summary of antibody workshop: The Role of Humoral Immunity in the Treatment and Prevention of Emerging and Extant Infectious Diseases.” *J Infect Dis*, 549-59.

Kumar, Dev, Surinder K Singla, Veena Puri, and Sanjeev Puri. 2015. “The Restrained Expression of NF-kB in Renal Tissue Ameliorates Folic Acid Induced Acute Kidney Injury in Mice.” *PLoS One*, e115947.

L'age-Stehr, J, H Teichmann, R K Gershon, and H Cantor . 1980. “Stimulation of regulatory T cell circuits by immunoglobulin-dependent structures on activated B cells.” *Eur J Immunol* 10 (1): 21-6.

Lanier, L L, N L Warner, J A Ledbetter, and L A Herzenberg. 1981. “Quantitative immunofluorescent analysis of surface phenotypes of murine B cell lymphomas and plasmacytomas with monoclonal antibodies.” *J Immunol*, 1691-7.

Laukkanen, A, L Emaus , V Pettila, and K M Kaukonen. 2013. “Five-year cost-utility analysis of acute renal replacement therapy: a societal perspective.” *Intensive Care Med* 39: 406-413.

LeBien, T W, and T F Tedder. 2008. “B lymphocytes: how they develop and function.” *Blood* 112 (5): 1570-80.

Lech, M, R Gröbmayr, M Ryu, G Lorenz, I Hartter, S R Mulay, H E Susanti, K S Kobayashi, R A Flavell, and H J Anders. 2014. “Macrophage phenotype controls long-term AKI outcomes--kidney regeneration versus atrophy.” *J Am Soc Nephrol* 25 (2): 292-304.

Lee, S, S Huen , H Nishio, H K Lee , B S Choi, C Ruhrberg , and L G Cantley. 2011. “Distinct macrophage phenotypes contribute to kidney injury and repair.” *J Am Soc Nephrol* 22: 317–326.

Lee, Sul A, Sanjeev Noel, Mohanraj Sadasivam, Abdel R A Hamad, and Hamid Rabb. 2017. “Role of Immune Cells in Acute Kidney.” *Nephron Clinical Practice* 137: 282-286.

Leung, K C, M Tonelli, and M T James. 2012. “hronic kidney disease following acute kidney injury-risk and outcomes.” *Nat Rev Nephrol* 9: 77–85.

Li, Jianhua , Erick Lu, Tangsheng Yi, and Jason Cyster. 2016. “EBI2 augments Tfh cell fate by promoting interaction with IL2-quenching dendritic cells.” *Nature*, 110–114.

Li, L, L Huang, S S Sung, P I Lobo, M G Brown, R K Gregg, V H Engelhard, and M D Okusa. 2007. “NKT cell activation mediates neutrophil IFN-gamma production and renal ischemia-reperfusion injury.” *J Immunol* 178 (9): 5899-911.

Lin, Wenyu , Daniela Cerny, Edmond Chua, Kaibo Duan, June Tai Jing Yi, Nurhidaya Binte Shadan, Josephine Lum, et al. 2014. “Human Regulatory B Cells Combine Phenotypic and Genetic Hallmarks with a Distinct Differentiation Fate.” *J Immunol* 193 (5): 2258-2266.

Linfert, D, T Chowdhry, and H Rabb. 2009. “Lymphocytes and ischemia-reperfusion injury.” *Transplant Rev.* 23 (1): 1-10.

Linterman, M A, W Pierson, S K Lee , A Kallies, S Kawamoto, T F Rayner, M Srivastava, et al. 2011. “Foxp3+ follicular regulatory T cells control the germinal center response.” *Nat Med* 17 (8): 975-82.

Liu, Xiaoming, Xuechao Jiang, Ronghua Liu, Luman Wang, Tingting Qian, Yijie Zheng, Yuting Deng, et al. 2015. “B cells expressing CD11b effectively inhibit CD4+ T-cell responses and ameliorate experimental autoimmune hepatitis in mice.” *Hepatology* 62 (5): 1563-1575.

Lo, L J, A S Go, and G M Chertow. 2009. “Dialysis-requiring acute renal failure increases the risk of progressive chronic kidney disease.” *Kidney Int* 76: 893–9.

Lobo, P I, A Bajwa, K H Schlegel, J Vengal , S J Lee, L Huang, H Ye, et al. 2012. “Natural IgM anti-leukocyte autoantibodies attenuate excess inflammation mediated by innate and adaptive immune mechanisms involving Th-17.” *Journal of Immunology* 188: 1675-1685.

Long, S A, M Rieck, S Sanda, J B Bollyky, P L Samuels, R Goland, A Ahmann, et al. 2012. “Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments Tregs yet transiently impairs β-cell function.” *Diabetes* 61 (9): 2340-8.

Lu, L H, D J Oh, B Dursun, Z He, T S Hoke, S Faubel, and C L Edelstein. 2008. “Increased macrophage infiltration and fractalkine expression in cisplatin-induced acute renal failure in mice.” *J Pharmacol Exp Ther* 324 (1): 111-7.

Luther, S A, T Lopez, W Bai, D Hanahan, and J G Cyster. 2000. “BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis.” *Immunity*, 471-81.

Ma, K, J Li, Y Fang, and L Lu. 2015. “Roles of B Cell-Intrinsic TLR Signals in Systemic Lupus Erythematosus.” *Int J Mol Sci*, 13084–13105.

Macgowan, D J. 1884. *Report on the health of Wenchow for the half-year ended 31 March 1884.* Imperial Maritime Customs Medical Reports.

Manns, B, C J Doig, H Lee, S Dean, M Tonelli , D Johnson, and C Donaldson. 2003. “Cost of acute renal failure requiring dialysis in the intensive care unit: clinical and resource implications of renal recovery.” *Crit Care Med* 31: 449-455.

Manohar, V, E Brown, V M Leiserson, and T M Chused. 1982. “Expression of Lyt-1 by a subset of B lymphocytes.” *J Immunol*, 532-8.

Manz, R A, M Löhning, G Cassese, A Thiel, and A Radbruch. 1998. “Survival of long-lived plasma cells is independent of antigen.” *Int Immunol*, 1703-11.

Matsumoto, M, A Baba, T Yokota, H Nishikawa, Y Ohkawa, H Kayama, A Kallies, S L Nutt , S Sakaguchi, and K Takeda. 2014. “Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation.” *Immunity* 14: 1040–1051.

Matsuoka, K, J Koreth, H T Kim, G Bascug, S McDonough, Y Kawano, K Murase, et al. 2013. “Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease.” *Sci Transl Med* 5 (179).

Matsushita, T, D Le Huu, T Kobayashi, Y Hamaguchi , M Hasegawa, K Naka, A Hirao, M Muramatsu, E Takehara, and M Fujimoto. 2016. “A novel splenic B1 regulatory cell subset suppresses allergic disease through phosphatidylinositol 3-kinase-Akt pathway activation.” *J Allergy Clin Immunol*, 1170-1182.

Matsushita, T, M Horikawa, Y Iwata, and T F Tedder. 2010. “Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis.” *J. Immunol* 2240–2252.

Mauri, C, and A Bosma. 2012. “Immune regulatory function of B cells.” *Annu Rev Immunol*, 221-41.

Mauri, C, D Gray, N Mushtaq, and M Londei. 2003. “Prevention of arthritis by interleukin 10-producing B cells.” *J Exp Med* 197 (4): 489-501.

Medicine, US National Library of. 2017. *Induction of Regulatory t Cells by Low Dose il2 in Autoimmune and Inflammatory Diseases (TRANSREG).* https://clinicaltrials.gov/ct2/show/NCT01988506.

Menard, L C, L A Minns, S Darche, D W Mielcarz, D M Foureau, D Roos, F Dzierszinski, L H Kasper, and D Buzoni-Gatel. 2007. “B cells amplify IFN-gamma production by T cells via a TNF-alpha-mediated mechanism.” *J Immunol*, 4857-66.

Menon, M, P A Blair, D A Isenberg, and C Mauri. 2016. “A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus.” *Immunity*, 683-697.

Miles, K, J Heaney, Z Sibinska, D S Salter, D Gray, and M Gray. 2012. “A tolerogenic role for Toll-like receptor 9 is revealed by B-cell interaction with DNA complexes expressed on apoptotic cells.” *Proc. Natl. Acad. Sci. USA.* 109: 887–892.

Miles, K, J Simpson, S Brown, G Cowan, D Gray, and M Gray. 2018. “Immune Tolerance to Apoptotic Self Is Mediated Primarily by Regulatory B1a Cells.” *Front Immunol* 8: 1952.

Mingari, M C, F Gerosa, G Carra, R S Accolla, A Moretta, R H Zubler, T A Waldmann, and L Moretta. 1984. “Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells.” *Nature*, 641–643.

Mizoguchi, A, E Mizoguchi, H Takedatsu , R Blumberg, and A K Bhan. 2002. “Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation.” *Immunity* 16 (2): 219-30.

Mohr, E, K Serre, R A Manz, A F Cunningham, M Khan, D L Hardie, R Bird, and I C MacLennan . 2009. “Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature.” *J Immunol*, 2113-2123.

Morris, Alan, and Göran Möller. 1968. “Regulation of cellular antibody synthesis effect of adoptively transferred antibody-producing spleen cells on cellular antibody synthesis.” *Journal of Immunology* 101 (3): 439-445.

Morrison, T E, J D Simmons, and M T Heise. 2008. “Complement receptor 3 promotes severe ross river virus-induced disease.” *J Virol* 82 (22): 11263-72.

Moser, K, K Tokoyoda, A Radbruch, I MacLennan, and R A Manz. 2006. “Stromal niches, plasma cell differentiation and survival.” *Curr Opin Immunol*, 265-270.

Moticka, Edward J. 2015. *Historical perspective on evidence based immunology.* Elsevier.

Müller, J, B Lunz, I Schwab, A Acs, F Nimmerjahn, C Daniel, and L Nitschke. 2015. “Siglec-G Deficiency Leads to Autoimmunity in Aging C57BL/6 Mice.” *J Immunol* 195 (1): 51-60.

Muraguchi, A, J H Kehrl , D L Longo, D J Volkman, K A Smith, and A S Fauci. 1985. “Interleukin 2 receptors on human B cells. Implications for the role of interleukin 2 in human B cell function.” *J Exp Med*, 181-97.

Muzio, M, D Bosisio, N Polentarutti, G D'amico, A Stoppacciaro, R Mancinelli, C van't Veer, et al. 2000. “Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells.” *J Immunol*, 5998-6004.

National Centre for Replacement, Refinement & Reduction of Animals in Research. 2019. *The 3Rs.* Accessed March 1, 2019. https://www.nc3rs.org.uk/the-3rs.

Needham, J. 1980. *China and the origins of immunology.* Hong Kong: University of Hong Kong.

Neta, R, and S B Salvin. 1974. “Specific suppression of delayed hypersensitivity: the possible presence of a suppressor B cell in the regulation of delayed hypersensitivity.” *J Immunol* 113 (6): 1716-25.

Neves, P, V Lampropoulou, E Calderon-Gomez, T Roch, U Stervbo, P Shen, A A Kühl, et al. 2010. “Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection.” *Immunity* 33 (5): 777-90.

Newcomer, W S, and J D Connally. 1960. “The bursa of Fabricius as an indicator of chronic stress in immature chickens.” *Endocrinology* 67: 264-6.

Ngo, V N, R J Cornall, and J G Cyster. 2001. “Splenic T zone development is B cell dependent.” *J Exp Med*, 1649-60.

Nitschke, L. 2015. “Siglec-G is a B-1 cell inhibitory receptor and also controls B cell tolerance.” *Ann N Y Acad Sci* 117-21.

Norberg, Renée, Gunnel Biberfeld, and Hans Wigzell. 1998. “Astrid Fagraeus, 1913–1997.” *Scandinavian Journal of Immunology* 47 (1): 91.

Nutt, S L, P D Hodgkin, D M Tarlinton, and L M Corcoran. 2015. “The generation of antibody-secreting plasma cells.” *Nat Rev Immunol*, 160-71.

Odorizzi , Pamela , and E John Wherry. 2012. “Inhibitory Receptors on Lymphocytes: Insights from Infections.” *J Immunol*, 2957–2965.

Oleska, Rew, and Sean M Bagshaw. 2014. “Acute kidney injury - epidemiology, outcomes and economics.” *Nature Reviews Nephrology* 10: 193-207.

Park, P, M Haas, P N Cunningham, L Bao, J J Alexander, and R J Quigg. 2002. “Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes.” *Am J Physiol Renal Physiol* 282 (2): 352-7.

Parnham, Peter. 2014. *The Immune System.* 4. Garland Science.

Perek, M, and A Eilat. 1960. “The bursa of fabricius and adrenal ascorbic acid depletion following ACTH injections in chicks.” *J Endocrinol* 20: 251-5.

Perez, R L, and J Roman. 1995. “Fibrin enhances the expression of IL-1β by human peripheral blood mononuclear cells. Implications in pulmonary inflammation.” *J Immunol* 154: 1879–1887.

Persy, V P, A Verhulst, D K Ysebaert, K E De Greef, and M E De Broe. 2003. “Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice.” *Kidney International* 63: 543-553.

Pfrengle, F, M S Macauley, N Kawasaki, and J C Paulson. 2013. “Copresentation of antigen and ligands of Siglec-G induces B cell tolerance independent of CD22.” *J Immunol*, 1724-31.

Piscitelli, S C, M J Wells , J A Metcalf, M Baseler, R Stevens, and R T Davey. 1996. “Pharmacokinetics and pharmacodynamics of subcutaneous interleukin-2 in HIVinfected patients.” *Pharmacotherapy* 16 (5): 754-9.

Poe, J C, and T F Tedder. 2012 . “CD22 and Siglec-G in B cell function and tolerance.” *Trends Immunol* 33 (8): 413-20.

Pritchard, Nicholas R, and Kenneth G C Smith. 2003. “B cell inhibitory receptors and autoimmunity.” *Immunology*, 263–273.

Rabb, H, C C Mendiola, J Dietz, S R Saba, T B Issekutz, F Abanilla, J V Bonventre, and G Ramirez. 1994 . “Role of CD11a and CD11b in ischemic acute renal failure in rats.” *Am J Physiol* 267: 1052-8.

Rabb, H, F Daniels , M O'Donnell, M Haq, S R Saba, W Keane, and W W Tang. 2000. “Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice.” *Am J Physiol Renal Physiol* 279 (3): F525-31.

Rauch, P J, A Chudnovskiy, C S Robbins, G F Weber, M Etzrodt, I Hilgendorf, E Tiglao, et al. 2012. “Innate response activator B cells protect against microbial sepsis.” *Science. 2012 Feb 3;*, 3 Feb: 597-601.

Renner, B, D Strassheim, C R Amura, L Kulik, D Ljubanovic, M J Glogowska , K Takahashi, M C Carroll, V M Holers, and J M Thurman. 2010. “B cell subsets contribute to renal injury and renal protection after ischemia/reperfusion.” *Journal of Immunology* 185 (7): 4393-400.

Reynaud, Claude-Agnès , and Jean-Claude Weill. 2012. “Gene profiling of CD11b+ and CD11b− B1 cell subsets reveals potential cell sorting artifacts.” 209 (3): 433.

Rezzonico, R, R Chicheportichex, V Imbert, and J M Dayer. 2000. “Engagement of CD11b and CD11c β2 integrin by antibodies or soluble CD23 induces IL-1β production on primary

human monocytes through mitogen-activated protein kinase-dependent pathways." *Blood* 95: 3868–3877.

Richards, James A, Martina Bucsaiova, Emily E Hesketh, Chiara Ventre, Neil C Henderson, Kenneth Simpson, Christopher O C Bellamy, et al. 2015. "Acute Liver Injury Is Independent of B Cells or Immunoglobulin M." *PloS ONE* 10 (9).

Robb, R J. 1984. "Interleukin 2: the molecule and its function." 5: 203–209.

Rosenberg, S A. 2014. "IL-2: the first effective immunotherapy for human cancer." *Journal of Immunology* 192: 5451–5458.

Rosser, E C, and C Mauri. 2015. "Regulatory B cells: origin, phenotype, and function." *Immunity* 42 (4): 607-12.

Rothstein, Thomas L, Daniel O Griffin, Nichol E Holodick, Tam D Quach, and Hiroaki Kaku. 2013. "Human B-1 cells take the stage." *Ann N Y Acad Sci* 1285: 97–114.

Rouschop, K M, J J Roelofs, N Claessen, P da Costa Martins, J J Zwaginga, S T Pals, J J Weening, and S Florquin. 2005. "Protection against renal ischemia reperfusion injury by CD44 disruption." *J Am Soc Nephrol* 16 (7): 2034-43.

Saadoun, D, M Rosenzwajg, F Joly, A Six, F Carrat, V Thibault, D Sene, P Cacoub, and D Klatzmann. 2011. "Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis." *N Engl J Med* 365 (22): 2067-77.

Sadlack, B, H Merz, H Schorle, A Schimpl , and A Feller. 1993. "Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene ." *Cell* 75 (2): 253-61.

Sakr, M, G Zetti, C McClain, J Gavaler, M Nalesnik, S Todo, T Starzl, and D Van Thiel. 1992. "The protective effect of FK506 pretreatment against renal ischemia/reperfusion injury in rats." *Transplantation* 53 (5): 987-91.

Schioppa, T, R Moore, R G Thompson, E Rosser, H Kulbe, S Nedospasov, C Mauri, L Coussens, and F R Balkwill. 2011. "B regulatory cells and the tumor-promoting actions of TNF-α during squamous carcinogenesis." *Proc. Natl. Acad. Sci. USA.* 108: 10662–10667.

Scott, Kirsten. 2018. "Conversation with author."

Shen, P, and S Fillatreau. 2015. "Antibody-independent functions of B cells: a focus on cytokines." *Nat Rev Immunol*, 441-51.

Shen, P, T Roch, V Lampropoulou, R A O'Connor, U Stervbo, E Hilgenberg , S Ries, et al. 2014. "IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases." *Nature* 507 (7492): 366-370.

Shimamura, T, K Hashimoto, and S Sasaki. 1982. "Feedback suppression of the immune response in vivo. II. Involvement of prostaglandins in the generation of suppressor-inducer B lymphocytes." *Cell Immunol* 69 (1): 192-5.

Shimamura, T, S Habu, K Hashimoto, and S Sasaki. 1984. "Feedback suppression of the immune response in vivo. III. Lyt-1+ B cells are suppressor-inducer cells." *Cell Immunol* 83 (1): 221-4.

Shimamura, Tadakatsu, Kazuo Hashimoto, and Shogo Sasaki. 1982. "Feedback suppression of the immune response in vivo: I. Immune B cells induce antigen-specific suppressor T cells." *Cellular Immunology* 68 (1): 104-113.

Sidman, CL, L D Shultz, R R Hardy, K Hayakawa, and L A Herzenberg. 1986. "Production of immunoglobulin isotypes by Ly-1+ B cells in viable motheaten and normal mice." *Science*, 1423-5.

Silver, S A, and G M Chertow. 2017. "The economic consequences of acute kidney injury." *Nephron* 137 (4).

Silver, S A, J Long, Y Zheng, and G M Chertow. 2017. "Cost of Acute Kidney Injury in Hospitalized Patients." *J Hosp Med* 12 (2): 70-76.

Simma, N, T Bose , S Kahlfuss, J Mankiewicz, T Lowinus, F Lühder , T Schüler, B Schraven, M Heine , and U Bommhardt. 2014. "NMDA-receptor antagonists block B-cell function but foster IL-10 production in BCR/CD40-activated B cells." *Cell Commun Signal* 12.

Singh, AP, A Junemann, A Muthuraman, A S Jaggi, N Singh, K Grover, and R Dhawan. 2012. "Animal models of acute renal failure." *Pharmacol Rep* 64 (1): 31-44.

Sivakumar, S. 2018. *Bio-Siva Homepage.* Accessed May 2018. http://biosiva.50webs.org/organs.htm.

Soiffer, R J, C Murray , R Gonin , and J Ritz . 1994. "Effect of low-dose interleukin-2 on disease relapse after T-cell-depleted allogeneic bone marrow transplantation." *Blood* 84 (3): 964-71.

Soiffer, R J, C Murray, K Cochran , C Cameron , E Wang , P W Schow , J F Daley, and J Ritz . 1992. “Clinical and immunologic effects of prolonged infusion of low-dose recombinant interleukin-2 after autologous and T-cell-depleted allogeneic bone marrow transplantation.” *Blood* 79 (2): 517-26.

Sokol , Caroline L, and Andrew D Luster. 2015. “The Chemokine System in Innate Immunity.” *Cold Spring Harbor Perspectives in Biology.*

Stallons, L Jay, Ryan M Whitaker, and Rick G Schnellmann. 2014. “Suppressed Mitochondrial Biogenesis in Folic Acid-Induced Acute Kidney Injury and Early Fibrosis.” *Toxicol Lett*, 326–332.

Stewart, J, G Findlay, N Smith, K Kelly, and M Mason. 2009. *Adding Insult to Injury.* National Confidential Enquiry into Patient Outcome and Death.

Sugama, K, K Suzuki, K Yoshitani, K Shiraishi, and T Kometani. 2013. “Urinary excretion of cytokines versus their plasma levels after endurance exercise.” *Exerc Immunol Rev*, 29-48.

Sun, J, J Wang, E Pefanis, J Chao, G Rothschild, I Tachibana, J K Chen, et al. 2015. “Transcriptomics Identify CD9 as a Marker of Murine IL-10-Competent Regulatory B Cells.” *Cell Rep*, 1110-1117.

Sun, Jianbo, Jiguang Wang, Evangelos Pefanis, Jaime Chao, Gerson Rothschild, Isao Tachibana, Jun Kui Chen, et al. 2015. “Transcriptomics Identify CD9 as a Marker of Murine IL-10-Competent Regulatory B Cells.” *Cell Reports* 13 (6): 1110–1117.

Susantitaphong, P, D N Cruz , J Cerda, M Abulfaraj, F Alqahtani, I Koulouridis, and Acute Kidney Injury Advisory Group of the America. 2013. “World incidence of AKI: a meta-analysis.” *Clin. J. Am. Soc. Nephrol.* 8: 1482–1493.

Suzuki, H, T M Kundig, C Furlonger, A Wakeham, E Timms, T Matsuyama, R Schmits, J J Simard, P S Ohashi, and H Griesser. 1995. “Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta.” *Science* 268 (5216): 1472-1476.

Tadagavadi, R K, and W B Reeves . 2010. “Renal dendritic cells ameliorate nephrotoxic acute kidney injury.” *Journal of the American Society of Nephrolgy* 21: 53–63.

Takada, M, A Chandraker, K C Nadeau, M H Sayegh, and N L Tilney. 1997. “The role of the B7 costimulatory pathway in experimental cold ischemia/reperfusion injury.” *J Clin Invest* 100 (5): 1199-203.

Tanaka, S, T Tanaka, T Kawakami, H Takano, M Sugahara, H Saito, Y Higashijima, J Yamaguchi, R Inagi, and M Nangaku. 2017. “Vascular adhesion protein-1 enhances neutrophil infiltration by generation of hydrogen peroxide in renal ischemia/reperfusion injury.” *Kidney International* 30041-30048.

Taylor, R L, and F M McCorkle. 2009. “A landmark contribution to poultry science—Immunological function of the bursa of Fabricius.” *Poultry Science* 88 (4): 816–823.

1652-1654. *The Plague of Athens, Michiel Sweerts.* https://en.wikipedia.org/wiki/Plague_of_Athens#/media/File:Plague_in_an_Ancient_City_LACMA_AC1997.10.1_(1_of_2).jpg.

Therapontos, Christina , Lynda Erskine, Erin R Gard, William D Figg, and Neil Vargesson. 2009. “Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation.” *Proceedings of the National Academy of Science.*

Thornton, M A, R Winn, C E Alpers, and R A Zager. 1989. “An evaluation of the neutrophil as a mediator of in vivo renal ischemic-reperfusion injury.” *American Journal of Pathology* 135 (3): 509-515.

Thucydides. 431 BCE. *The History of the Peloponnesian War.* Translated by Richard Crawley.

Tiselius, A, and E A Kabat. 1939. “AN ELECTROPHORETIC STUDY OF IMMUNE SERA AND PURIFIED ANTIBODY PREPARATIONS.” *J Exp Med* 69 (1): 119-31.

Todd, J A, M Evangelou, A J Cutler, M L Pekalski, N M Walker, H E Stevens, L Porter, et al. 2016. “Regulatory T Cell Responses in Participants with Type 1 Diabetes after a Single Dose of Interleukin-2: A Non-Randomised, Open Label, Adaptive Dose-Finding Trial.” *PLoS Med* 13 (10).

Toivanen, Paavo . 1998. “Bursa of Fabricius.” In *Encyclopedia of Immunology (Second Edition)*, by Peter J Delves , 393-396. Elsevier.

Truman, L A, M L Pekalski, P Kareclas, M Evangelou, N M Walker, J Howlett, A P Mander, et al. 2015. “Protocol of the adaptive study of IL-2 dose frequency on regulatory T cells in

type 1 diabetes (DILfrequency): a mechanistic, non-randomised, repeat dose, open-label, response-adaptive study." *BMJ Open* 5 (12).

Uchino, S. 2006. "The epidemiology of acute renal failure in the world." *Curr. Opin. Crit. Care* 12: 538–543 .

2017. *United Network for Organ Sharing.* https://unos.org/us-organ-transplants-set-record-in-2016/.

van de Veen, W, B Stanic, G Yaman, M S Wawrzyniak , S Söllner , D G Akdis , B Rückert , C A Akdis, and M Akdis. 2013. "IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses." *J Allergy Clin Immunol* 131 (4): 1204-12.

Varki, A, and T Angata. 2006. "Siglecs--the major subfamily of I-type lectins." *Glycobiology* 16 (1): 1R-27R.

Varrier, Matt, Lui G Forni, and Marlies Ostermann. 2015. "Long-term sequelae from acute kidney injury: potential mechanisms for the observed poor renal outcomes." *Critical Care* 19: 102.

Venkatachalam, M A, K A Griffin, R Lan, H Geng, P Saikumar, and A K Bidani. 2010. "Acute kidney injury: a springboard for progression in chronic kidney disease." *Am J Physiol Renal Physiol* 298: F1078–94.

Verbinnen, B, K Covens, L Moens, I Meyts, and X Bossuyt. 2012. "Human CD20+CD43+CD27+CD5−B cells generate antibodies to capsular polysaccharides of Streptococcus pneumoniae." *J Allergy Clin Immunol* 130: 272–275.

Vinuesa, C G, M A Linterman, C C Goodnow, and K L Randall. 2010. "T cells and follicular dendritic cells in germinal center B-cell formation and selection." *Immunol Rev*, 72-89.

von Spee-Mayer, C, E Siegert, D Abdirama, A Rose, A Klaus, T Alexander, P Enghard , et al. 2016. "Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus." *Ann Rheum Dis* 75 (7): 1407-15.

Wan, B, L Hao, Y Qiu, Z Sun, Q Cao, Y Zhang, T Zhu, H Wang, and Y Zhang. 2006. "Blocking tumor necrosis factor-alpha inhibits folic acid-induced acute renal failure." *Exp Mol Pathol*, 211-6.

Wang, C Y, R A Good, P Ammirati, G Dymbort, and R L Evans. 1980. “Identification of a p69,71 complex expressed on human T cells sharing determinants with B-type chronic lymphatic leukemic cells.” *J Exp Med*, 1539-44.

Wang, J X, A M Bair , S L King, R Shnayder, Y F Huang , C C Shieh, R J Soberman, R C Fuhlbrigge, and P A Nigrovic . 2012. “Ly6G ligation blocks recruitment of neutrophils via a β2-integrin-dependent mechanism.” *Blood* 10 (7): 1489-98.

Wang, R X, C R Yu, I M Dambuza, R M Mahdi, M B Dolinska, Y V Sergeev, P T Wingfield, S H Kim, and C E Egwuagu. 2014. “Interleukin-35 induces regulatory B cells that suppress autoimmune disease.” *Nat Med* 20 (6): 633-41.

Weber, G F, B G Chousterman, I Hilgendorf, C S Robbins, I Theurl, L M Gerhardt, Y Iwamoto, et al. 2014. “Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis.” *J Exp Med*, 1243-56.

Whitney, G, S Wang, H Chang, K Y Cheng, P Lu, X D Zhou, W P Yang, M McKinnon, and M Longphre. 2001. “A new siglec family member, siglec-10, is expressed in cells of the immune system and has signaling properties similar to CD33.” *Eur J Biochem*, 6083-96.

Willerford, D M, J Chen, J A Ferry, L Davidson, A Ma, and F W Alt. 1995. “Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment.” *Immunity* 3 (4): 521-30.

Wilson, F P. 2017. “Information Technology and Acute Kidney Injury: Alerts, Alarms, Bells, and Whistles.” *Adv Chronic Kidney Dis* 24 (4): 241-245.

Wolf, S D, B N Dittel , F Hardardottir, and C A Janeway . 1996. “Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice.” *J Exp Med* 184 (6): 2271-8.

Wright, Christopher . 2006. *British and Irish paintings in public collections.* New Haven and London: Yale University Press for the Paul Mellon Centre for Studies in British Art. http://catalogue.wellcomelibrary.org/record=b1203489.

Wright, S D, and M T Jong . 1986. “Adhesion-promoting receptors on human macrophages recognize Escherichia coli by binding to lipopolysaccharide.” *J Exp Med* 164 (6): 1876-88.

Xiao, S, C R Brooks, C Zhu, C Wu, J M Sweere, S Petecka , A Yeste, F J Quintana, T Ichimura, and R A Sobel. 2012. “Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice.” *Proc. Natl. Acad. Sci. USA.* 109: 12105–12110.

Yamate, J, A Ishida, K Tsujino, M Tatsumi, S Nakatsuji, M Kuwamura, T Kotani , and S Sakuma. 1996. “Immunohistochemical study of rat renal interstitial fibrosis induced by repeated injection of cisplatin, with special reference to the kinetics of macrophages and myofibroblasts.” *Toxicol Pathol* 24 (2): 199-206.

Yanaba, K, J D Bouaziz, K M Haas, J C Poe, M Fujimoto, and T F Tedder. 2008. “A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses.” *Immunity* 28: 639–650.

Yang, Min, Lingyun Sun, Shengjun Wang, King-Hung Ko, Huaxi Xu, Bo-Jian Zheng, Xuetao Cao, and Liwei Lu. 2010. “Cutting Edge: Novel Function of B Cell-Activating Factor in the Induction of IL-10–Producing Regulatory B Cells.” *The Journal of Immunology* 184: 3321-3325.

Yang, S H, J P Lee, H R Jang, R H Cha, S S Han, U S Jeon, D K Kim, J Song, D S Lee, and Y S Kim. 2011. “Sulfatide-reactive natural killer T cells abrogate ischemia-reperfusion injury.” *J Am Soc Nephrol* 22 (7): 1305-14.

Yikrazuul. 2009. *N-Acetylneuraminic acid; Neu5Ac or NANA; N-acetylneuraminate.* 20 December. Accessed May 2018. https://en.wikipedia.org/wiki/N-Acetylneuraminic_acid#/media/File:Beta_N-Acetylneuraminic_Acid.svg.

Yokota, N, M Burne-Taney, L Racusen, and H Rabb. 2003. “Contrasting roles for STAT4 and STAT6 signal transduction pathways in murine renal ischemia-reperfusion injury.” *Am J Physiol Renal Physiol* 285 (2): F319-25.

Yoshizaki, A, T Miyagaki, D J DiLillo, T Matsushita, M Horikawa, E I Kountikov, R Spolski, J C Poe, W J Leonard, and T F Tedder. 2012. “Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions.” *Nature* 8 (491): 264-8.

Ysebaert, D K, K E De Greef , S R Vercauteren, M Ghielli, G A Verpooten, E J Eyskens, and M E De Broe. 2000. “Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury.” *Nephrol Dial Transplant* 15 (10): 1562-74.

Yu, A, L Zhu, N H Altman, and T R Malek . 2009. “A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells.” *Immunity* 30 (2): 204-17.

Zeng, X, G M McMahon, S M Brunelli, D W Bates, and S S Waikar. 2014. “Incidence, outcomes, and comparisons across definitions of AKI in hospitalized individuals.” *Clin J Am Soc Nephrol* 9: 12-20.

Zhang, D, X Hu, L Qian, S H Chen, H Zhou, B Wilson, D S Miller, and J S Hong. 2011. “Microglial MAC1 receptor and PI3K are essential in mediating β-amyloid peptide-induced microglial activation and subsequent neurotoxicity.” *J Neuroinflammation* 8 (1): 3.

Zhang, M, W G Austen, I Chiu, E M Alicot, R Hung, M Ma, N Verna, et al. 2004. “Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury.” *Proc Natl Acad Sci* 101 (11): 3886-91.

Zhang, Yong-Hong , Ming He, Yan Wang, and Ai-Hua Liao. 2017. “Modulators of the Balance between M1 and M2 Macrophages during Pregnancy.” *Front Immunol.*

Zhou, Hui, Jieying Liao, Jim Aloor, Hui Nie, Belinda C Wilson, Michael B Fessler, Hui-Ming Gao, and Jau-Shyong Hong. 2013. “CD11b/CD18 (Mac-1) Is a Novel Surface Receptor for Extracellular Double-stranded RNA to Mediate Cellular Inflammatory Responses.” *J Immunol* 190 (1): 115–125.

Zhou, X, X Zang, Y Guan, T Tolbert, T C Zhao, G Bayliss, and S Zhuang. 2018. “Targeting enhancer of zeste homolog 2 protects against acute kidney injury.” *Cell Death Dis* 9 (11): 1067.

Zorn, E, E A Nelson, M Mohseni, F Porcheray , H Kim, D Litsa , R Bellucci , et al. 2006. “IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo.” *Blood* 108 (5): 1571-9.

Zorn, Emmanuel , Mehrdad Mohseni, Haesook Kim, Fabrice Porcheray, Allison Lynch, Roberto Bellucci, Christine Canning, Edwin P Alyea, Robert J Soiffe, and Jerome Ritz. 2009. “Combined CD4+ donor lymphocyte infusion and low-dose recombinant IL-2 expand FOXP3+ regulatory T cells following allogeneic hematopoietic stem cell transplantation.” *Biol Blood Marrow Transplant* 15 (3): 382–388.

Zouggari, Yasmine , Hafid Ait-Oufella, Philippe Bonnin, Tabassome Simon, Andrew P Sage, Coralie Guérin, José Vilar, et al. 2013. “B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction.” *Nature Medicine* 19 (10): 1273–1280.