Investigation of the therapeutic potential of ES-62 in a murine model of SLE

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B.Sc. M.Res. Ph.D

A thesis submitted in fulfillment of the requirements
for the degree of Doctor of Philosophy

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Abstract

Autoimmune inflammatory disorders such as systemic lupus erythematosus (SLE) remain debilitating conditions, as many patients are refractory to existing conventional and biologic therapies or suffer serious adverse effects, such as susceptibility to catastrophic infection. Therapies based on the actions of parasite-derived immunomodulators that dampen inflammation to promote the survival of the parasite without seriously immunocompromising the host, may therefore provide alternative strategies for the development of novel and safer drugs. One such molecule, ES-62, protects against disease in mouse models of rheumatoid arthritis and asthma; in both of these pathologies, suppression of disease is due to modulation of pathogenic IL-17A responses. As IL-17A has been implicated in the pathogenesis of SLE, in this thesis, the therapeutic potential of ES-62 is explored in the MRL/*lpr* mouse model of SLE.

SLE is characterized by autoantibody responses to dsDNA, as well as other nuclear and cytoplasmic antigens, which result in the deposition of autoantibody-immune complexes that cause localized inflammation in tissues with dense capillary networks, most often the skin, joints and kidneys. The MRL/*lpr* mouse is genetically predisposed to develop lupus-like pathology displaying many of the characteristics of human disease, including the major cause of morbidity, glomerulonephritis. Twice weekly treatment of MRL/*lpr* mice with ES-62 significantly suppressed the development of proteinuria, a direct measure of renal dysfunction. Despite drastic improvement in renal function, the kidneys from ES-62 treated mice did not show substantial improvement in histopathology as indicated by the overall levels of glomeruloproliferation, cellular infiltration and complement or immunoglobulin deposition in the kidneys. However, exposure to ES-62 did reduce the expression of complement (C3aR and C5aR) and immunoglobulin receptors (FcγRI (CD64)), thus rendering renal cells hypo-responsive to these pro-inflammatory stimuli. Moreover, by modulating MyD88 signaling, ES-62 likely suppresses renal cell responsiveness to chronic DAMP and IL-1 signals as well as potentially promoting glomerular barrier stability.

Consistent with their hypo-responsive phenotype, renal fibroblasts from ES-62 treated mice produced less MCP-1 in response to TLR stimulation and this was associated with reduced infiltration into the kidney by effector T and B cells and granulocytes; along with the ability of the parasite product to modulate the production of the pro-inflammatory cytokines IL-17A and IL-22. ES-62 suppressed the production of IL-22 both prior to and following onset of disease suggesting a key role for this cytokine in lupus pathogenesis, a proposal confirmed by neutralization studies which demonstrated that IL-22 played an essential role in the development of disease in the MRL/*lpr* mouse. This was further supported by studies showing that recombinant IL-22 significantly accelerated and exacerbated disease. By contrast, despite suppressing early IL-17A responses, the production of IL-17A was significantly increased in ES-62 treated mice during the established phase of disease, suggesting that IL-17A may promote pathogenesis during the initiation of pathology, yet act to resolve aberrant inflammation in the kidney. This potential dual role for IL-17A in the regulation of kidney inflammation was corroborated by studies using neutralizing antibodies and recombinant IL-17A, as the early neutralization of IL-17A production slowed the onset and severity of proteinuria and the late administration of rIL-17A suppressed disease severity.

Aberrant B cell responses drive pathogenesis both in murine models of SLE and also in human disease: reflecting this, B cell depleting therapies have proved successful in the clinic. Thus, the effects of ES-62 on the population dynamics of effector and regulatory B cell subsets were investigated and these studies revealed that ES-62 induced a hypo-responsive B cell phenotype that was associated with modulated development, migration and/or activation of pathogenic effector B cells. Furthermore, the proportion of IL-10 producing 'regulatory' B cells were significantly elevated in the ES-62 treated MRL/*lpr* mice during the established phase of disease. Crucially, the protection afforded against the development of proteinuria by ES-62 was mimicked by the adoptive transfer of B cells from ES-62 treated MRL/*lpr* mice: moreover, such protection was associated with modulation of the IL-17A/IL-22 axis, as observed in MRL/lpr mice treated with ES-62.

Together with previous reports on the therapeutic potential of ES-62 in arthritis and asthma, these studies suggest that therapies based on the parasite product have a future in the clinic. ES-62 itself is not suitable as a therapy, due to it immunogenic nature and the complexity of its biosynthesis: thus small molecular analogues (SMAs) of the parasite product have been synthesized. Two of these were tested in the MRL/*lpr* mouse and found to suppress the development of proteinuria, even when administered after the onset of pathology. This protection, as with that afforded by ES-62, was associated with a modulation of MyD88 signaling in the kidney and indicates that novel drugs, based on the safe modulation of the immune system by the parasite derived product, ES-62, have the potential to treat lupus nephritis in SLE patients.

Author's declaration

The work presented in this thesis represents the original work carried out by the author and has not been submitted in any form to another university. Where use has been made of materials provided by others, due acknowledgement has been made.

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September 2013

To the late nighters and weekend writers,
keep fighting the good fight.

Acknowledgements

First and foremost, I would like to thank my supervisor, Professor Maggie Harnett: you have been such an inspiration to work with that I feel honored to have learned my trade in your lab. I think the character and spirit of the Harnett lab at Glasgow has been, and continues to be, extremely warm and welcoming; this permeation over the years can only be a testament to the positive support and guidance that you have selflessly provided. Furthermore, I would like to thank Professor Billy Harnett for additional scientific support, mentoring, sharing a beer and taking my jokes whenever Liverpool beat Manchester United. It has been great getting to know you both and working with you over the past 4 years, I look forward to our future collaborations.

My heartfelt thanks and gratitude go to the members of the Harnett labs, both old and new, who have made me feel welcome and appreciated for the entirety of my time in the lab. I've enjoyed my time with you in Glasgow and look forward seeing you all in the future. I would particularly like to thank those of you who have been closest to me over the years (Miguel, Rusty, Dim, Verica and Jen) as well as those non-Harnetts that have been there all the way (Vuk, Laura and Sharon) our trips to the hills, the pub and Newcastle have kept me sane over the years; although the latter may have made the rest of you insane!

Special thanks has to go to the University of Glasgow for hosting he, and to the Wellcome Trust for providing an exceptional program that has allowed to me experience life and science in this great city, not to forget the summer working in the South of France. Furthermore, I would like to thank the trust for funding my scientific travel to the dull destinations of Whistler and Hawai; and for supporting my bench work, which has kept me occupied and off the streets for the past 4 years.

On the topic of bench work, I would like to thank some of the most important members of the lab that are often overlooked in acknowledgements, I can't list you all but I have to mention the Ranin electronic pipettes: without you, I couldn't have done it. On a more serious note, there are people in the department that have provided support and technical assistance over the years: mainly Jim, Shauna, Robin and Dianne in the GBRC. I would also like to thank Tony, Sandra, Joanne and Denis at the CRF for looking after the lads.

I would like to thank my family and friends, with special thanks to parents and sister, for providing me with support and inspiration; I would be nowhere today without their abiding love, dedication and tolerance of my disappearance into the world of science for weeks on end and for always phoning when the football or Strictly is on.

And finally, some quotes that I feel are relevant to science but would never be seen in another thesis:

“Even the bravest men are shocked by sudden terrors”
Tacitus on receiving reviewer’s comments.

“A meal that ends without cheese is like a beautiful woman with one eye”
Jean Anthelme Brillat-Savarin after he lost some data that was not essential but would have been nice to have.

“When bread is baked some parts are split at the surface, and these parts which thus open, and have a certain fashion contrary to the purpose of the baker’s art, are beautiful in a manner, and in a peculiar way excite a desire for eating.”
Marcus Aurelius on find an outlier that subsequently ruined a t-test.

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List of abbreviations

AHR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
ANA	Anti-nucleic acid
BCR	B cell receptor
BM	Bone marrow
BMM	Bone marrow derived macrophage
Breg	Regulatory B cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CIA	Collagen induced arthritis
DC	Dendritic cell
DN	Double negative
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbance assay
ES	Excretory secretory
FBS	Fetal bovine serum
H & E	Haematoxylin and eosin
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
i.p.	Intra-peritoneal
i.v.	Intra-venous
kDa	Kilo Daltons
Lpr	Lymphoproliferative
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NK	Natural killer
OVA	Ovalbumin
PAMP	Pattern associated molecular pattern
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PMA	Phorbal 12 myristate 13-acetate
pLN	Peripheral LN
RA	Rheumatoid arthritis
s.c.	Sub cutaneous
SLE	Systemic lupus erythematosus
SMA	Small molecular analogue
TCR	T cell receptor
TLR	Toll like receptor

1. General introduction

1.1. Immunity: a double-edged sword.

The immune system has evolved to protect us from disease by developing efficient mechanisms to eliminate pathogens whilst limiting host pathology resulting from inflammation. The advent of increased sanitation and widespread vaccination uptake has resulted in reduced exposure to infectious agents, consequently, aberrant or inappropriately strong immune responses are increasingly prevalent, resulting in a variety of pathologies and underlining the need for highly regulated immune responses. Such aberrant hyper-immune responses can lead to conditions associated with autoimmunity or allergy: hence, it is now often thought of as a double-edged sword. Increasingly, a major focus in translational medicine is to identify novel safe drug targets that allow the suppression of inflammatory disease, without compromising the capacity of the immune system to clear invading microorganisms and cancerous cells. Relating to this, a core aim of this thesis has been to exploit the helminth-derived immunomodulator ES-62, to identify key pathogenic inflammatory events in one such autoimmune inflammatory disorder, systemic lupus erythematosus (SLE, lupus), in order to increase our fundamental understanding of the etiology of this disease and potentially identify novel, safer anti-inflammatory drug targets.

1.2. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE, lupus) is a systemic autoimmune inflammatory disease characterized by the production of antibodies recognizing cytoplasmic and nuclear self-antigens, characteristically double stranded DNA (dsDNA). Complexes of self-reactive antibodies and host antigens circulate throughout the body and become trapped in organs with dense capillary networks such as the kidney, joints, skin and cardiovascular system: the deposition of these immune complexes cause local inflammation and damage. The kidney is a major site of disease and glomerulonephritis is one of the most serious manifestations of lupus (1,2).

The production of anti-dsDNA autoantibodies is associated with disease severity (3). These antibodies usually bind to DNA released from necrotic cells as this disorder is also associated with defects in the machinery of apoptotic cell clearance. However, in the kidney, circulating DNA can embed in the glomerular

matrix due to charge interactions with collagen type IV or heparin sulphate at the glomerular basement membrane, providing a reservoir of antigen for circulating anti-dsDNA antibodies (4,5). Inflammation in the kidney is driven by the activation of antibody (FcR) receptors(6), complement receptors (7,8) and toll-like receptors (TLR) (3,5,6,9); with the subsequent proinflammatory cytokine response inducing the recruitment of immune system cells into the kidney that further drive pathology by the production of antibodies, cytokines and the attempted clearance of apoptotic or antigenic debris (2,10).

It is currently estimated that 5 million people are affected with SLE worldwide; as with many autoimmune diseases, it is most common and severe in women (11). Lupus is monitored in the clinic by the SLE disease activity index (SLEDAI), a method by which clinicians can score the severity of disease by assessing specific cell counts, anti-DNA antibody and complement levels in the blood; fever; chest pain; mucosal inflammation; inflammatory rash; proteinuria; haematuria; muscle weakening; arthritis; vasculitis and CNS complications (12). SLE is typically treated by general immunosuppressive regimes and hence, there is an urgent unmet clinical need for more specific and less toxic drugs. Several promising drugs that have entered clinical trials, failed to show a significant benefit: it is now widely accepted that this was due to poor characterization and stratification of the heterogeneous SLE patient population preventing establishment of an appropriate clinical trial (13). The precise etiology of SLE is as of yet unknown but deregulation of multiple aspects of the immune system have been implicated in this complex disease (10). Therefore, to develop the required drugs and get them into the clinic, a better understanding of the immune system and its role in this disease is required.

1.3. Animal models of SLE

There are multiple mouse models of SLE that can either develop disease spontaneously or be induced. The relevance of these models is complicated by our lack of understanding of the origin of this autoimmune disease, which to date has advanced by investigating observations made in patients and these models (14). The classical spontaneous models are those provided by NZB/NZW F1, MRL/*lpr* and BXSB/*Yaa* mice. The New Zealand Black (NZB) and New Zealand

White (NZW) strains of mice are both prone to develop mild autoimmunity, however, the F1 cross phenotype exhibits exacerbated disease and these mice die of renal failure within 12 months. NZB/NZW F1 progeny develop several of the pathologies associated with SLE, such as lymphadenopathy, splenomegaly and ANA production, however, they do not produce autoantibody responses to RNA-containing immune complexes (14). Likewise, the MRL strain of mice, which have a complex background due to the crossing of the LG/J mice with C3H/Di, C57BL/6 and AKR/J strains, contains several genes associated with susceptibility in SLE patients (14). Consistent with this, MRL mice exhibit all of the major symptoms presented by SLE patients. MRL/*lpr* mice have a mutation on chromosome 19 that alters the transcription of the FAS receptor and these mice develop an accelerated version of the disease, relative to the parental MRL/MP strain, and usually die of renal failure within 5 months (14). The BXSB/*Yaa* strain develops a lupus-like disease due to duplications of at least 16 genes that contribute to SLE, the most prominent of which is TLR7(14). More recently described spontaneous models of SLE include the FcγR2b$^{-/-}$mouse (15), which exhibits hyper-reactive B cell responses, and the Ro52$^{-/-}$ mouse that develops a lupus-like disease following tissue injury (16). As well as these spontaneous models, there are a number of inducible models of SLE that, for example, employ administration of pristine or induction of chronic graft versus host disease regimens (14).

1.4: The innate immune system

1.4.1. First line of defense

The innate immune system provides a natural form of protection and is present, in some form, in all eukaryotic organisms. It provides the first line of defense against microorganisms and, in addition to barrier functions; it involves the activation of a range of polyspecific extracellular and intracellular receptors. This leads to the recognition and/or phagocytosis of foreign particles, resulting in the release of pro-inflammatory mediators, cellular infiltration and the induction of inflammation. In addition, via antigen presentation cells, the innate immune system can trigger the activation of antigen specific adaptive immune responses. In SLE, prolonged activation of the innate immune system propagates the inflammation associated with disease pathology (17).

1.4.2. Receptors of the innate immune system

Our understanding of the innate immune system was revolutionized by the seminal findings of Janeway and Madzhitov that immune responses are initiated by the recognition of conserved pathogen-associated molecular patterns (PAMPs) present on microorganisms, by pattern recognition receptor (PRR). PRRs are germ-line encoded receptors expressed on the cell surface or in intracellular vesicles. Upon recognition of PAMPs, the innate immune response is often sufficient to neutralize and clear the threat of invading pathogens, however, in more complex scenaria; PRR activation can result in the triggering of the adaptive immune response. PAMPs are present on a variety of molecules expressed by pathogens such as bacterial lipopolysaccharide (LPS), fungal carbohydrates and viral nucleic acids (18). There are several families of PRRs including the cytosolic NOD-like receptors, C-type lectin receptors and RIG-1-like receptors, which recognize bacterial products, β-glucans and nucleic acids respectively (19). However, by far the most studied and well defined family of PRRs are the toll-like receptors (TLR; Fig. 1.1)(20).

1.4.3. Toll like receptors

TLR signaling is initiated by ligand-mediated homo- or hetero-dimerization of TLR-ligand-binding subunits: all TLRs, except TLR3, signal via the adaptor protein MyD88 to activate the NF-κB pathway and hence drive inflammation (19) (Fig. 1.1). Deregulation of TLR signaling is associated with the development of autoimmune diseases (21). The expression of TLRs is increased on monocytes, T and B cells from SLE patients, and their stimulation with endogenous antigen leads to the elevated production of pro-inflammatory cytokines (22). Moreover, infection often precedes autoimmune responses and has been proposed to trigger disease flares via TLR signaling in chronic autoimmune disease.

1.4.4. Danger hypothesis

The "Danger Hypothesis" proposes that the immune system recognizes endogenous antigens that are produced by dying, damaged or corrupted cells (23), and in an analogous manner to PAMPs, these damage/danger associated molecular patterns (DAMPs or Alarmins) are detected by PRRs. Although infection in SLE patients likely exacerbates disease, the ability of PRRs to recognize

DAMPs probably provides the major mechanism by which PRRs contributes to disease severity (24). One such DAMP that is considered to contribute to disease in SLE patients is the high mobility group box 1 (HMGB1) protein. Circulating HMGB1 levels are elevated in SLE patients and correlate with disease. Although early reports that HMGB1 stimulated TLR4 reflected sample contamination (25), HMGB1 has since been shown to stimulate immune system cells via TLR9 and TLR2 in addition to RAGE receptors (7,8,26,27).

1.4.5. Apoptosis

Cell death is a necessary and natural process that occurs during pathological and physiological circumstances. The resulting debris must be cleared through a controlled process such as apoptosis (controlled non-inflammatory cell death or 'suicide') to avoid overzealous immune responses occurring. Such self-harming responses are triggered by tissue damage and/or cellular necrosis (uncontrolled inflammatory cell death) (28). The innate immune system acts to regulate this by responding to DAMPs expressed on apoptotic cells: an example of this is phosphatidylserine, a lipid that is normally expressed on the inner layer of the phospholipid bilayer but expressed on the surface of apoptotic cells (28). Reflecting this role of the innate system in apoptosis, a major factor in the pathogenesis of SLE is the defective apoptotic clearance of cell debris and the subsequent over exposure of self-antigens, which, along with a breakdown in immune tolerance, drives the activation and generation of damaging autoimmune responses. Apoptosis *per se* is enhanced in SLE patients (29) yet factors that control the clearance of cell debris: such as MFG-E8, an integrin that allows phagocytes to bind to apoptotic cells; are reduced (30) leading to defective clearance.

1.4.6. Complement

A fundamental component in the clearance of apoptotic debris is the complement system, which comprises of a cascade of more than 25 soluble serum proteins that recognize pathogenic, immunogenic and apoptotic material (Fig. 1.2). The activation of complement initiates a series of events involving the neutralization or opsonization of dangerous material and targeting of phagocytosis (31). Defects in the classical complement cascade have long been associated with SLE as this

pathway is important for the clearance of apoptotic debris (32): the alternative pathway on the other hand is increasingly recognized as pathogenic in SLE patients and mouse models of the disease
(2,10,17,33-36).

1.4.7. Type 1 interferons

Type 1 interferons (IFN) are a family of cytokines best known for their antiviral capabilities. They are expressed by a wide variety of cells and are induced by PRR activation. In some autoimmune diseases they are protective as they can suppress certain aspects of the immune response (37) but they are generally considered to be pathogenic in SLE where there is a clear type 1 IFN signature in SLE patients, two thirds of which express high serum levels of the cytokine that correlates with SLEDAI (38). Such high type 1 IFN expression is associated with enhanced B cell survival and auto-antibody production (39) and consistent with they key role of B cells in SLE, studies in mice have revealed that IFNα can only exacerbate autoimmunity in strains of mice that are genetically susceptible to develop lupus (40).

1.4.8. Cells of the innate immune system

1.4.8.1. Phagocytes

Phagocytosis is a process by which extracellular material is internalized and then degraded as an initial attempt to clear cell debris and limit infection. Neutrophils are the first cells are recruited to the site of inflammation; these short-lived, terminally differentiated cells designed to phagocytize and kill infectious agents and further promote inflammation by secreting cytokines and chemokines; however, in autoimmune disease the excessive activation of neutrophils can cause extensive collateral damage to the host (41). One example of this is through a process unique to neutrophils called NETosis, whereby the neutrophils expel their ‘sticky’ nucleic material as neutrophil extracellular traps (NETs). Although in a normal or healthy context this is an effective way to deal with multiple pathogens at once, in SLE patients where the clearance of this nuclear material will be defective, perhaps due to reduced DNase 1 levels, NETosis simply increases the amount of nucleic self-antigens exposed to the immune system (42,43).

Phagocytosis is not restricted to neutrophils and in the case of "professional antigen-presenting cells" of the innate system, macrophages and dendritic cells (DC), the peptides resulting from the proteolytic degradation associated with phagocytosis are presented to T cells via MHC Class II molecules (Fig. 1.3). These professional antigen-presenting cells shape the subsequent type of adaptive immune response by producing specific combinations of cytokines, chemokines and other inflammatory mediators. This response depends on the additional signals they receive at the time of phagocytosis: for example, in the context of the gut where many harmless foreign particles are found (commensal microorganisms and food particles), a phagocyte would express regulatory cytokines, such as IL-10, to prevent an immune response: however, if an antigen were sampled at the time of a bacterial infection or cell damage then these cells would be licensed to secrete proinflammatory cytokines, such as IL-6, to trigger an immune response.

Moreover, as tissue resident cells, macrophages are often the first phagocytes to respond to cell death and therefore play a pivotal role in the clearance of self-antigens. In SLE, macrophages are defective in clearing apoptotic cell debris *in vitro* (44) resulting in necrosis and release of a proinflammatory DAMPs, which further activate the innate immune system (44). Similarly, monocytes, which normally express high levels of scavenger receptors that can bind PAMPs and DAMPs (45), are reduced in number and defective in clearing apoptotic debris in SLE patients, yet produce elevated levels of the proinflammatory cytokines, TNFα and IL-6, compared to monocytes from healthy controls (46,47). Furthermore, although these cells express comparable levels of the receptor for the regulatory cytokine IL-10, monocytes from SLE patients are less sensitive to IL-10 compared to those from healthy controls and this IL-10-hyporesponsive phenotype is enhanced in the presence of immune complexes (48). Complementing this, and consistent with their role in initiating and controlling T cell responses, both in terms of determining the type of T cell response as well as neutralizing the threat of auto-reactive T cells by inducing anergy or cell death, DC are reported to be hyperactive in SLE, producing pro-inflammatory cytokines, for example type 1 IFN, in response to circulating complexes of autoantibodies and nucleic acids (49).

1.4.8.2. Innate lymphoid cells

There has been increasing recognition of innate lymphoid cells and reflecting this several populations have been recently grouped into the family of "innate lymphoid cells" (ILC). For example, ILC1 (which include natural killer (NK) cells) produce high levels of IFNγ and are involved in the clearance of cells infected with intracellular pathogens including bacteria and viruses. Interestingly, NK cells have been implicated as protective in SLE and a decline in their numbers inversely correlates with SLEDAI (50). The relevance of other populations of the ILC family: the lymphatic tissue inducer (LTi), ILC2 (natural helper or nuocytes), ILC17 and ILC22 cells has not been examined in SLE. Invariant natural killer T cells (iNK T cells), which respond to glycolipid antigens presented by CD1d, a non-classical MHC molecule, by producing a variety of cytokines are functionally and numerically reduced in the blood of SLE patients: however they are found to be concentrated at the site of cutaneous inflammation (51). The significance of these cells in SLE is yet to be determined (52). Other lymphoid cells with "innate-like" functions, γδT cells and B1 B cells, are discussed in more detail in 1.5.3.1 and 1.5.5.4.8.

1.5. The adaptive immune response

1.5.1. Innate versus adaptive responses

Whereas the innate immune response offers a rapid, non-specific response to contain infection, the adaptive immune system provides a slower but more specific response combining effector and memory cells that can target specific pathogens more effectively, particularly on re-infection. The principal cells of the adaptive immune response are the B and T cells, which develop from a common progenitor in the bone marrow (53), to provide the humoral and cellular arms of the adaptive immune response, respectively. In SLE, regulation of both of these cell type responses is dysfunctional.

1.5.2. Effector regulatory balance

It is essential during the adaptive immune response that regulatory cells, which suppress and control the effector immune response, also develop to ensure a controlled immune response that will clear immunogenic material whilst causing minimal collateral damage to the host cells. Both of these effector and regulatory

cell populations must interact harmoniously to maintain homeostasis. Autoimmune disease occurs when effector responses expand to the extent that regulatory cells can no longer control them; this may be due to defective regulatory function or aberrant and/or excessive levels of immunogen. By contrast, if the regulatory network is too strong,, cancer or chronic infection can occur (54).

1.5.3. T lymphocytes

1.5.3.1. T cell development and activation

T cell development occurs in the thymus and involves proliferation of progenitors, rearrangement of antigen receptor (TCR) genes, and positive and negative selection of cells expressing viable TCR (55). Selection is required as the specificity of the newly generated TCR is random; T cells therefore undergo a negative selection process to prevent the development of self-reactive T cells. Negative selection is controlled by stromal cells in the thymus that express self-antigens; thus, any $CD4^+CD8^+$ double positive (DP) thymocytes that recognise these antigens in the context of self MHC with strong affinity will be removed by either deletion, receptor editing or anergy. Cells expressing a TCR that only weakly recognises self-antigen will be positively selected for further differentiation (55). Mature T cells therefore exit the thymus as either MHC class II restricted $CD3^+CD4^+$ T helper cells or MHC class I restricted $CD3^+CD8^+$ cytotoxic T cells (56). More recently, it has emerged that DP thymocytes recognizing antigen with intermediate affinity may differentiate into natural regulatory T cells (Tregs) (57). When a mature naive T cell recognizes antigen presented by an APC, it will move to the T cell zone of a secondary lymphoid organ, express co-stimulatory molecules such as CD40 ligand (CD40L) and ICOS, in order to interact with B cells specific for the same antigen. The majority of T cells express a $\alpha\beta$ TCR however, a small proportion (5%) of T cells express a $\gamma\delta$ TCR. These $\gamma\delta$ T cells are innate-like T cells with limited TCR repertoire diversity and are therefore not classed as ILC. $\gamma\delta$ T cells may contribute towards the pathogenesis of AI by activating B cells, conventional $\alpha\beta$ TCR T cells and secreting pro-inflammatory cytokines (58).

1.5.3.2. TCR signaling

The cytoplasmic domain of the TCR does not have any capacity to signal and therefore is required to form an antigen signaling receptor complex with the accessory signal transducing molecules CD3γ, CD3δ, CD3ε and CD3ζ, which have the ability to signal through immunoreceptor tyrosine-based activation motifs (ITAMs; (59)). The CD4 and CD8 molecules expressed on the surface of naïve T cells recognize non-polymorphic regions of the MHC class II and MHC class I molecules respectively, and by doing so, strengthen the bond between TCR and MHC molecule (60). The CD4 and CD8 molecules aid in TCR signaling further by recruiting LCK, a tyrosine kinase important in phosphorylation of the ITAMs in the TCR signaling complex (61). TCR signaling is insufficient to activate T cells and co-receptor signaling is required for full T cell activation (62). T cells from SLE patients usually exhibit amplified TCR signaling that is rerouted through alternative signaling pathways (63). For example, rather than simply utilizing the CD3ζ: ZAP70 pathway, the TCR in T cells from SLE patients additionally associates with the signaling via the common γ-chain: SYK pathway, which normally transduces FcR signals, resulting in stronger signaling through the TCR and thus reducing the requirement of co-receptor signaling and cytokines for T cell activation (64).

1.5.3.3. Co-receptor signaling

To ensure the full activation or priming of naïve T cells, 3 signals from APC are required: 1) TCR recognition of antigen presented by MHC molecules, 2) production of pro-inflammatory cytokines to induce the particular functional phenotype of the cells and 3) co-stimulation via interactions between co-receptors on APC and T cells (65). APC, mainly DC, that have been activated via PRR and cytokine receptor signaling, will migrate to the secondary lymphoid organs and express CD80/86 and ICOS-L molecules that will interact with "costimulatory" CD28 and ICOS molecules on the T cells, respectively. This signaling amplifies and ‘fine tunes’ that of the TCR signal, allowing the T cell to respond (66). Consistent with their hyper-responsive phenotype, the level of expression of stimulatory co-receptors is usually found to be elevated on the APC and/or T cells from SLE patients (67). Moreover, although not all co-receptors transduce positive signals, the negative receptor CTLA-4, which binds to CD80/86 molecules with higher affinity than CD28 to prevent excessive T cells responses is expressed at

lower levels on the surface of T cells in lupus patients (68). Defective CTLA4 signaling has also been reported in SLE prone mice (69).

1.5.3.4. T cell responses

$CD8^+$ cytotoxic T cells circulate through the blood and secondary lymphoid organs (lymph nodes and spleen) surveying antigens presented by APC. Upon priming, the expanded population of $CD8^+$ effector cells will search for and kill target cells via the release of cytolytic molecules (granzymes and perforin) and activate the innate immune system by secreting pro-inflammatory cytokines such as IFNγ and TNFα, in order to drive the clearance of infected cells (65). IFNγ has been proposed to be one of the major pathogenic cytokines in SLE and consistent with this, IFNγ-producing $CD8^+$ T cells are found in the urine of patients during a lupus flare have been associated with disease (70,71).

Like $CD8^+$ T cells, $CD4^+$ helper T cells circulate throughout the body but alternatively respond to antigens presented by MHC class II molecules. Depending on the cytokine signals they receive at the time of MHC:peptide complex recognition, $CD4^+$ helper T cells can develop into at least four effector (Th1, Th2 or Th17) or regulatory populations that shape the immune response in a specific manner (72). SLE patients present with a mixed $CD4^+$ T helper cell phenotype (73) reflected by the elevated levels of a variety of cytokines, including IL-18, IL-12, IL-17A and IL-4 often observed in SLE patients (74).

1.5.3.5.1. Th1 responses

Th1 cells are generated to help combat intracellular pathogens and are often associated with cytotoxic responses: as they result in the recruitment of $CD8^+$ T cells and macrophages, which kill infected cells and phagocytose the resulting cell debris respectively (75). The main Th1 polarizing cytokine, IL-12p70, is composed of two subunits, p35 and p40(76), and binds to a specific heterodimeric receptor containing the IL-12Rβ1 and IL-12Rβ2 subunits (77). This interaction activates a JAK: STAT4 pathway resulting in the expression of t-Bet, the signature transcription factor associated with Th1 responses (78). The signature cytokine of the Th1 phenotype, IFNγ, is elevated in the serum (70) and urine of patients with SLE (79); copy number variations of *il-12* and *t-bet* are also elevated in patients

and correlate with disease severity (80). Indeed, Th1 induction has been shown to be essential for the development of disease in the MRL/*lpr* mouse model of SLE (81).

1.5.3.5.2. Th2 responses

Th2 cell responses are important in the protection against extracellular pathogens as they promote B cell responses by the production of IL-4, IL-5, IL-9, IL-13 and IL-25 (IL-17E) (82,83). The transcription factor that drives the development of Th2 cells is GATA3, and although the exact mechanisms of Th2 activation *in vivo* are not yet known, the *in vitro* expression of GATA3 can be induced by: IL-4 activation of STAT6, IL-2 activation of STAT5, WNT activation by β-catenin, T cell factor 1 and also by Notch signaling (78,84). Reflecting their mixed inflammatory phenotype, Th2 related cytokines have also been found to be elevated in SLE patients and although they have not received as much focus as other T helper cell responses, it is increasingly recognized that Th2-associated IgE, basophil and cytokine responses may play an important pathogenic role in some SLE patients (85,86).

1.5.3.5.3. Th17 and IL-17 responses

1.5.3.5.3.1. Development

Th17 cells are one of the most recent T helper cell phenotypes to be characterized and their discovery led to a readjustment of the T helper cell paradigm as it became apparent that T cell responses can still be made in the absence of Th1 or Th2 promoting factors (87). Indeed, many responses or diseases previously characterized as exhibiting a Th1 phenotype have since been reclassified as being Th17-dependent. Th17 cells have been reported to produce a number of cytokines belonging to the IL-17 family (IL-17A-F, Table 1.1), which are important in protection against bacterial and fungal infections. These responses are also implicated in the etiology of inflammatory disease (88,89). Th17 cells are also capable of producing and inducing several other cytokines, such as IL-21 and IL-22, which are involved in the activation and recruitment of other cells to the site of inflammation (90).

Recent comprehensive analysis has established that development of Th17 cells requires three individual stages: (i) 'induction' occurs within the first 4 hours following stimulation with TGFβ1 and IL-6, the latter of which stimulates the expression of the Th17-associated transcription factor RORγt; (ii) 'amplification' occurs between 4-20 hours and during this period, the cells begin to express mRNA of both pro- and anti-inflammatory cytokines such as IL-17A and IL-10 respectively; and finally, (iii) 'activation' occurs following expression of the IL-23 receptor (after 48 hours) as signaling via this receptor permits the cells to become fully activated Th17 cells that express pro-inflammatory cytokines and chemokines, but not IL-10 (91). However, FAS and GM-CSF signaling are also required for full differentiation of Th17 cells and whilst IL-17A production can be stimulated by either IL-1β or IL-23, optimal cytokine release occurs following stimulation with both of these proinflammatory cytokines (92).

1.5.3.5.3.2. Innate sources of IL-17

Although IL-17 responses are often generically described as Th17 responses, IL-17A can still be made in T cell-deficient $RAG^{-/-}$ mice, suggesting alternative sources of this cytokine (93). Furthermore, IL-17A responses can be detected within 8 hours following bacterial challenge (94), a time frame that suggests an innate, rather than adaptive immune source, as the latter usually requires approximately 3-5 days (24). It is now clear that several populations of the innate immune system can produce IL-17A, such as ILC (NK and LTi cells), iNK T cells, γδT cells and $CD11b^{+}$ $GR1^{+}$ myeloid cells, the latter including neutrophils (24,95-98).

1.5.3.5.3.3. Transcription factors

All IL-17A producing cells express RORγt, the transcription factor that controls the development of Th17 cells (99), however, expression of RORγt is not essential for production of this cytokine by non-Th17 cells (60,81,82)(24) and in the absence of RORγt, other transcription factors drive IL-17A responses. One such transcription factor is the aryl hydrocarbon receptor (AHR). Th17 cells, γδ T cells and ILC are among some of the cells that express AHR, which appears to be important for effector responses of fully activated Th17 cells, including the production of IL-22. AHR allows cells to respond to environmental stimuli, suggesting that AHR may

provide molecular evidence for the epidemiological link between the development of autoimmune disease and environment toxins (100,101).

1.5.3.5.3.4. IL-17 in autoimmunity

IL-17A exhibits multiple functional outcomes that allow this cytokine to orchestrate an immune response: thus, for example, in SLE IL-17A acts in synergy with IL-1, IL-6 or TNFα in the recruitment of neutrophils to sites of inflammation (102) and with B cell activating factor (BAFF) to promote the survival and activation of B cells (103). It may also act to recruit other effector cells, such as Th1 cells, to sites of inflammation (104). In addition, IL-17A has been implicated in the regulation of mucosal barrier integrity during pathogenesis and homeostasis (105), immune homeostasis (106) and the induction of innate anti-microbial agents such as β-defensins (24).

IL-17A has been proposed to be crucial for the development of autoimmune disease and consistent with this, has been shown to play roles in both initiating and perpetuating disease in previously considered Th1-biased inflammatory (models of) diseases (107). Thus, early IL-17A responses have been reported to be important in the development of disease in a collagen-induced model of rheumatoid arthritis (CIA) (108) and cooperation between IL-17A-producing $\gamma\delta$ T cells and Th17 cells enhances disease in a murine model of uveitis (109). IL-17A is also essential for the development of experimental autoimmune encephalitis (EAE), a model of multiple sclerosis (110). Interestingly, therefore, given its mixed inflammatory phenotype, IL-17A is consistently found to be elevated in the serum of SLE patients compared to healthy controls (111), IL-17A levels are also found to be enhanced in strains of mice that spontaneously develop a lupus-like disease (112) and IL-17 signaling is essential for disease in the FcγR2b$^{-/-}$ mouse model of SLE (113). Consistent with this, IL-17A producing cells have also been observed in the kidneys of patients and murine models of SLE (111,114,115).

IL-17 appears to act as a master regulator of inflammation as it also appears to be crucial to the development of Th2-based pathologies (116) and it has been proposed that IL-17 may also play protective roles in the resolution of inflammation, as reported in murine models of colitis (117) and asthma (118).

1.5.3.5.4. Th22 and IL-22 responses

1.5.3.5.4.1. Development and source

IL-22 is a cytokine of the IL-10 family that can be produced by cells of both the innate (ILC) and adaptive (γδ, $CD4^+$ and $CD8^+$ T cells) arms of the immune system (106) as indicated by its production in $Rag^{-/-}$ mice (95,119,120). Interestingly, therefore, it is unusual in that whilst being produced exclusively by leukocytes it acts predominantly on non-haemopoietic cells (120). The IL-22 receptor is a heterodimer consisting the β chain of the IL-10 receptor and a specific IL-22-binding receptor molecule (121) and the bioavailability of IL-22 is controlled, at least in part by a soluble IL-22 binding protein (122). Two cell phenotypes that produce IL-22 exclusively, NK22 and Th22 cells, have been reported (123,124). As with Th17 cells, Th22 cells can be induced by IL-23, IL1β and IL-6, the latter being required for the expression of the IL-22R (125): however, whereas TGFβ is also required for the development of Th17 cells, TGFβ inhibits the development of Th22 cells (126). Moreover, Th22 cells express the RORγt and AHR transcription factors but whilst expression of AHR is essential for IL-22 production, that of RORγt is not (99,101).

1.5.3.5.4.2. Function in autoimmunity

IL-22 can be functionally related to IL-17 in that both cytokines exhibit effects on epithelial cells, including epithelial barrier formation (127); however, IL-17 and IL-22 are generally non-redundant and are functionally distinct (106). IL-22 can often act in an anti-inflammatory or tissue repair manner (106), although by working in synergy with other pro-inflammatory cytokines, such as TNFα or IFNγ, IL-22 can enhance inflammation (128,129). Thus, during mucosal inflammation, IL-22 is important in maintaining epithelial barrier integrity and promotes the resolution of damage by triggering epithelial cell growth (128,130) and hence acts in a protective manner during lung inflammation (131,132). In support of this protective role against mucosal inflammation, the transfer of IL-22 producing cells is protective in murine models of IBD (133) and there is also evidence for IL-22 being protective in mouse models of MS, IBD and hepatitis (134,135). Depending on the immunological context however, IL-22 may also be pathogenic in autoimmune disease (128,136) and has been reported to play both pathogenic and protective roles in an ovalbumin-induced model of airway hyper-responsiveness or asthma.

Thus, IL-22$^{-/-}$ mice do not develop airway hyper-responsive and neutralization of IL-22 in wild-type mice, at the time of immunization, prevented disease. By contrast, neutralization of IL-22 during antigen challenge, enhanced airway inflammation whilst at this late stage of disease administration of recombinant cytokine suppressed disease. These data suggested both a pathogenic role for IL-22 during disease initiation and a protective role during antigen challenge (118). Indeed, there is increasing evidence that the IL-17A:IL-22 balance may regulate pathology, as such relationships have also been reported in experimental models of arthritis (Pineda, Harnett & Harnett, unpublished data) (106).

This potential dual functionality of IL-22 may explain why, in SLE, the limited studies on the role of IL-22 in pathogenesis have been contradictory. For example, levels of Th22 cells have been associated with SLEDAI (137,138) and the copy number variations of *il-22* are increased in SLE patients (139). Furthermore, treatment with hydroxychloroquine was found to decrease serum IL-22 levels (140) and TGFβ levels are low in SLE patients, perhaps suggesting that Th22, rather than Th17 or Tregs, cells may be pathogenic in SLE (141). By contrast, it has been suggested that Th22 levels are an indicator only of cutaneous lupus (142) whilst others have reported that IL-22 is decreased in the serum of SLE patients, suggesting a potential protective role for this cytokine in SLE (143,144).

1.5.3.5.5. Regulatory T cells

1.5.3.5.5.1. Development

Regulatory T cells (Treg) can suppress and therefore homeostatically limit immune responses. Thus the loss of Tregs can result in immunopathology, autoimmunity and allergy. Tregs provide a major component of the mechanisms contributing to the maintenance of peripheral tolerance of T cells, the process by which auto-reactive T cells that escape central tolerance in the thymus or encounter harmless foreign antigen are either removed by apoptosis or rendered anergic. The induction of Tregs requires IL-2 and TGFβ, which stimulates the induction of the Treg signature transcription factor, FoxP3. It is now clear that there are several distinct populations of regulatory T cells. For example, FoxP3+ naturally occurring regulatory T cells (nTregs) develop in the thymus and are selected by their relatively strong reactivity to self-antigens presented by stromal cells during T cell

development. By contrast, induced FoxP3+ Tregs, IL-10-secreting T_R1 cells and TGFβ-secreting Th3 cells develop in the periphery following recognition of a particular antigen in the presence of Treg-inducing cytokines such as TGFβ (145,146). Moreover, it appears that regulatory T cells can suppress immune responses by at least four mechanisms: (i) production of the regulatory cytokines IL-10 and TGFβ; (ii) cytolysis via production of granzymes and perforin; (iii) disruption of metabolism by consumption of survival/growth factors or alternatively, by production of anti-inflammatory metabolites such as adenosine nucleosides; and (iv) by modulation of DC function via their expression of CTLA-4, the negative signaling co-receptor molecule (57).

1.5.3.5.5.2. Tregs in autoimmunity

Several groups have reported an inverse correlation between the level of circulating Tregs and SLEDAI (147-151) and consistent with this, Treg levels were found to be elevated in patients following treatment with corticosteroids (152,153). Moreover, although Tregs from SLE patients were capable of suppressing immune responses *in vitro,* the effectiveness of this suppression was reduced compared to the capacity of Tregs from healthy controls (154,155). It is not clear, however, whether this ineffective suppression is due to a Treg defect or because the T cells from SLE patients are resistant to Treg mediated suppression (156). Nevertheless, Treg deficiencies in mice result in a lupus-like disease including glomerularnephritis (157) and several studies have reported a Treg defect in mouse models of SLE (158).

1.5.4. B cells

1.5.4.1. B cell development

B cells are a fundamental component of disease pathology in SLE patients (3); underlining this, therapies targeting B cells have proved effective in the clinic. In addition to producing the autoantibodies that are characteristic of lupus, B cells also present antigens and secrete cytokines that shape the immune response. The expansion of B cells in SLE patients correlates with SLEDAI (159) whilst renal infiltration by B cells is a predictor of severe disease and clinical outcome (160). B cells are also essential for pathology in mouse models of SLE (161-163).

B cell development starts in the bone marrow where common lymphoid precursors develop into pre-pro-pre-B cells, pro-B cells, pre-B cells and finally immature B cells (Fig. 1.4). During development, the B cell will rearrange the variable (V), diversity (D) and joining (J) segments of its immunoglobulin genes to form a viable B cell receptor (BCR). BCR form an antigen signaling complex with the signal transducing molecules, Igα and Igβ (164), which contain cytoplasmic ITAM signaling domains that are phosphorylated by the protein tyrosine kinase SYK (165) (Fig. 1.5). The rearrangement of the Ig genes is a random process and 75% of early B cells are auto-reactive. Several mechanisms exist to prevent the release of these cells into the periphery. For example, strong recognition of self antigen expressed by stromal cells in the bone marrow will induce cell death of IgM+ immature B cells by apoptosis whereas an intermediate BCR signal will induce receptor editing (166). Alternatively, self-reactive B cells can enter a state of anergy where they will not respond to antigen (167).

BCR signal strength further determines the peripheral development of B cells (168) with B cell survival requiring weak 'tonic' BCR signaling(169). Consistent with a pathogenic role of B cell hyper-responsiveness, BCR signaling is elevated in SLE patients (170) whilst inhibition of BCR signaling prevents disease development in a lupus mouse model (171). Furthermore, mice that do not express negative regulators of BCR signaling, such as Lyn and SHP-1, develop a lupus like disease and supporting the physiological relevance of such processes, the expression of these signal transducers is reduced in B cells from SLE patients (172).

1.5.4.8. Peripheral B effector cell development

1.5.4.8.1. The spleen

The spleen has two major functions in biology (Fig. 1.6): in the red pulp, blood is filtered to remove old or damaged cells and antigens: whereas in the white pulp, immature (transitional) B cells differentiate into mature-naïve, effector and memory B cells (Fig. 1.7). The white pulp has three main architectural features: the periarteriolar lymphoid sheath, or the T cell zone; the B cell zone containing B cell follicles and the marginal zone (MZ), where blood borne antigens are sampled by specialist macrophages and the MZ B cell subset (Fig. 1.6) (173).

1.5.4.8.2. Transitional B cells

B cells expressing a viable BCR leave the bone marrow via the circulatory system in an immature form termed transitional type 1 (T1; $CD19^{+}CD21^{-}CD23^{-}IgM^{+}IgD^{-}AA4.1^{+}$) and whilst they home to the spleen, they lack the ability to recirculate (Fig. 1.7) (174). The survival of T1 B cells and their further maturation is heavily dependent on BAFF survival signals (175). In the spleen, T1 B cells quickly differentiate into a second transitional state termed transitional type 2 (T2; $CD19^{+}CD21^{-}CD23^{+}IgM^{+}IgD^{-}AA4.1^{+}$): these cells enter the B cell follicle where they acquire the expression of IgD and CD23 and the ability to circulate around the body(176). The development of T2 B cells into either marginal zone precursor cells (MZP; $CD19^{+}CD21^{+}CD23^{+}IgM^{+}IgD^{+}AA4.1^{-}$) or follicular type 1 (Fo1; $CD19^{+}CD21^{low/-}CD23^{+}IgM^{low}IgD^{+}AA4.1^{-}$) B cells is dependent on four factors: BCR signal strength, BAFF survival signals, Notch signaling and NFκB activation (174).

1.5.4.8.3. Marginal zone B cells

MZP B cells reside in the B cell follicles of the spleen where they develop into marginal zone B cells (MZ; $CD19^{+}CD21^{+}CD23^{-}IgM^{+}IgD^{-}AA4.1^{-}$). MZ B cells represent approximately 15% of all B cells (177) and are found between the white and red pulp in the marginal sinuses of the spleen: they sample antigens in the blood and respond to lipid and T-independent antigens. MZ B cells capture antigens that have been opsonized with antibodies or complement via receptors expressed on their surface. They also express high levels of CD1d, a non-classical MHC molecule that allows the cells to present lipid antigens to iNKT cells (178) as well as high levels of TLRs allowing them to respond to PAMPS. MZ B cells constantly shuttle between the marginal zone and B cell follicles where they present this captured antigen to local follicular DC (179,180). MZ B cells can also respond to antigen directly and develop into short-lived antibody producing cells, which migrate into the red pulp and produce antibodies. However, recent studies suggest that a proportion of activated MZ B cells may also enter the B cell follicle and undergo further development in germinal centers (181). It is likely that they contribute to the pathogenesis of SLE due to their ability to sense blood borne antigens, such as immune complexes, and consistent with this, the proportion of MZ B cells in the B cell follicle is elevated in lupus prone mice (182,183). It is difficult to examine the role of MZ B cells in patients, as these cells do not leave

the spleen. Moreover, there is also evidence that MZ B cells may be protective in SLE prone mice as stimulation of TLR9 with DNA results in elevated IL-10 production that can suppress Th1 responses (184) although it is unclear whether immune complexes containing DNA would have the same protective effect.

1.5.4.8.4. Follicular B cells

As with MZP B cells, Fo1 B cells are also derived from T2 B cells. Fo1 B cells comprise the major phenotype of mature B cells in adults, representing approximately 70% of all B cells. They circulate around the body in the blood and home to B cell follicles in the secondary lymphoid organs, which are situated adjacent to T cell zones to allow interactions between B cells and T helper cells. Whilst Fo1 B cells are responsible for T cell dependent responses in the B cell follicles, they are capable of producing T independent responses outside of the follicles and in the bone marrow (174). A second follicular B cell population exists, termed follicular type 2 B cells (Fo2; $CD19^{+}CD21^{low/-}CD23^{+}IgM^{+}IgD^{+}AA4.1^{-}$) that develop independently of antigen and likely form a pool of cells that can replenish the marginal zone B cell population in the event that it is destroyed by a blood borne pathogen (185).

1.5.4.8.5. B cell activation

Circulating Fo1 B cells migrate around the body in search of antigen. B cells can recognize antigens that are soluble or membrane bound such as those tethered to the surface of cells via complement or antibody receptors (186). Signaling events at the immunological synapse, where the BCR has recognized membrane-bound antigen, induce localized spreading of the B cell plasma membrane to maximize the number of BCR: antigen interactions. The signaling following such polarization of the BCR triggers cytoskeleton rearrangements and the release of lytic enzymes from the B cell, which release the antigen from the presenting cell. The B cell then internalizes the antigen complexed to its BCR and processes it via the endosomal MHC class II pathway (Fig. 1.3). Activated B cells will then migrate towards the T cell: B cell border in a secondary lymphoid organ in search for T cell help. If the newly acquired antigen, presented by the B cell in the form of MHC-peptide, is recognized by the TCR of an activated T cell, the T cell will release pro-inflammatory cytokines that will fully activate the B cell. At this point the B cell will

migrate into the B cell follicle and enter the germinal center reaction (186-188). Co-receptor expression by B cells dictates their activation and pro-inflammatory status, it is important for efficient activation by T cells; in SLE patients, CD71, CD80 and CD86 are all elevated on circulating B cells (189).

1.5.4.8.6. Germinal center reaction

The germinal center (GC) has two histologically distinct regions known as the dark and lights zones. The dark zone is densely packed with rapidly proliferating B cells known as centroblasts, which are drawn to the dark zone via CXCR5 homing towards CXCL13 that is produced by local stromal cells. In the dark zone centroblasts proliferate and undergo affinity maturation involving a process known as somatic hypermutation (SHM), which introduces mutations into the IgV region of already rearranged immunoglobulin genes to modulate the affinity of the BCR for a specific antigen. Following SHM, centroblasts leave the dark zone and enter the light zone by down regulating CXCR5 and up regulating CXCR4, which homes towards CXCL12 that is produced by cells in the light zone. In the light zone of the GC, centrocytes expressing the newly mutated BCR with high affinity are selected by competitive binding to antigens expressed by, or tethered to, the follicular DC (FDC). TLR4 signaling by FDC is essential for the onset of the GC reaction and affinity maturation (190). Following such affinity maturation and selection, the centrocytes undergo a process known as class switch recombination (CSR), which sees the immunoglobulin genes of the GC B cell can switch isotype with the heavy chain of the BCR being irreversibly changed from α or μ heavy chains to γ, α or ε to provide IgG, IgA or IgE isotypes respectively. This process depends on the composition of cytokines produced in the context of particular antigens, for example, high levels of IL-4 will promote the development of IgE responses (187). The high levels and random nature of somatic hypermutation in the GC results in the development of autoreactive cells: normally these cells are cleared efficiently but in SLE patients, who experience enhanced lymphocyte activation and proliferation, the GC is a major source of autoantigens (191). Furthermore, the specific loss of FAS-mediated cell death in the GC leads to the development of a lupus like disease in mice (192).

1.5.4.8.7. Plasma cell development

GC B cells that undergo strong BCR stimulation in the light zone develop into antibody producing plasma cells or memory B cells (187). Some memory B cells appear to be long lived and can survive without tonic BCR stimulation by their specific antigen; upon further challenge these memory B cells reactivate to provide a rapid humoral immune response. For plasma cell development, the GC B cell is required to down-regulate the expression of B cell lineage transcription factors Pax5, Bcl-6 and mTA3 and up-regulate the plasma cell specific transcription factor, BLIMP1 along with IRF4 and XBP1. The GC B cells first develop into plasmablasts which are rapidly proliferating cells that can also produce antibody before becoming long-lived plasma cells, which are non-dividing, terminally differentiated antibody producing cells. (193). Plasmablasts can either develop into long-lived plasma cells locally or migrate to another part of the body such as a site of inflammation or stromal niche such as the bone marrow.

Plasmablasts are defined as short-lived antibody producing cells: their development can be promoted by IFNα and perhaps consistent with this, their production of anti-dsDNA antibodies are associated with flare responses in SLE patients (194,195). The levels of plasmablasts correlate with SLEDAI and are raised during flares in SLE patients, this may suggest that they are one of the main effector B cell population driving disease in SLE patients. Indeed, although the levels of long-lived plasma cells are also elevated in SLE patients during infection, their levels do not correlate with SLEDAI (196). However, it should be noted that some groups have reported that elevated levels of plasma cells do correlate with SLEDAI and anti-dsDNA titres, although in this case these cells were not differentiated from plasmablasts (159)

The GC reaction is not essential for the generation of antibody producing cells. Indeed, the first antibody producing cells to respond to antigen are the innate B1 or innate-like MZ B cells, which usually develop into loci of antibody producing cells outside of the B cell follicle. However, it has been reported recently that both B1 (see 1.5.4.8.8. below) and MZ B cells can also enter the GC reaction. The antibodies produced by the initial extra-follicular responses are of the IgM isotype that usually have low affinity for antigens. The role of these early 'natural'

antibodies is to facilitate the immune response, for example, by allowing APC to collect antigen via antibody receptors. Moreover, and as stated above, circulating Fo1 and memory B cells can also develop into antibody producing cells outside of the GC. Thus, when a Fo1 B cell receives T cell help it will either enter the GC and begin affinity maturation or develop directly into short lived plasmablasts depending on the strength of BCR signaling: weak BCR signals send the cells into the GC for affinity maturation whereas strong BCR signals allow the B cells to develop into plasmablasts outside of the B cell follicle, thus providing a rapid source of antibodies (177).

1.5.4.8.8. B1 B cells

B1 B cells are a population of innate B cells that represent less than 3% of the B cell lineage. They were initially identified as a population of B cells expressing CD5, hitherto thought to be a T cell marker, that were elevated in lupus prone mice (197). B1 B cells have therefore been associated with autoimmunity but they are also present in normal mice and healthy individuals and represent a major B cell population in the pleural and peritoneal cavities in mice but are rare in the secondary lymphoid organs (198). B1 B cells comprise two major phenotypes that can be distinguished by their expression of CD5 into B1a ($CD5^+$) and B1b ($CD5^-$) subclasses, which have similar characteristics but develop differently (199). Despite first being identified in lupus-prone mice, the role of B1 cells in autoimmunity, particularly in SLE, is controversial. For example, B1b cells have been suggested to play an important role in the development of nephritis in BAFF transgenic mice, which develop a lupus like disease (200). However, although CD5 expression is elevated on B cells from SLE patients, recent studies have revealed that expression of CD5 on B cells can result in the recruitment of the negative regulatory signal transducer, SHP-1 to the BCR, perhaps suggesting a protective role for CD5 (201). Furthermore, $CD5^+$ B cells are known to act as "regulatory" B cells (see 1.5.4.9), producing IL-10 and thereby suppressing pro-inflammatory immune responses (202).

1.5.4.3. BAFF and APRIL

Signaling induced by the B cell activating factor (BAFF) is important for the survival, differentiation and activation of B cells; especially those of the immature

"transitional" B cell phenotype in the periphery(175). BAFF, and its closely related homologue APRIL, both belong to the TNF family of cytokines. Both BAFF and APRIL exist in membrane bound forms or can be cleaved at a furin site to produce soluble cytokines that normally exist as trimers. BAFF binds to three receptors, BAFF-R, TACI and BCMA whereas APRIL can bind to TACI and BCMA (203). APRIL is not essential for the development of pathology in lupus prone mice (204) and inversely correlates with SLEDAI (205-207). Although levels of BAFF are generally considered to be elevated in SLE patients and contribute to pathogenesis (208,209), BAFF-R expression is reduced (210).

A pathogenic role has been proposed for BAFF in SLE, as high concentrations of this cytokine may act to promote the survival of auto-reactive B cells; indeed BAFF has been reported to act in synergy with IL-17 to promote the survival and proliferation of B cells (103). Perhaps consistent with this, expression of the IL-17A receptor is elevated on circulating B cells from SLE patients (211). Consistent with the proposed pathogenic role for BAFF, BAFF-transgenic mice develop a lupus like disease (212) and BAFF-R signaling drives a lupus like pathology in BAFF-transgenic mice, in a manner that is dependent on TLR signaling via the adaptor protein MyD88 (200,213). Although blockade of BAFF prevents pathology in lupus prone mice (214), a recent study with lupus-prone mice that do not express BAFF-R, TACI or BCMA revealed that disease can occur in the absence of any individual BAFF/APRIL receptor (215,216). Nevertheless, a BAFF targeting therapy was the first drug specific for the treatment of SLE granted FDA approval in 50 years (217).

1.5.4.4. TLR signaling in B cells

TLR signaling appears to be instrumental in the fine-tuning of B cell responses and appears to be involved in most aspects of B cell biology through to the development into antibody secreting cells; TLR signaling may also influence the class of antibody produced (218). Indeed, it has been proposed that certain molecules are autoantigens in SLE because they can activate the innate immune system, in a sense, acting like auto-adjuvants. Expression of TLR9 is elevated in circulating B cells from SLE patients and such increased expression is associated with disease (219,220) although the mechanisms involved are complex. For example, although the development of anti-dsDNA antibodies is abrogated in $tlr9^{-/-}$

lupus prone mice, disease is exacerbated as TLR9 signaling also acts to suppress TLR7 activation (221,222). By contrast, whilst TLR7-deficient mice do not develop lupus-like pathology, the Yaa mouse develops lupus like disease, due to overexpression of TLR7 (223,224).

1.5.4.6. Antibodies

A major role of B cells is to produce a soluble form of their BCR called antibodies; these molecules bind to antigens or cells and neutralize, opsonize or target the cells for deletion. Each antibody contains two pairs of polypeptide chains, a heavy chain and a smaller light chain linked via a hinge region to provide flexibility, with each pair connected by disulphide bonds (Fig. 1.8). The Fab region contains the variable antigen binding domains and this is connected to the Fc domain, which binds to antibody Fc receptors (FcR). There are 5 forms of antibodies termed isotypes: IgG, IgD and IgE are all monomeric proteins whereas IgA molecules form dimers and IgM molecules form pentamers although the BCR expressed by immature B cells contains a IgM monomer. There are 4 IgG subclasses in mice: IgG1, IgG2a, IgG2b and IgG3 and 2 IgA subclasses (225). SLE patients develop autoantibody responses against several nuclear and cytoplasmic antigens such as Ro, La and Sm (195), but the autoantibodies most characteristic of SLE in patients are those raised against dsDNA, the levels of which correlate with SLEDAI and are associated with disease flares (3,226).

1.5.4.7. Antibody receptors

Antibodies: either free, complexed with antigen or attached to cells can be detected by the immune system via a series of Fc binding receptors (FcR) for IgM, IgA, IgG and IgE. For example, there are 4 classes of FcR that recognize IgG antibodies in mice: three activating receptors, FcγR1, FcγR3 and FcγR4 and one inhibitory receptor called FcγR2b. Other antibody receptors also exist such as CD23, which binds IgE with low affinity whereas FceRI is the high affinity receptor associated with mast cell function and allergic responses(227,228). FcγR are expressed by many cells and their signaling can induce both activating and tolerogenic responses. While innate immune effector cells such as DC, macrophages and neutrophils, express both activating and inhibitory FcγR, B cells

only express the inhibitory FcγR2b and it is unclear whether T cells express FcR (229).

Each FcγR has an extracellular antibody binding α chain that signals through an adjacent ITAM-containing adaptor molecule; this is normally the FcRγ chain but can sometimes be replaced by other adaptor molecule such as CD3ζ. An exception is FcγR2b, which signals directly through an immunoreceptor tyrosine inhibitory motif (ITIM). Binding of immune complexes to FcR results in the receptor crosslinking that is required for signaling, which in the case of the activating receptors can result in cell activation, the release of pro-inflammatory mediators and proliferation. By contrast, cross-linking of FcγR2b on B cells results in cell death by apoptosis and provides a mechanism of negative feedback inhibition of B cell activation and antibody production (230). Consistent with this, FcγR2b is required to prevent the generation of self-reactive B cells as knockout mice develop lupus-like systemic autoimmunity (229), FcγR2b polymorphisms are also associated with SLE (231).

1.5.4.9.5. Antibody independent roles of B cells

B cells are essential for the development of disease in lupus prone mice: however, B cells engineered to lack the ability to secrete antibody still have the capacity to induce disease, providing evidence for antibody-independent roles of B cells in mediating disease (232,233). The exact mechanisms involved are not clear but B cells appear to be important for the activation of T cells in autoimmunity via cell contact dependent mechanisms such as the presentation of antigen and expression of co-stimulatory molecules. Moreover, they can influence T cell responses by their production of cytokines. When cultured in polarizing conditions, B cells have been shown to produce cytokines associated with Th1 and Th2 responses, these cells have been termed Be1 and Be2 respectively, and are thought to promote the effect of B cells during an immune response (234). Recently, B cell production of IL-6 has been indicated as important for the development of autoimmunity (235): furthermore, B cells have been reported to produce IL-17 in response to infection with Trypanosoma: these B cells do not express RORγt and are activated by CD45 and BCR signaling (187,236,237). Finally, and consistent with the finding that B cells, that cannot secrete antibody

are still able to drive disease pathogenesis in mouse models of SLE, in patients with autoimmune disease undergoing B cell depletion therapy, beneficial effects are noticeable before antibody levels drop (217,238,239).

1.5.4.9. Regulatory B cells

1.5.4.9.1. Historical overview

As with T cells, several populations of B cells with regulatory cell characteristics have been identified. The lack of a signature regulatory B cell transcription factor has restricted the advance of our understanding, although it appears that some B cells may express FoxP3 (240). Janeway and colleagues first proposed the existence of a regulatory B cell population in 1996 by showing that mice which lacked B cells developed severe disease in the EAE model of MS, this was subsequently shown to be due to a loss immune regulation by IL-10 producing regulatory B cells (Bregs) (241,242). Since then, Bregs have been shown to be effective at suppressing disease in mouse models of IBD, RA and SLE (243-245). Consistent with this, Breg subsets have been identified in humans that are defective in patients suffering from MS or SLE (246,247). Although regulatory B cells (Bregs) express some of the phenotypical characteristics of B1 B cells, such as CD5, which are known to produce IL-10 (199). Bregs can also express phenotypic markers of B2 cells, such as CD21 and CD23. Thus it is unclear whether Bregs develop from innate or adaptive progenitor cells, nevertheless, several distinct B cell populations have been reported to express IL-10 with T2, MZP and MZ cells being reported to be protective in models of lupus (244), RA(248) and colitis (249) respectively.

1.5.4.9.2. Breg development

Several aspects of B cell biology have been identified as potential triggers for the development of Bregs. Thus, although BCR, CD40 and TLR signaling are important for the development of antibody producing cells, the same signals may also influence the development of Bregs (250). For example, the importance of BCR signaling in the development of Bregs was highlighted in mouse models of autoimmunity, where in EAE the loss of CD19 signaling augmented Breg responses (251) whilst B cells expressing a BCR specific for an irrelevant protein (hen egg lysosome) failed to suppress disease (242). Furthermore, in the CIA

model, IL-10 production was stimulated by antigen (252). Likewise, CD40 signaling was shown to be required for B cell-mediated suppression of disease in the EAE, CIA and MRL/*lpr* mouse models: such CD40 stimulation can be provided *in vivo* by either innate (iNKT) or adaptive ($CD4^+$ T helper) cells (242). Moreover, as B cells can also express CD40-ligand, in addition to expressing CD40 (253), it is also possible that the development of Bregs can be driven by B cells in an antigen independent manner as shown in the MRL mouse (253-255). This process clearly requires tight control as continuous expression of CD40-L by B cells results in a lupus-like disease (250,256). Likewise, the final signal required to induce IL-10 production by B cells is provided by TLR signaling which is generally thought to promote immune responses. Nevertheless, B cells deficient in TLR2, TLR4 or MyD88 do not resolve EAE (257) and TLR9 stimulation has been reported to induce B cell IL-10 production (178). It may be that TLR signaling is different in B cells, compared to other cells such as DCs, and it may be that a certain type of TLR signaling in B cells promotes a regulatory phenotype, however, clearly much is left to be discovered relating to the activation and development of Bregs (242).

1.5.4.9.3. Bregs control innate and adaptive responses

IL-10 from Bregs can potentially inhibit T cell responses by suppressing DC priming of Th1 or Th17 cells. Supporting this, DC from B cell-deficient mice were found to produce more IL-12 (258). Furthermore, IL-10 from B cells has been shown to suppress IL-12 and IL-6 production by DC (257). Alternatively, B cells can suppress T cell responses by activating Tregs and consistent with this, Breg-deficient mice exhibit a reduction in *foxp3* mRNA (259). Moreover, adoptive transfer of B cells can also suppress the spontaneous IBD that develops in lymphopaenic mice, this suggests that Bregs can inhibit both innate and adaptive immune responses (260). Nevertheless, although IL-10 is protective and regulates disease in murine models of lupus (261) it may also be pathogenic via the activation of B cells. Thus, injection of NZB/NZW lupus-prone mice with IL-10 exacerbates disease, neutralization delays disease onset (262). Reflecting this, polymorphisms that lead to elevated IL-10 production have been reported in SLE patients (263) whilst blocking of IL-10 reduces disease severity in SLE patients (264).

1.6. Treating SLE

1.6.1. Steroidal and non-steroidal anti-inflammatory drugs

SLE has traditionally been treated with a variety of steroidal and non-steroidal anti-inflammatory drugs. Corticosteroids are the most widely used short-term therapy for treating SLE patients; however, due to the toxicity associated with prolonged use they are generally reserved for the treatment of patients during flare responses (265,266). These drugs have multiple effects on both innate and adaptive immune responses including suppression of pro-inflammatory cytokine production and lymphocyte proliferation as well as the induction of apoptosis in pathogenic lymphocytes and macrophages (267,268). Likewise, Cyclophosphamide, which is a very effective agent at causing cell death, is used cautiously during the most severe of SLE manifestations (269). A less toxic alternative is mycophenolate mofetil (MMF) which is an inhibitor of DNA synthesis (270) and inhibits lymphocyte proliferation: it is well tolerated with relatively mild, but uncomfortable, side effects such as vomiting diarrhea and increased susceptibility to infection (271). Two other commonly used drugs are azathioprine and methotrexate which both suppress nucleotide synthesis, therefore suppressing the rapid expansion of immune cells such as lymphocytes (272,273). By contrast, the anti-malarial drugs, chloroquine and hydroxychloroquine that have long been prescribed for SLE patients appear to act by suppressing phagocytosis and TLR activation (274,275). Antimalarials have proved particularly useful in treating disease flare and suppressing the acceleration of disease in early onset lupus patients (276,277).

1.6.2. Biologics

Some of the most effective and recently developed successful therapies for treating autoimmune diseases, including SLE, come in the form of therapeutic antibodies. There are currently 25 antibodies approved for use in patients with a further 240 in development for diseases as diverse as autoimmunity, cancer, organ transplantation and infection (278). They are a mainstay of modern biotech companies and in the USA generate more than $27 billion each year (279). Therapeutic antibodies work by blocking the action of a specific molecule, such as preventing a cytokine from binding its receptor or targeting a specific cell for destruction. They can be engineered to either contain toxic enzymes or signaling

molecules to kill cells and they can also target cells for destruction naturally by activating complement dependent cytotoxicity. Finally, therapeutic antibodies can act as agonists by stimulating receptors (225,280).

Therapeutic antibodies targeting B cells have received much focus over the past 5 years. The surprise success of Rituximab, a drug initially developed for the treatment of cancer, in the treatment of rheumatoid arthritis provided the rationale for testing in other autoimmune diseases (275,281). Rituximab is a chimeric anti-CD20 antibody that depletes circulating B cells, but not plasma cells. Consistent with this, the beneficial effects of rituximab are not necessarily associated with a loss of autoantibodies and several theories have been suggested as to why exactly it is protective in autoimmune diseases. Possible explanations are that the antibody-labeled B cells distract macrophages and neutrophils away from target organs; that the treatment suppresses the antibody-independent inflammatory functions of B cells or that by depleting the B cells, the effector: regulatory balance can be reset naturally (54,275).

Rituximab is therapeutic in some SLE patients and consequently, its off-label use is common: however, two large phase 2/3 clinical trials failed to meet their primary endpoints (282). The general consensus appears to be that both of these trials were poorly designed, with poorly stratified patient cohorts, and although subsequent analysis suggests that Rituximab was in fact beneficial, this drug has not been approved for use in SLE patients (283,284). Nevertheless, clinical trials utilizing other B cell-targeting agents, for example, anti-CD19 and anti-CD22 antibodies are currently underway (275). Clinical trials with Belimumab, an anti-BAFF antibody that clears the B cell survival factor from circulation, have been more successful. Belimumab was shown to be moderately successful in treating SLE patients and became the first drug to receive FDA approval specifically for the treatment of SLE patients in 50 years. Nevertheless, this drug is only effective in approximately 14% of patients (38,285).

As cytokines drive many aspects of SLE, targeting them has also long been thought of as a viable option for treatment. Consistent with this, treatment with monoclonal antibodies specific for IL-6 (Toclizumab) (286) and IL-10 (264) have

shown moderate results in phase 1 trials: however, trials using an anti-TNFα antibody (Infliximab) were terminated prematurely as some patients experienced exacerbated disease (287). Moreover, reflecting the clear type I IFN signature in SLE patients, an anti-IFNα therapy is to be tested in such patients (288) (38). Likewise, targeting of co-stimulatory molecules has also been attempted as their expression is usually elevated in SLE patients (254,289). However, although an early phase 1 trial of anti-CD40 in SLE patients showed promise (290), a phase 2 trial did not show any benefit (291). Moreover, Abatacept, which is a fusion protein of the CTLA-4 extracellular domain and an Fc portion of immunoglobulin that is approved for the treatment of RA, showed a modest benefit to SLE patients with mild disease in a phase 2 trial although the primary endpoints were not met (292).

Although such biologics are good at targeting cell surface or soluble molecules, this strategy is less effective at targeting intracellular signaling pathways (293) as small chemicals that can easily pass through the lipid bilayer can modulate these targets more efficiently. In SLE, the signaling molecules involved in the hyper-activity of immune response cells, particularly T and B cells, have been targeted. For example, Fostamatinib which acts to inhibit SYK, a critical component of BCR and TCR signaling in SLE patients, suppresses disease in mouse models of lupus and may be beneficial in patients when clinical trials begin(294). Fostamatinib is currently going through a phase 2 clinical trial for the treatment of rheumatoid arthritis and is showing promise with, perhaps surprisingly given the importance of its target, relatively few side effects (295).

1.7. Developing drugs from parasites

1.7.1. Parasites versus autoimmunity

There is a clear emerging socioeconomic driving factor(s) underpinning the increasing prevalence of autoimmune diseases, as they occur significantly less in developing countries relative to industrialised nations. Over the past 30 years, countries such as Hungary and South Korea have experienced a rise in autoimmune disease as their economies have developed (296,297): one possible environmental factor that is likely to be altered during the development of a nation is improved sanitation and a subsequent reduced exposure to pathogens, such as helminths (298). This correlation has been captured by the hygiene hypothesis,

which states that increased sanitation and clean living in modern society has reduced our exposure to microorganisms that would normally train and hone our immune responses, resulting in elevated levels of autoimmune and allergic disease (299,300). Indeed, the incidence levels of autoimmunity and allergy, which are high in developed nations, inversely correlate with that of helminth infection, which is endemic in developing countries. Furthermore, the levels of autoimmunity or allergic disease in the offspring of immigrants that have traveled from developing to developed nations is much higher compared with disease rates in the country of origin (301). An exception to this observation may be SLE, which does not seem to be defined by socioeconomic factors.

1.7.2. Helminth immune suppression

Helminths are parasitic worms that are capable of inducing debilitating disease such as severe cutaneous lesions, elephantiasis and blindness (302). Nematodes have unusually long life spans for parasites and have been reported to survive for up to 10 years: one explanation for this is their ability to suppress the host immune response (303). Thus through selective modulation of the immune response, helminths can prevent cytolytic immune responses that would normally clear the worm whilst ensuring that the host remains relatively healthy with an active immune system (304,305). Thus, it is plausible that the immune response evolved to optimally function with background immune modulation by helminths: therefore, when helminths are removed from the environment the immune system may automatically become hyperactive (306). This theory can only partially explain the relationship between helminths and autoimmunity, however, as if this was the case, one would expect the prevalence of immune mediated inflammatory disease to be much higher in developed societies. It is more likely therefore, that this contributes as another environmental factor in the development of autoimmunity.

1.7.3. Treating autoimmunity with helminths

The unique relationship between helminths and the mammalian immune system, in particular the ability of worms to modulate the immune response, has led researchers around the world to investigate the potential application of these parasites or their immunomodulatory products in the treatment of immune mediated disease, such as allergy and autoimmunity (298). Various helminth

species have been shown to protect against disease in several mouse models of intestinal inflammation, allergic disease, and autoimmunity (298,307,308). This has culminated in clinical trials examining the therapeutic potential of helminths in the treatment of autoimmune disease, most recently the use of *Trichuris suis* in IBD patients. The administration of live parasites is potentially dangerous as these are live pathogens and can elicit immune responses that can exacerbate disease or cause other pathologies. *T. suis* was chosen for the treatment of humans as it is a porcine helminth and observations with pig farmers, who by the nature of their profession are chronically exposed to *T. suis*, revealed that this parasite can colonize humans but only briefly (309). Patients received live *T. suis* in an open label phase 1 clinical trial and the authors reported an 80% improvement; however, subsequent placebo-controlled double-blind clinical trials showed more modest, but significant, improvements (298). A recent trial examining the potential of the human parasite *Necator americanus* in Crohn's disease has also looked promising. Due to the risk of infection low parasite numbers were used, nevertheless, patients with moderate, but not mild or inactive, disease showed some improvement (310).

Perhaps the most optimal and safe way to utilize the potential of the parasites to modulate the immune system would be to identify the specific components responsible for the immunomodulation. Such molecules may be therapeutically and economically viable options for the treatment of autoimmune and allergic disease: alternatively they may be used as blue prints for the design of novel therapeutics. It is also important to realize the potential of such parasite-derived immunomodulators as tools to dissect the immune system and hence identify safe mechanisms of immune suppression that have been selected by nature throughout evolution (311).

1.7.4. Helminth immunomodulators

Helminth infections naturally induce a Th2 and regulatory phenotype in order for the worms to 'hide their tracks' and repair damaged tissue as they migrate throughout the host (306,308). Such Th2 responses are not pathogenic however, as they are limited or modified by the parasite (312).

Helminth-derived immunomodulatory molecules can suppress the immune system in a variety of ways, for example, cystatins from *Onchocerca volvulus* and *Acanthocheilonema viteae* may suppress inflammatory macrophages via mechanisms involving the scavenger receptor, CD36 and the TGFβ receptor (313,314); whilst calreticulin from *Heligmosomoides polygyrus,* via interacting with scavenger receptor A in mice, can skew Th2 responses (315); and *Dirofilaria immitis*-derived antigen binds to CD40 on B cells stimulating the production of antigen non-specific IgE, thus saturating IgE receptors, which has been shown to prevent anaphylaxis (316). Several parasite-derived immunomodulators act by modulation of TLR signaling, which usually promotes Th1/Th17 responses (308). Suppressing TLR signaling therefore prevents the generation of immune responses that would clear the helminth (306). However, as such strong immune responses also underpin the pathologies of autoimmune disease, immunomodulatory molecules from *Schistosoma mansoni, Ascaris lumbricoides, A.viteae* and *Fasciola hepatica* act, at least in part, to protect against development of such inflammatory disorders in mouse models by modulation of TLR signaling (311).

1.7.5. ES-62: a parasitic helminth-derived immunomodulator

One such immunomodulator that acts via subversion of TLR signaling is ES-62, an excretory-secretory (ES) protein from the murine filarial nematode *A. viteae.* ES-62 has conserved orthologues with the human filarial nematodes *Brugia malayi* and *O. volvulus* (317) and is a tetrameric glycoprotein comprising 63 kDa subunits (317). Although the molecule contains a putative peptidase site that is yet to be fully validated, the major immunomodulator functions of ES-62 are due to the phosphorylcholine moieties present on the molecule (318). Indeed the addition of PC moieties to irrelevant proteins such as BSA, or free PC, can replicate many of the immunomodulatory effects of ES-62 (319,320).

TLR4 is required both for the internalization of ES-62 by macrophages (but not B cells) and its immunomodulatory activity and consistent with this, it appears to act to subvert both immunoreceptor and TLR signaling through a MyD88 dependent mechanism. The mutated, LPS-unresponsive TLR4 molecule expressed by C3H/HeJ mice is sufficient for ES-62 entry and immunomodulation (321,322).

Consistent with its proposed role in subverting TLR-mediated pro-inflammatory signalling, ES-62 suppresses Th1 polarising responses following TLR2, TLR4 and TLR9 stimulation (322).

1.7.5.1. Immunomodulation by ES-62

Over the past 15 years ES-62 has been shown to modulate, both directly and indirectly, the pro-inflammatory responses of several cell populations. The first indication that ES-62 was a putative immunomodulator came from experiments that showed the parasite product to suppress B cell activation and proliferation *in vitro* (318,323) and *in vivo* (324). This was achieved by an uncoupling of the BCR from the MAP kinase signaling pathways and recruitment of the negative signaling molecules, SHP-1 (tyrosine phosphatase) and Pac-1 (MAP kinase phosphatase) (325). This anti-proliferative phenotype was complemented by the finding that ES-62 increased spontaneous IL-10 production by peritoneal B1 B cells suggesting a potential anti-inflammatory role for the molecule (326). The key role of the PC moiety of ES-62 in the induction of IL-10 was indicated by the finding that whereas native ES-62 induces IgG1 but not IgG2a antibody responses, recombinant ES-62, which does not contain PC moieties, induces both IgG1 and IgG2a responses: interestingly, this PC-mediated blocking of potentially pathogenic IgG2a responses was dependent on IL-10 (327).

ES-62 can suppress TCR signaling and induce anergy in Jurkat T cells by modulating the activation of the TCR tyrosine kinases Lck, Fyn and ZAP-70 (328). Perhaps reflecting this, ES-62 was also found to suppresses the clonal expansion and modulated the functional phenotype of antigen specific T cells (329). However, this does not appear to be via direct effects on naive T cells but rather by modulation of APC function (Whelan et al 2000). Thus, DC and macrophages stimulated with TLR ligands, such as LPS, usually elicit Th1 responses via the production of IL-12 and IFNγ. However, ES-62 suppresses the ability of DCs and macrophages, even following LPS stimulation, to promote Th1 responses (330,331). ES-62 also modulates the secretion of other proinflammatory cytokines by macrophages, most notably IL-12p70, IL-23, IL-6 and TNFα, suggesting a potential suppression of both Th1 and Th17 responses by the parasite product (331,332) Strikingly, *in vivo* exposure is sufficient to render bone marrow

progenitors prone to an anti-inflammatory phenotype on subsequent ex vivo differentiation of DC or macrophages (333). More recently, transfer studies suggest that ES-62 may also influences T cell responses by modulating antigen specific T cell B cell interactions (334).

1.7.5.3. Therapeutic potential of ES-62 in the treatment of autoimmunity

The immunomodulatory actions of ES-62 presumably reflect the strategies developed by the parasite to evade the host immune response and survive without causing pathology. Investigation of whether such anti-inflammatory actions could be exploited therapeutically for the treatment of inflammatory disorders in humans revealed that ES-62 can suppress pathogenic inflammation in mouse models of both allergic and autoimmune diseases. Thus, ES-62 was found to suppress disease in the collagen induced arthritis (CIA) model of rheumatoid arthritis by suppressing collagen-specific Th1-associated cytokine production (TNFα, IL-6 and IFNγ), lymphocyte proliferation and IgG2a production (335). The effect on lymphocyte proliferation and cytokine production was later revealed to be due to the PC moiety whereas the reduction in collagen-specific antibody production was not dependent on the presence of PC (319). More recently, it has become increasingly clear that pathogenesis of many autoimmune pathologies such as those observed in CIA are mediated by IL-17 responses, and reflecting this, in addition to suppressing Th1 responses, ES-62 was found to down-modulate pro-inflammatory IL-17 production in the paw and draining LN as well as disrupting the activation of Th17 or IL-17$^+$ $\gamma\delta$ T cells by DC (336).

As infection with helminths usually skews the immune system to a more Th2 orientation (306), and indeed ES-62 itself elicits Th2 responses (330), it was at first rather surprising that ES-62 could also prevent development of Th2-biased allergic inflammation (228). However, the mechanisms of action underpinning this have now been partially elucidated and reflect functional desensitization of mast cell responses by down-regulation of key elements downstream of FcεR1 and TLR4 signaling (228,337) and also by increasing IFNγ to counter-regulate Th17 and (consequent) Th2 responses (338).

The realization that IL-17 can promote both Th1/Th17 and Th2 inflammation(339,340) provides a rationale for the therapeutic potential of ES-62 in both autoimmune and allergic disorders. Indeed, the therapeutic potential of ES-62 appears to be restricted to diseases with a Th17 element as Th1 type-1 diabetes like disease in the NOD mouse (W. Harnett & A. Cooke, unpublished observations) and the Th1 immune pathology induced by *Toxoplasma gondii* or *Plasmodium chabaudi* or other filarial nematode antigens are unaffected by ES-62 (341-343) (W. Harnett unpublished). Likewise, and important in the consideration of the therapeutic potential of ES-62, the parasite molecule did not prevent Th1 immune responses to the BCG vaccine suggesting that patients receiving drugs developed from ES-62 would still be responsive to vaccination (344).

1.7.5.4. Thesis Aims: ES-62 and the MRL/*lpr* mouse

Previous studies evaluating the therapeutic potential of ES-62 in the MRL/*lpr* mouse model of SLE have revealed that prophylactic treatment with the parasite product suppresses the development of proteinuria. Despite such a clear improvement in disease outcome, surprisingly no obvious improvement in renal histology was observed and cellular infiltration; C3 and antibody deposition of the kidneys appeared unaltered. Likewise, no clear immunomodulatory effect in this model of a mixed Th1/Th2 phenotype could be detected nor any suppression of the associated lymphadenopathy and splenomegaly. Thus, the core aims of the thesis were to identify and characterize the protective mechanisms of action of ES-62 in the MRL/*lpr* mouse model of lupus. In particular, although still controversial in SLE, given the increasing recognition of the importance of IL-17 in the pathogenesis of autoimmune disease it was planned to focus on the effects of ES-62 on Th17, and associated cytokine responses such as IL-22, in the MRL/*lpr* mouse. As a major role of IL-17 appears to relate to the recruitment of pro-inflammatory effector cells to sites of inflammation, it was planned to relate any modulation of IL-17 responses to renal pathology. Moreover, given the effects of ES-62 on B cell responses and recent reports that regulatory B cells were protective in mouse models of arthritis, at least in part by suppressing Th17-associated responses (345), it was also planned to determine whether exposure to ES-62 resulted in resetting of the effector/regulatory balance in the MRL/*lpr* mouse.

IL-17 cytokine	Cell type	Implicated in
IL-17A	Th17, LTi, NK, iNKT, mast cells, neutrophils and γδ T cells.	Elevated in RA, IBD, psoriasis, MS and atherosclerosis.
IL-17B	Chondrocytes and neurons.	Reduced in psoriasis.
IL-17C	Epithelial cells.	Elevated in psoriasis.
IL-17D	Undefined.	Reduced in psoriasis.
IL-17E (IL-25)	Memory Th2 cells, eosinophils, basophils, mast cells and epithelial cells.	Elevated in asthma and atopic dermatitis but reduced in IBD.
IL-17F	Th17, LTi, NK, iNKT, neutrophils and γδ T cells.	Elevated in RA, IBD and psoriasis.
IL-17 receptor	**Cell type**	**Implicated in**
IL-17RA	Ubiquitous.	Elevated in RA and IBD.
IL-17RB	c-kit$^+$lin$^-$ cells, Th2 and Th9 cells, fibroblasts and basophils.	Elevated in asthma and atopic dermatitis.
IL-17RC	Adipocytes, chondrocytes, fibroblasts and epithelial cells.	Elevated in RA.
IL-17RD	Epithelial and endothelial cells.	Undefined.
IL-17RE	Undefined.	Undefined.

Table 1.1: The IL-17 family of cytokines, their receptors and implications in disease. LTi, lymphoid tissue inducer cells; NK, natural killer cells; iNKT, invariant natural killer cells; RA, rheumatoid arthritis; IBD, irritable bowel disease and MS, multiple sclerosis. Adapted from Pappu *et al.* 2011(89)

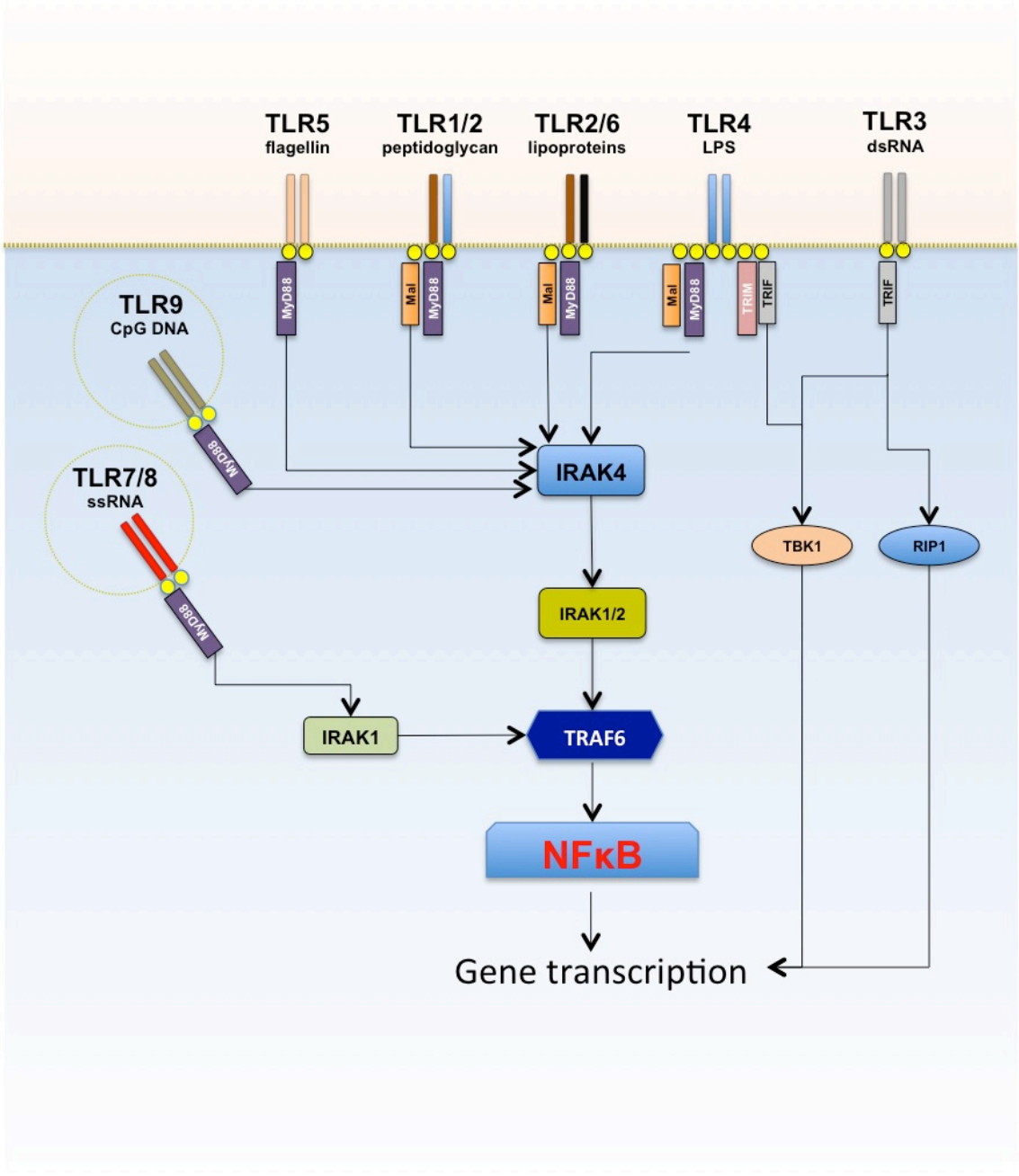

TLR5
flagellin
TLR1/2
peptidoglycan
TLR2/6
lipoproteins
TLR4
LPS
TLR3
dsRNA
MyD88
Mal
MyD88
Mal
MyD88
Mal
MyD88
TRIM
TRIF
TRIF
TLR9
CpG DNA
MyD88
TLR7/8
ssRNA
MyD88
IRAK4
TBK1
RIP1
IRAK1/2
IRAK1
TRAF6
NFκB
Gene transcription

Figure 1.1: TLR signaling. TLRs are composed of extracellular binding domains consisting of multiple leucine rich repeats (colored elongated rectangles) and intracellular Toll/IL-1R (TIR) domains (yellow circles). Upon ligation by PAMPs or DAMPs, TLRs form homodimers (TLR3, TLR4, TLR5 and TLR9) or heterodimers (TLR1/2, TLR2/6 and TLR7/8) and recruit the appropriate adaptor proteins, MyD88, MAL, TRIM or TRIF. These adaptor molecules then signal through the IRAK/TRAF, TBK or RIP1 pathways to induce the activation of transcription factors and subsequent gene expression.
Adapted from Liew *et al.,* Nature Reviews Immunology (2005)(19).

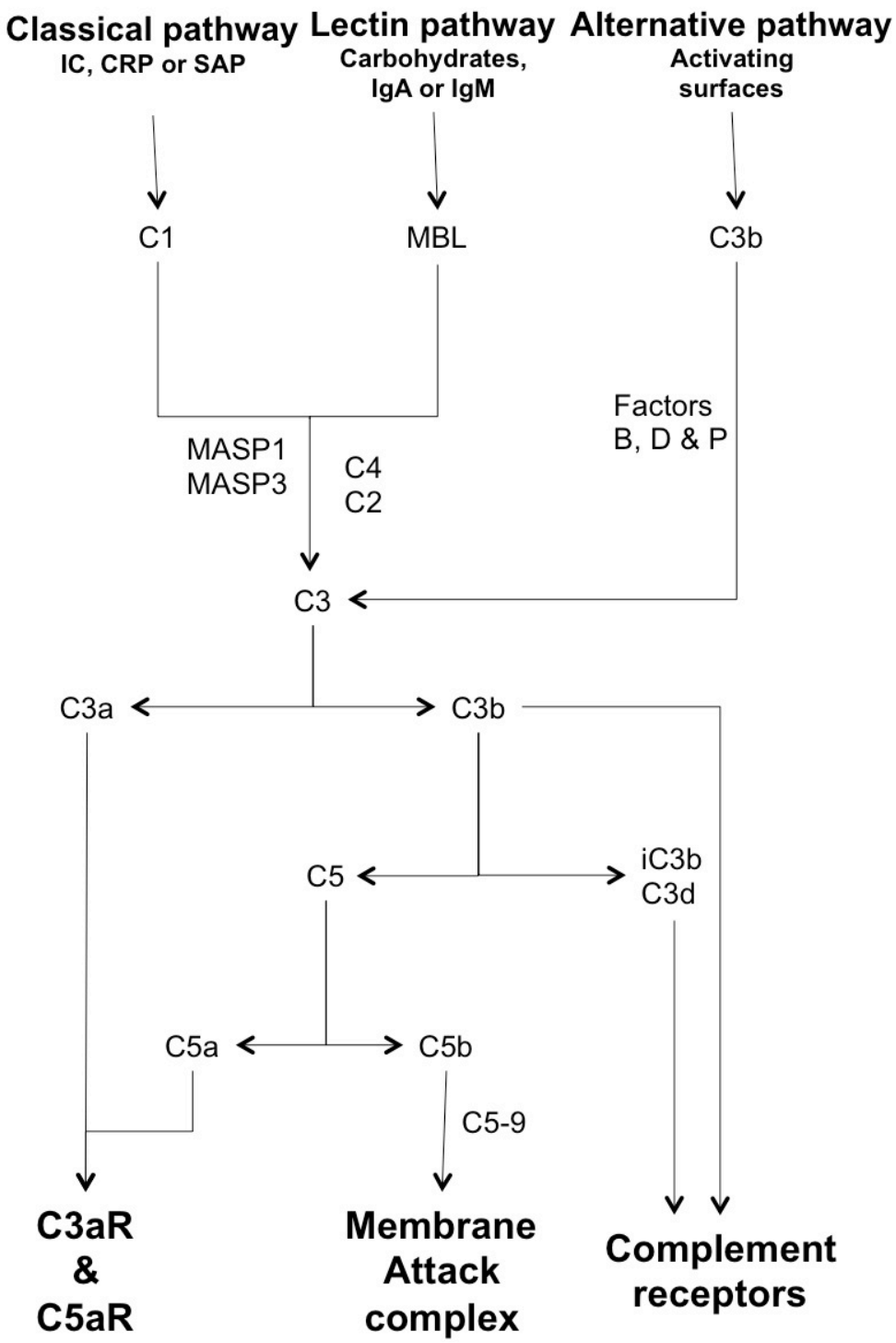

Classical pathway
IC, CRP or SAP
Lectin pathway
Carbohydrates, IgA or IgM
Alternative pathway
Activating surfaces
C1
MBL
C3b
Factors B, D & P
MASP1 MASP3
C4 C2
C3
C3a
C3b
C5
iC3b C3d
C5a
C5b
C5-9
C3aR & C5aR
Membrane Attack complex
Complement receptors

Figure 1.2: Complement cascade. C3 is the central component of the complement cascade and this can be activated by the (i) classical pathway, in response to immune complexes (IC), C-reactive protein (CRP) or serum amyloid protein (SAP); (ii) lectin pathway, by specific carbohydrates, including sugar groups on IgA or IgM and the (iii) alternative pathway, by direct binding of C3b to activating surfaces that initiates a cascade that results in the formation of the membrane attack complex (MAC) and the activation of complement receptors on a variety of cells.

Adapted from Sacks *et al.,* Nature Reviews Immunology (2012)(346).

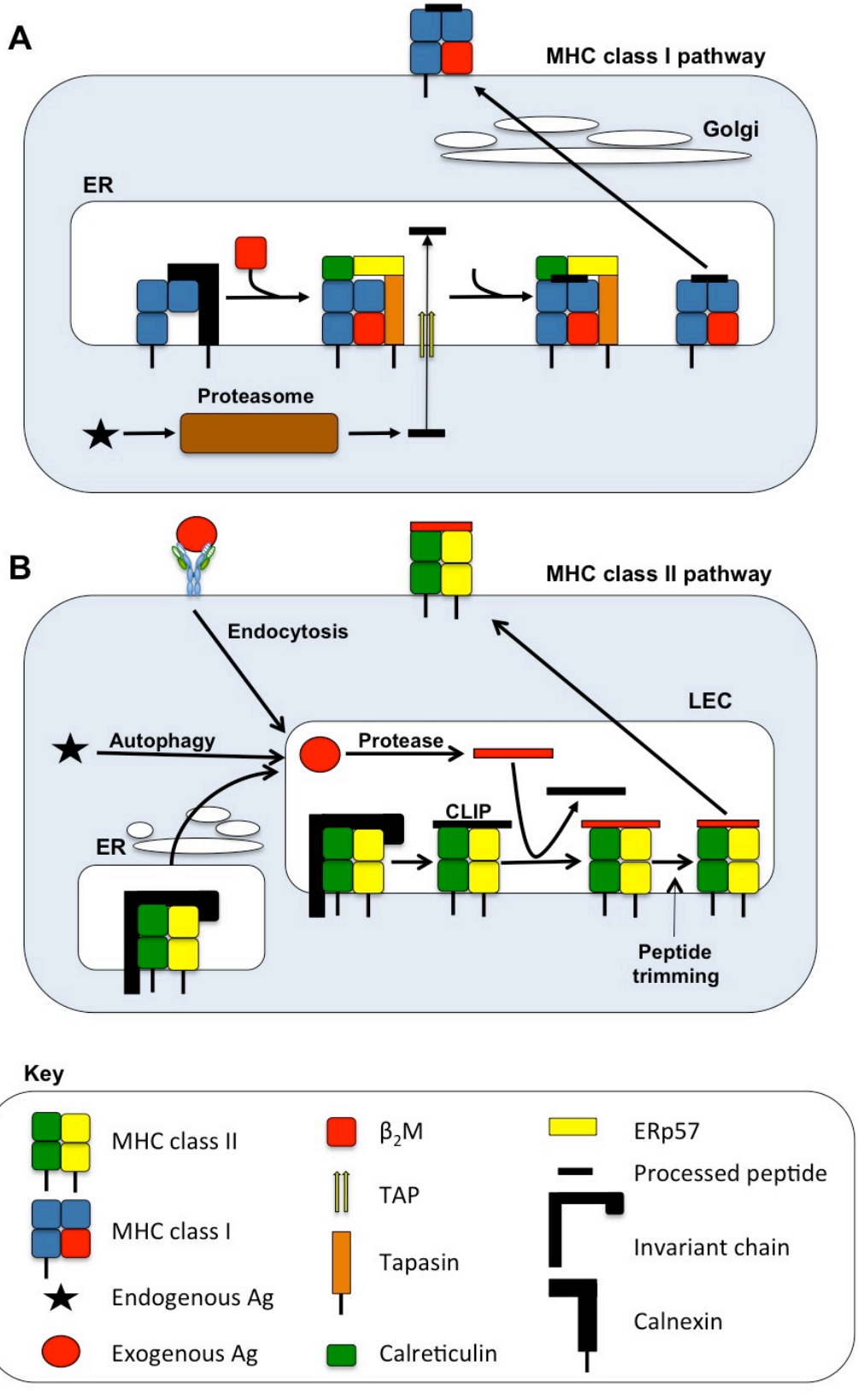

A
MHC class I pathway
Golgi
ER
Proteasome
B
MHC class II pathway
Endocytosis
LEC
Autophagy
Protease
CLIP
ER
Peptide trimming
Key
MHC class II
MHC class I
Endogenous Ag
Exogenous Ag
β2M
TAP
Tapasin
Calreticulin
ERp57
Processed peptide
Invariant chain
Calnexin

Figure 1.3: Antigen presentation via MHC. The MHC class I pathway (A) presents endogenous peptides that have been processed via the proteasome, and enter the endoplasmic reticulum via the TAP transporter to be loaded onto MHC class I molecules, held in the correct conformation by a complex of the MHC molecules (α1-3 and β_2M) and chaperone molecules (tapasin, calreticulin and ERp57). Binding peptide induces release of the chaperone molecules and the MHC class I-peptide complex is then trafficked to the cell surface via the golgi apparatus. The MHC class II pathway (B) presents exogenous peptides that have entered the cell via endocytosis, or endogenous peptides that have been generated via autophagy. MHC class II molecules are assembled in the endoplasmic reticulum with CLIP blocking the peptide-binding groove. They are then exported through the golgi apparatus to vesicles, that fuse with late endosomal compartments that contain peptide fragments that are then loaded onto the MHC class 2 complex before being exported to the cell surface.
Adapted from P. E. Jensen, Nature Immunology (2007)(347).

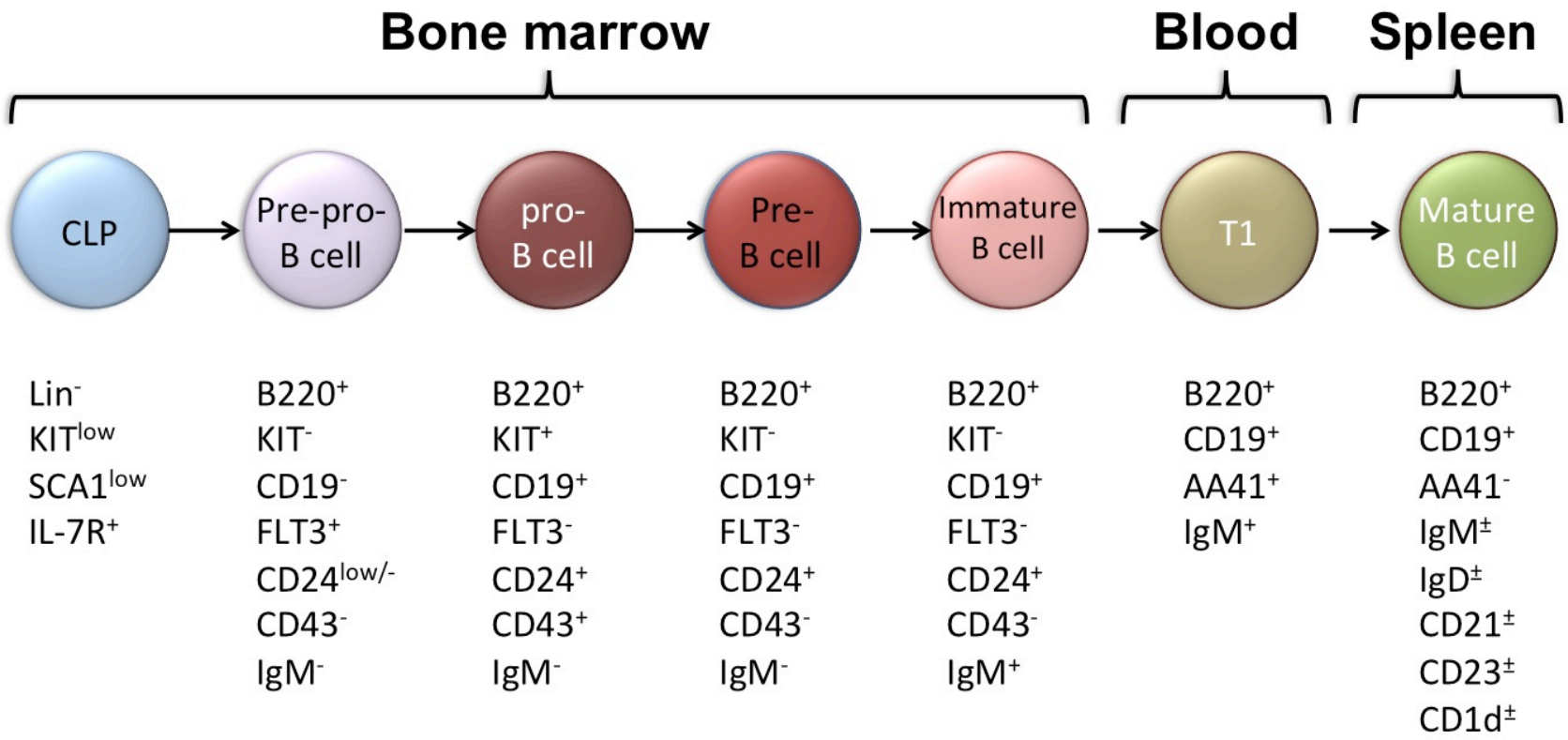
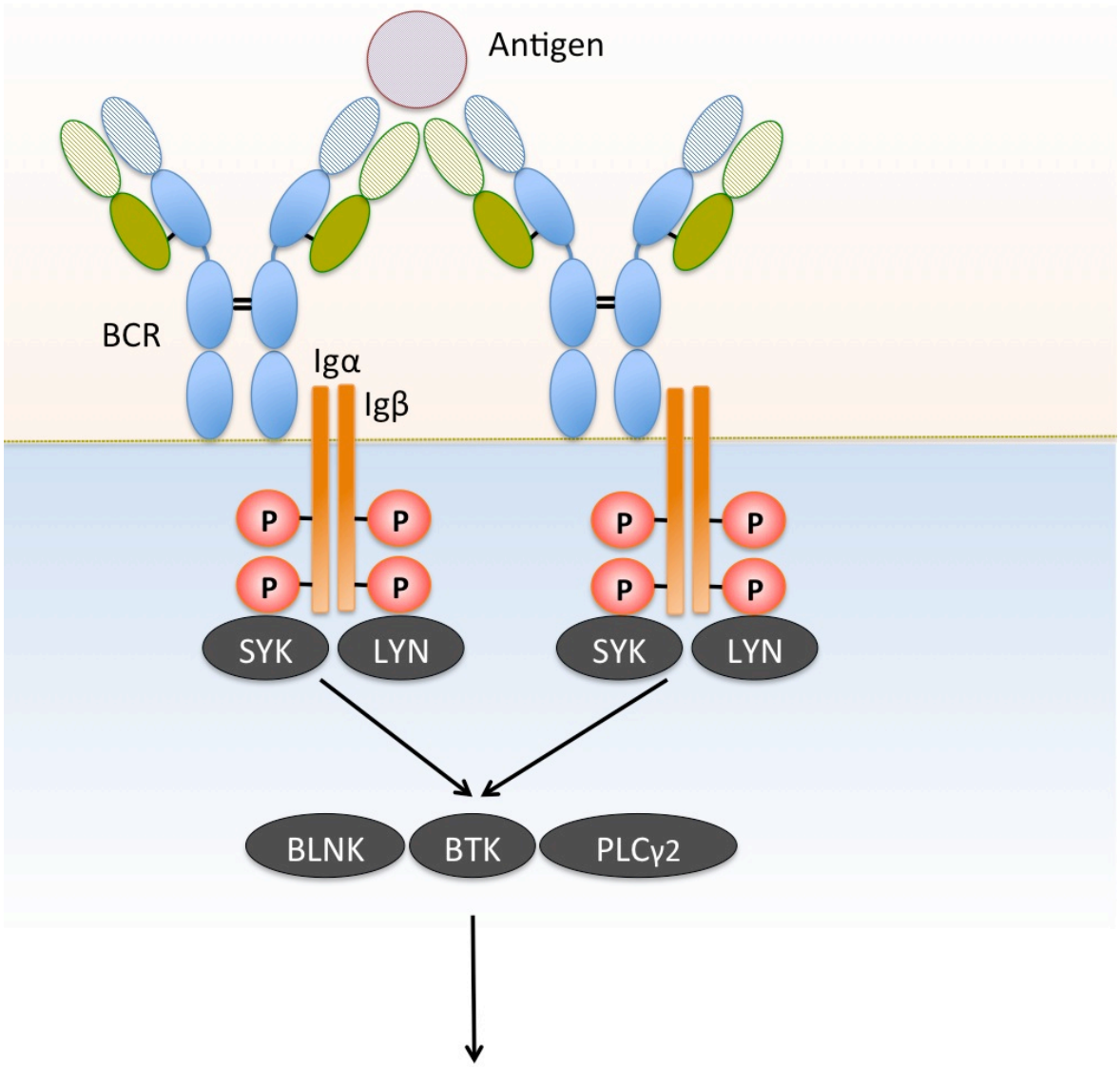

Bone marrow
Blood
Spleen
CLP
Pre-pro-B cell
pro-B cell
Pre-B cell
Immature B cell
T1
Mature B cell
Lin- KITlow SCA1low IL-7R+
B220+ KIT- CD19- FLT3+ CD24low/- CD43- IgM-
B220+ KIT+ CD19+ FLT3- CD24+ CD43+ IgM-
B220+ KIT- CD19+ FLT3- CD24+ CD43- IgM-
B220+ KIT- CD19+ FLT3- CD24+ CD43- IgM+
B220+ CD19+ AA41+ IgM+
B220+ CD19+ AA41- IgM± IgD± CD21± CD23± CD1d±

Figure 1.4: Early B cell differentiation. The early stages of B cell development occur in the bone marrow where, arising from haemopoietic stem cells, common lymphoid precursors (CLP) commit to developing into immature B cells through the pre-pro-, pro- and pre-B cell stages until they finally develop into IgM-expressing immature B cells, at which point they leave the bone marrow and enter the circulation to migrate to the spleen where they further mature via transitional stages to mature B cells. During the bone marrow stages, the immunoglobulin genes are rearranged to form a viable B cell receptor (BCR).
Adapted from T. Nagasawa, Nature Review Immunology (2006)(348).



Proliferation, activation and antibody production

Figure 1.5: B cell receptor signaling. The BCR forms an antigen receptor complex with Igα (CD79a) and Igβ (CD79b) molecules which recruit SYK and LYN. These adaptor molecules activate downstream signaling elements such as BLNK, BTK and PLCγ_2 which promote cell survival, proliferation and development via activation of the PI-3kinase, PLCγ and MAP kinase signaling pathways.
Adapted from Choi *et al.*, Cancer Journal (2013)(349).

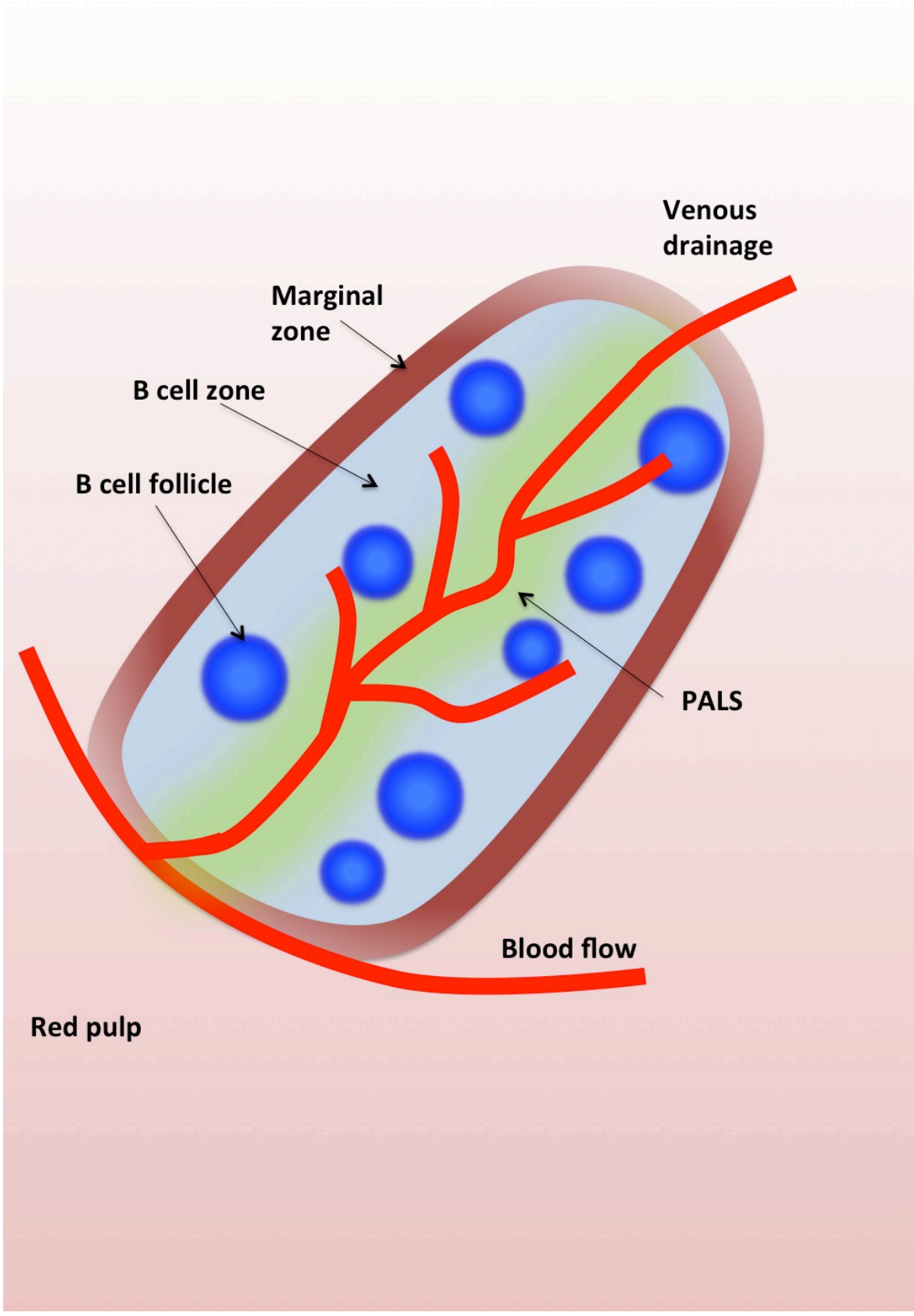

Venous drainage
Marginal zone
B cell zone
B cell follicle
PALS
Blood flow
Red pulp

Figure 1.6: The spleen. The spleen is a secondary lymphatic organ with two major functions; the filtration of blood and detection of circulating foreign or immunogenic material, which occurs in the red-pulp; and the peripheral maturation of immature B cells, which occurs in the white pulp. The structure of the white pulp can be separated into three distinct structures: (i) the periarteriolar lymphoid sheath (PALS), which contains T cells; (ii) the B cell zone, where B cell follicles and germinal centers form and (iii) the marginal zone, which contains specialized B cell and macrophage populations that filter the blood in the red-pulp for antigens. Adapted from Pillai *et al.*, Annual Review Immunology (2005)(173).

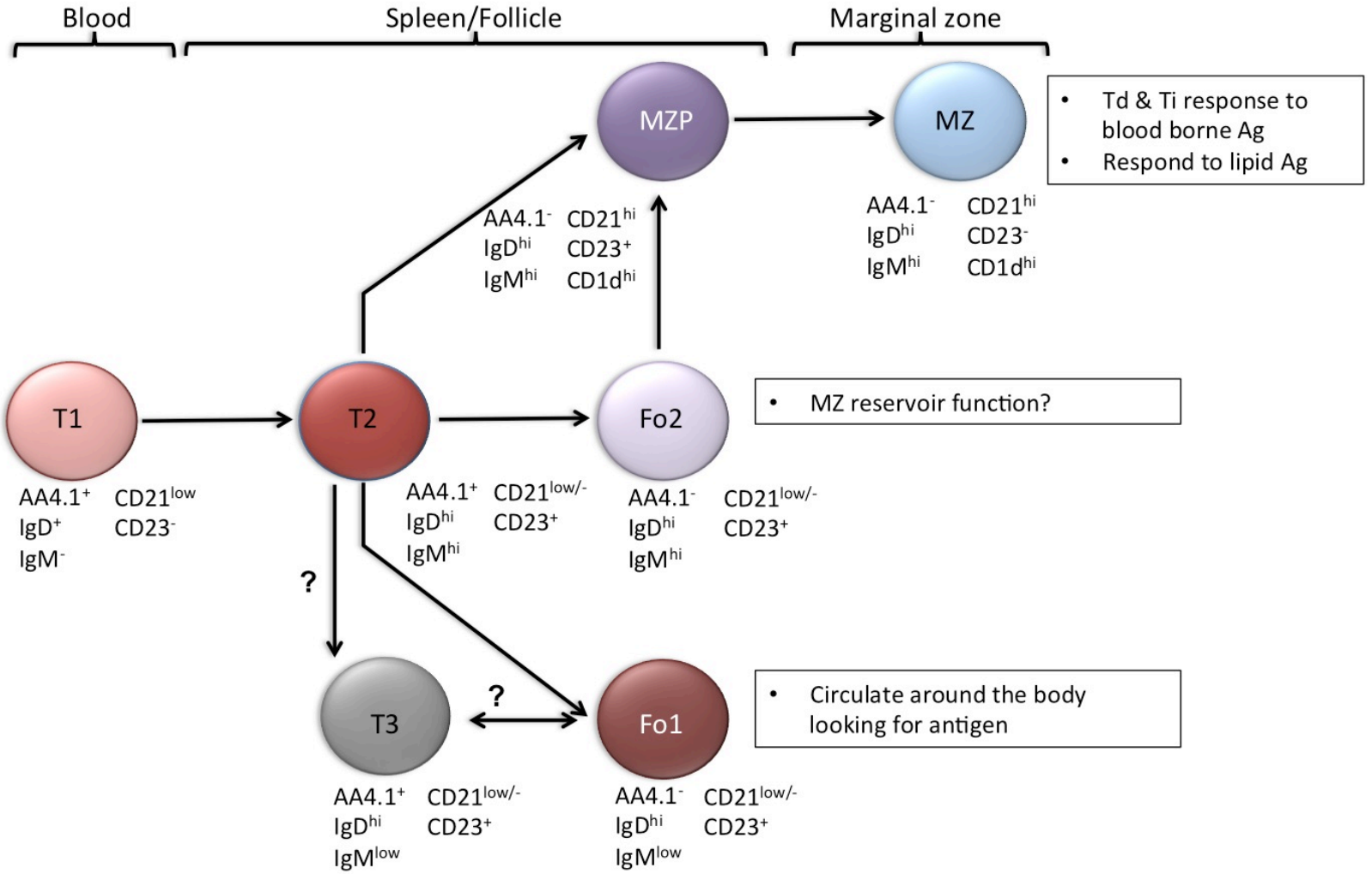

Blood
Spleen/Follicle
Marginal zone
MZP
MZ
• Td & Ti response to blood borne Ag
• Respond to lipid Ag
AA4.1- CD21hi
IgDhi CD23+
IgMhi CD1dhi
AA4.1- CD21hi
IgDhi CD23-
IgMhi CD1dhi
T1
T2
Fo2
• MZ reservoir function?
AA4.1+ CD21low
IgD+ CD23-
IgM-
AA4.1+ CD21low/-
IgDhi CD23+
IgMhi
AA4.1- CD21low/-
IgDhi CD23+
IgMhi
?
T3
?
Fo1
• Circulate around the body looking for antigen
AA4.1+ CD21low/-
IgDhi CD23+
IgMlow
AA4.1- CD21low/-
IgDhi CD23+
IgMlow

Figure 1.7: Peripheral B cell development. Immature B cells that have developed in the bone marrow enter the spleen via the blood (Fig. 1.4); these T1 B cells lack the ability to recirculate and, once in the spleen (Fig. 1.6), undergo several stages of development into mature B cell phenotypes. As T1 B cells ($CD19^+CD21^-CD23^-IgM^+IgD^-AA4.1^+$) enter the white pulp, they pass through the PALS into the B cell zone where they first express surface IgD and acquire the ability to recirculate and are termed T2 B cells ($CD19^+CD21^-CD23^+IgM^+IgD^+AA4.1^+$). Following strong BCR signaling, T2 B cells will develop into follicular type 1 B cells (Fo1; $CD19^+CD21^{low/-}CD23^+IgM^{low}IgD^+AA4.1^-$), which circulate around the body searching for antigen presented by APC. Following weak BCR signaling, T2 B cells will develop into marginal zone precursor B cells (MZP; $CD19^+CD21^+CD23^+IgM^+IgD^+AA4.1^-$), which reside in the B cell zone before developing into marginal zone B cells (MZ; $CD19^+CD21^+CD23^-IgM^+IgD^-AA4.1$). MZ B cells reside in the marginal zone of the spleen (Fig. 1.6) where they sample antigens passing through the blood of the splenic red pulp and respond to T-dependent and T-independent antigens. If a T2 B cell receives no BCR stimulation but can compete successfully for BAFF, it develops into a follicular type 2 B cell (Fo2; $CD19^+CD21^{low/-}CD23^+IgM^+IgD^+AA4.1^-$). Little is known about Fo2 B cells but they may act as reserve cells to replenish the marginal zone under certain stimuli such as when the MZ B cell population is rapidly depleted by a blood borne pathogen. Transitional 3 cells (T3 $CD19^+CD21^{low/-}CD23^+IgM^+IgD^+AA4.1^+$) are a third transitional population that exists in the secondary lymphoid organs, they are thought represent a population of anergic B cells however their differentiation is yet to be delineated.

Adapted from Pillai *et al.,* Nature Reviews Immunology (2009)(174).

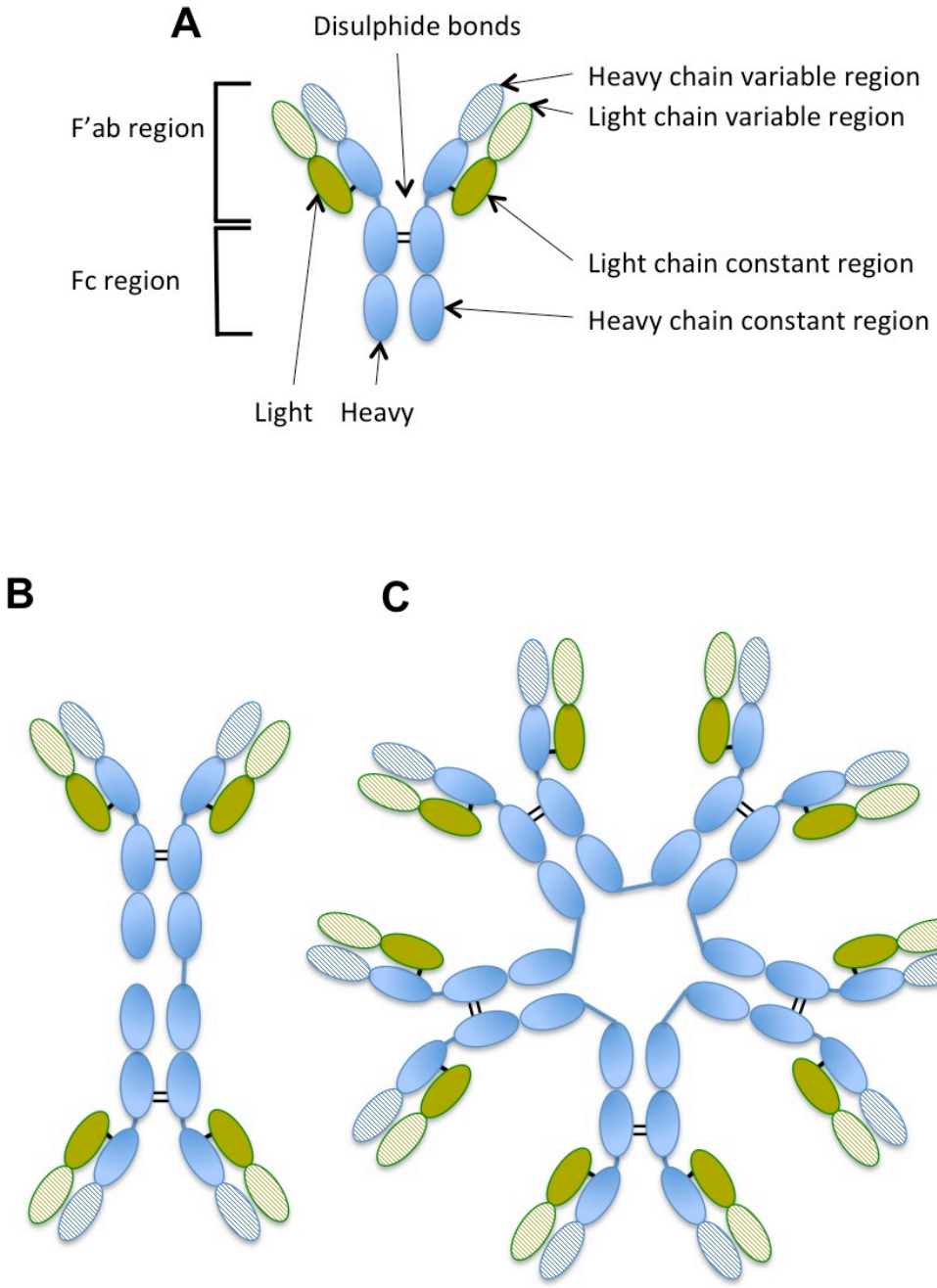

A
Disulphide bonds
Heavy chain variable region
Light chain variable region
F'ab region
Fc region
Light chain constant region
Heavy chain constant region
Light
Heavy
B
C

Figure 1.8: Antibodies. Antibodies are secreted forms of the B cell antigen receptor (Fig. 1.5) that exhibit varying degrees of antigen specificity depending on the type of cell that secretes them. An antibody comprises 4 polypeptide chains, 2 heavy (blue) and 2 light (green), and can be separated into two fragments: the antigen binding F'ab fragment and the antibody receptor binding Fc fragment (A). This structure is maintained by several disulphide bonds (black lines). The end domains of each F'ab fragment contain the variable regions (striped domains) of the protein, which bind to specific epitopes (A). Antibodies can be produced in 5 classes: IgG, IgD and IgE are monomers with a structure similar to that presented in 'A'; IgA is produced as a dimer as represented in 'B'; and IgM can be produced as either a monomer or pentamer as represented in 'A' and 'C' respectively.

2. Materials and Methods

2. 1. Mice

Animals were maintained in the Biological Services Units of the Universities of Glasgow and Strathclyde in accordance with the Home Office UK Licenses PPL60/3580, PPL60/3119, PPL60/4300, PPL60/3791, PPL60/3810, PIL60/12183, PIL60/12950, and PIL60/9576 and the respective Ethics Review Boards of The University of Glasgow and Strathclyde University.

2.2. Purification of ES-62

ES-62 was purified as previously described (335), briefly: ES-62 was purified, using endotoxin free reagents, from spent culture medium (endotoxin-free RPMI 1640; Life Technologies, Paisley, U.K. with added glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml)) of adult *A. vitea*. Larval forms were removed by filtering the media through 0.22 μm filter (Sigma-Aldridge, Poole, U.K.). The media was then transferred to a stirred ultracentrifugation unit containing a YM10 membrane (Amicon, Stonehouse, U.K.). The sample was then further concentrated using a Centricon micro-concentrators with a 30kDa cutoff membrane (Amicon) before being applied to a sepharose column fitted to an isocratic fast protein liquid chromatography system (Pharmacia). 95% of protein-containing eluted fractions were shown to contain a single protein band that was confirmed to be ES-62 by SDS-page. Endotoxin levels were confirmed to be <0.003 U/ml using the Endosafe Kit (Charles River Laboratories, Kent, UK) (319,336,338)

2.3.1. MRL/*lpr* mouse model of SLE

Male MRL/MP and male or female MRL/*lpr* mice were obtained from Harlan Olac, Bicester, UK at 5-8 weeks of age and maintained until 20-22 weeks of age. Proteinuria was monitored twice weekly using Multistix (Bayer, Cambridge, UK). ES-62 (2 μg; which reflects the endogenous concentration of ES-62 in a mouse during a natural infection with *A. vitea* (335)) or PBS (100 μl) was administered subcutaneously twice weekly from week 7 after birth; recombinant IL-17A (1 μg (118)), recombinant IL-22 (1 μg (118)) or neutralizing anti-IL-22 (100 μg (118)) was administered twice weekly in 100 μl of sterile PBS from 12-20 weeks; neutralizing anti-IL17A (100 μg (118)) was administered twice weekly in 100 μl of sterile PBS from 7-12 weeks of age. Mice were sacrificed using CO_2 and exsanguination by

cardiac puncture using a 23-gauge needle and 1 ml syringe. Serum was collected from blood using pediatric blood tubes (LSL, UK). Spleens, kidneys, peripheral (popliteal, inguinal, brachial, axillary, cervical), renal lymph nodes and bones were harvested.

2.3.2. Collagen induced mouse model of rheumatoid arthritis

Collagen induced arthritis (CIA) was induced in 8-week old male DBA/1 mice. Each mouse received 100 μg of bovine type II collagen (CII, MD Biosciences) in complete freunds adjuvant containing 4mg/ml of *Mycobacterium tuberculosis* (CFA, MD Biosciences) by intradermal injection at the base of the tail on day 0 and 200 μg of CII in PBS via inter-peritoneal injection on day 21. Mice were monitored daily for signs of arthritis, which was measured as a total score from 4 limbs where: 0 = normal, 1 = digit(s) involvement, 2 = erythema, 3 = erythema and swelling, 4 = extension/loss of function. ES-62 (2 μg) or PBS control was administered subcutaneously on days -2, 0 and 21. Mice were sacrificed using CO_2 and exsanguination by cardiac puncture using a 23-gauge needle and 1 ml syringe. Spleen, paws and paw-draining LN were harvested.

2.3. Cell culture

Lymphoid tissue was dissected, mechanically disrupted and passed through a nitex gauze (Cadisch Precision Meshes Ltd., UK) to obtain a single cell suspension. Spleen samples were treated with 5 ml of red cell lysis buffer (eBioscience) for 5 minutes on ice before being diluted 10-fold in PBS and centrifuged at 400 xg, 4ºC for 5 minutes. Viable cell counts were obtained using cells stained with Trypan blue (0.4%, Sigma) and a haematocytometer (Digital Bio.). B2 B cells were purified as previously described by depletion of $CD43^+$ cells using anti-CD43 antibodies conjugated to magnetic micro beads (Miltenyi)(230), double negative T cells were purified by depletion of $CD19^+$ $CD4^+$, $CD8^+$ and $CD11b^+$ cells (Miltenyi). B cells were cultured in RPMI containing 5% FCS, 50 μM β2-Mercaptoethanol, 1% L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 10 U/ml penicillin and 10 μg/ml of streptomycin; and T cells were cultured in RPMI containing 10% FCS, 1% L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 10 U/ml penicillin and 10 μg/ml of streptomycin.

Kidneys were minced in 1 ml of RPMI medium before being passed through nitex gauze and centrifuged at 400xg, 4ºC for 5 minutes. The supernatant was stored for further analysis of the renal interstitial fluid and the cell pellet was resuspended in 5 ml of red cell lysis buffer and incubated on ice for 5 minutes before being diluted 10-fold in PBS and centrifuged at 400 xg, 4ºC for 5 minutes. Cells were then passed through a cell strainer (BD) to remove any fat and tissue debris still present in the sample.

Renal fibroblasts were differentiated by culturing kidney cells ($2x10^6$) for two weeks in RPMI containing 20% FCS, 1% L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids,10 U/ml penicillin and 10 μg/ml of streptomycin; media was replaced twice weekly and the phenotype of the fibroblasts was confirmed by flow cytometry as being $CD54^+$ and $CD106^+$ (Fig. 2.1).

2.4. Purification of lymphocytes

B2 B cells were purified from whole spleen samples by magnetic bead sorting according to manufacturers guidelines (Miltenyi). Briefly, single cell suspensions of splenocytes were incubated with anti-CD43 magnetic micro-beads for 15 minutes before being washed and massed through an LD column. The purified $CD43^-$ cell fraction was on average 86% positive for $CD19^+$ cells; which included transitional, follicular, marginal zone precursor, marginal zone and germinal center B cells. Double negative T cells were also purified by depletion of $CD4^+CD19^+CD8^+CD11b^+$ cells from the pLN of 21-week MRL/*lpr* mice. Population purity was confirmed by flow cytometry.

2.5. Generation of bone marrow derived macrophages and DC

To retrieve bone marrow cells, harvested femurs and tibias were cleaned of all tissue and the ends of the bones were removed. A 25G needle was then inserted into each bone and 10 ml of media was flushed through into a 50 ml falcon tube. Large pieces of bone marrow were then dissociated by gentle pipetting through a 21G needle. The sample was then centrifuged at 400xg (4^oC) for 5 minutes and the cell pellet was resuspended in 5 ml of cold red cell lysis buffer (eBiosciences) and incubated on ice for 5 minutes. RPMI (35ml) was then added and the cells were centrifuged at 400xg (4^oC) for 5 minutes. The cell pellet was then

resuspended in 5 ml of culture medium and passed through a cell strainer. Culture media contained RPMI supplemented with 10% FCS, 1% L-Glutamine, 10 U/ml penicillin and 10 μg/ml of streptomycin, 1% pyruvate acid, 1% non-essential amino acids (all Life Technologies, UK) and either recombinant GM-CSF (10 μg/ml) for DC development or 20% L929-conditioned media, which contains the M-CSF required to develop macrophages. The cells were counted and their concentration adjusted to $2x10^6$ cells/ml: 1ml of this cell stock was added to a non-tissue culture-treated petri dish along with a further 9 ml of culture media. DC or macrophage cultures were incubated at 37°C with 5% CO_2 for 6 days with the media being replaced on the third day. On the sixth day the cells were harvested by gentle scraping.

2.6. Flow cytometry

Cells ($1x10^6$) were seeded onto round bottom 96-well plates and washed with PBS at 400xg for 5 minutes at 4ºC and stained with the Live/Dead® fixable aqua dead cell dye (Invitrogen) following the manufacturers protocol. Cells were then resuspended in FACs staining buffer (PBS containing 1% FCS and 2.5 mM EDTA at 4ºC) and Fcγ Receptors were blocked by incubation with 100μl of αCD16/CD32-specific antibody containing 2.4G2 supernatant for 15 minutes. Primary antibody cocktails with relevant isotype controls (Table 2.1) were added to the wells and incubated for 30 minutes at 4ºC. The cells were then washed twice before incubation with secondary antibodies or streptavidin conjugated fluorophores, if necessary. The cells were washed a final 3 times before being passed through a nitex gauze into labeled FACs tubes. FACs analysis was carried out on a BD LSR II.

For the detection of intracellular cytokines or transcription factors, commercial kits were utilized according to the manufacturers' protocols. Cytokine production was detected by intracellular cytokine staining as previously described (336). Briefly, lymphocytes (10^6/ml) were stimulated with 50 ng/ml PMA (Sigma-Aldrich, UK) plus 500 ng/ml ionomycin (Sigma-Aldrich, UK) and 10 μg/ml LPS (*E. coli* O111:B4, Sigma-Aldrich, UK (350)) for 1 h before addition of 10 μg/ml Brefeldin A (Sigma-Aldrich, UK), cells were the incubated for a further 5 h at 37°C with 5 % CO_2. Cells were fixed and washed several times in permeabilization buffer before primary

antibodies were added in permeabilization buffer, incubated at 4^{o}C for 30 minutes. Cells were then washed three times with permeabilization buffer and finally with FACs staining buffer.

2.7. Analysis of peripheral B cell subsets

The study of peripheral B cell subsets is complicated by the multiple stages of development from immature transitional cells to functionally mature marginal zone, follicular or antibody producing B cells. For this analysis the phenotypic markers defined by Allman and Pillai (351) were used (Table 2.2); however, the gating strategy suggested was modified to improve cell subset resolution and experimental efficiency as follows (Fig. 2.2).

Cell populations were initially selected on the basis of size and granularity using the forward scatter (FSC) and side scatter (SSC) parameters respectively (Fig. 2.2A). Doublets, events where two cells are connected and pass through the laser beam at the same time, were excluded by comparing FSC-Height and FSC-Area (Fig. 2.2B). As antibodies can bind non-specifically to dead or dying cells, which is partially due to expelled DNA during cell death, these cells were excluded from analysis using the Live/Dead® fixable aqua dead cell dye (Invitrogen), which binds to free amines on the cell surface: as dead cells have compromised cell membranes the dye can permeated the cell and bind to free amines inside the cell, thus dead cells have a higher staining intensity (Fig. 2.2C).

The conventional method for the separation of marginal zone precursor (MZP), marginal zone (MZ) and follicular B cells is to separate CD19^{+} cells by CD21 and CD23 expression (Fig. 2.3A). Using this method, the position of the MZP gate is difficult to accurately define and the clarity of this population varies between experiments, mouse strains and disease states. To improve the resolution of these populations, CD19^{+}CD23^{-} and CD19^{+}CD23^{+} cells were initially separated (Fig. 2.3B), with the CD19^{+}CD23^{+} gate containing the MZP (CD21^{hi}CD1d^{hi}), follicular and transitional (Fo & T; CD21$^{low/-}$CD1d$^{int/-}$) B cells (Fig. 2.3C); whereas the CD19^{+}CD23^{-} gate contains the MZ (CD21^{+}IgM^{+}) and T1 (CD21^{-}IgM^{+}) B cells (Fig. 2.3D). MZP, MZ and Fo have specific IgM and IgD expression profiles. Using the conventional method of separating these subsets, MZ B cells are IgM^{hi}IgD$^{low/-}$,

MZP are $IgM^{hi}IgD^{hi}$ and Fo are $IgM^{low/-}IgD^{hi}$ (Fig. 2.3E); these populations are more clearly defined using the modified protocol (Fig. 2.3F).

The follicular population identified (Fig. 2.3C) is a hetrogenous population that contains the functionally distinct follicular type 1 (Fo1: $IgD^{hi}IgM^{low/-}AA4.1^{-}$) and follicular type 2 (Fo2: $IgD^{hi}IgM^{hi}AA4.1^{-}$) B cells as well as the transitional 2 (T2: $IgD^{hi}IgM^{hi}AA4.1^{+}$) and transitional 3 (T3: $IgD^{hi}IgM^{low/-}AA4.1^{+}$) populations. These populations are first separated on the basis of their IgM and IgD expression (Fig. 2.3G) and then by the expression of the immature marker AA4.1 (Fig. 2.3H & I).

For the identification of germinal centre (GC) B cells, $CD19^{+}CD43^{-}$ cells (Fig. 2.4A) were examined for the expression of the GC cell specific marker GL7 along with the pan B cell marker CD24 to ensure that no contaminating non-B cells are selected in the initial gate (Fig. 2.4B). The presence of FAS is then confirmed (Fig. 2.4C) however, in this study FAS is treated as a redundant marker and was not included in the analysis of GC B cells, as essentially all of the $CD19^{+}CD43^{-}$ $CD24^{+}GL7^{+}$ cells are positive for FAS (~90%) and FAS is not expressed in MRL/*lpr* mice.

GC B cells develop into antibody producing cells. The phenotype of these cells is usually defined by the ability to produce antigen specific antibodies and their rate of proliferation: plasmablasts are rapidly dividing antibody-producing cells whereas plasma cells are sessile antibody producers. The most commonly used method to identify plasma cells by flow cytometry is to compare the expression of CD138 to that of B220 (Fig. 2.5A) (352) or CD19 (Fig. 2.5B) (353) as plasma cells are generally thought to be $CD138^{+}CD19^{-}B220^{-}$. This method of separation is not appropriate for the MRL/*lpr* mouse model for three reasons: firstly, the majority of CD138 is expressed by the $B220^{+}CD4^{-}CD8^{-}$ double negative (DN) T cell population (McGrath, Harnett & Harnett unpublished data and data not shown), which accounts for up to 80% of lymphocytes from 16 weeks of age; secondly, as several populations of $B220^{+}$ and $CD19^{+}$ B cells, myeloid cells and T cells express CD138, a dump channel to exclude cells positive for CD4, CD8, GR1, F4/80, CD11b and CD11c (Fig. 2.5C) is applicable to all plasma cell analysis by flow cytometry; and finally, as the MRL/*lpr* mouse develops a spontaneous disease that

is not driven by any specific auto-antigen, it is difficult to identify antigen specific antibody producing cells. The $CD138^+$ populations were therefore characterized as precisely as possible, initially by selecting the $Dump^-CD138^+$ cells (Fig. 2.5C) as this excludes any CD4, CD8, GR1, F4/80, CD11b and CD11c positive cells from the analysis and provides a clear population that can be further dissected on the basis of CD19 and B220 expression (Fig. 2.5D). Based on the current literature the $B220^-CD19^-$ population contains fully differentiated plasma cells, the $B220^{low/-}$ $CD19^+$ population contains either short-lived plasma cells or plasmablasts and the $B220^+CD19^+$ population contains either pre-plasma cells or plasmablasts (352,354). Examination of the expression of B220 and CD19 on the populations isolated by conventional plasma cell gating reveals a mixed population (Fig. 2.5E) where the $CD138^+B220^{low/-}$ population (Fig. 2.5A) contains $CD19^+$ and $CD19^-$ populations (Fig. 2.5E), the $CD138^+CD19^-$ population (Fig. 2.5B) contains $B220^+$ populations (Fig. 2.5E), the $CD138^{hi}CD19^{hi}$ population (Fig. 2.5D) contains $B220^+$ and $B220^{low}$ populations (Fig. 2.5E) and the $CD138^{low}CD19^+$ population (Fig. 2.5D) are all $B220^{hi}$ (Fig. 2.5E).

The final population examined represents B1 B cells. These innate B cells develop independently of B2 B cells and are generally defined as being $CD19^{hi}CD43^+IgM^{hi}IgD^-$ and can be separated into $CD5^+$ B1a cells and $CD5^-$ B1b cells (199) (Fig. 2.5F & G).

2.8. Organ histology

Whole organs from MRL/*lpr* mice were harvested and fixed with 10% Formalin for 24 hours followed by 30% sucrose for an additional 24 hours before being snap frozen in OCT embedding media (Raymond A Lamb) and stored at -80^{o}C. Sections, 7μm thick, were cut from the frozen tissues using a Cryostat (Thermo Scientific), and mounted onto Super Frost® Plus slides (VWR). Slides were wrapped in metallic foil and stored at -20^{o}C. Haematoxylin and eosin (H&E) staining was done as described by Fischer *et al*, 2008. Briefly, sections were removed from -80^{O}C storage and equilibrated to room temperature before being uncovered and fixed in ice-cold acetone/ethanol (3:1). The slides were then air dried and stained with Harris haematoxylin (a nuclear stain) and 1% eosin (a connective tissue stain). Alternatively, to detect basement membranes in the

kidney glomerulus, kidneys were stained with periodic acid and Schiffs stain (PAS). Briefly, fixed sections were oxidized by submersion in periodic acid for 10 minutes before being washed for 5 minutes and then submerged in Schiff's solution for 20 minutes. Sections were then washed in slightly warm running water until the stain had receded enough so that the basement membrane was visible around the glomerulus by light microscopy. Cell nuclei were then briefly stained with Harris haematoxylin (5-10 seconds) and washed. H & E or PAS stained sections were dehydrated in increasing concentrations of ethanol and xylene before being mounted in DPX and covered with a glass cover slip. Sections were visualized using an Olympus BX41 light microscope.

2.9. Immunohistochemistry and ANA staining

For immunohistochemistry, tissue sections were fixed in ice-cold acetone for 10 minutes before being air-dried for a further 30 minutes; tissues were outlined with a wax pen and allowed to air-dry for 1 hour before being rehydrated in PBS-tween (PBS-T) for 5 minutes and blocked in 1% BSA in PBS-T for 1 hour. Slides were washed three times in PBS-T for 5 minutes before being incubated with primary antibody for 2 hours at room temperature. Slides were then washed three times in PBS-T and then transferred to a dark staining box and incubated with secondary fluorochrome conjugated antibodies for 2 hours at room temperature. Slides were washed three times in the dark and then incubated with DAPI for 5 minutes. Slides were then washed for a final time before being mounted in ProlonGold (Invitrogen) and covered with a glass cover slip. Serum anti-nucleic acid antibodies (ANA) were detected using Hep-2 slides (Antibodies incorporated, CA) in accordance with manufacturers guidelines with the following modifications. Briefly, slides were incubated with 40 μl of diluted serum samples (1:40) for 30 minutes in a moist staining box before being washed thoroughly with PBS. Slides were then incubated with affinity purified rabbit-anti-mouse-IgG (heavy and light chain) antibodies (diluted 1:200 in PBS; Jackson Laboratories) diluted in PBS and incubated for a further 30 minutes in a moist staining box. Slides were then washed thoroughly once again with PBS before being incubated with a FITC-conjugated goat-anti-rabbit-IgG antibody (diluted 1:200 in PBS) for 30 minutes. Slides were washed a final time in PBS before being mounted with the mounting solution provided and covered with a glass cover slip. Slides were viewed using a

Axiovert S100 fluorescent microscope (Zeiss). Fluorescence staining intensity was subjectively assessed and where appropriate scored in the nucleus and cytoplasm as: none (0), ± (1), positive (2) and bright (3) (Fig. 2.6).

2.10. ELISAs

Cytokines were detected by ELISA using 96-well plates (Corning) and the following commercially available and validated kits: TNFα, IL-1β, IL-6, IL-12p70, IL-27, MCP-1 and IL-10 (all from eBioscience, CA); IFNγ (BD, NJ); IL-17A (BioLegend, CA); IL-22 and IL-23 (R&D, MN). Serum antibody concentrations were determined by sandwich assay. Mouse serum was serially diluted in PBS (10^3-10^{10}) in a 96-well plate and incubated over night at 4^{o}C. Samples were then washed, and non-specific binding blocked using 10% FCS in PBS before being incubated with biotinylated mouse- anti-IgG1, -anti-IgG2a, -anti-IgM or -anti-IgE (all from BD, NJ, and diluted 1:200) for 1 hour at room temperature. Samples were washed and incubated with streptavidin conjugated horseradish peroxidase for 30 minutes before being washed one final time and then incubated with TMB substrate and stop solutions (KPL, MD). Absorbance read at 450nm using a Tecan Sunrise plate reader.

2.11. Western blot

Cells were washed with cold PBS twice and centrifuged at 400xg for 5 minutes at 4^{o}C, the cell pellet was air-dryed and disrupted (by vortex) in 30-50μl of modified RIPA cell lysis buffer (50 nM Tris buffer (pH 7.4), 150 mM sodium chloride, 2% (v/v) NP40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 1x Halt^{TM} protease inhibitor and 1x Halt^{TM} phosphatase inhibitor (Pierce)) and incubated for 30 minutes on ice. Cell debris was then separated from the protein extract by centrifugation at 16,000xg for 15 minutes at 4^{o}C. The supernatant was then removed and protein concentration was measured using the BCA protein assay kit (Pierce). Proteins were separated by gel electrophoresis using the NuPAGE Novex system (Invitrogen). Protein extract (20-50 μg) was heated with NuPAGE LDS sample buffer and NuPAGE reducing agent for 10 minutes in a heat block set at 72^{o}C. Sample (20 μl) was then injected into pre-cast 4-12% Bis-Tris gradient gels along with 5 μl of Kaleidoscope protein ladder (Bio-Rad). Gels were run in NuPAGE MOPS buffer supplemented with antioxidant at 180V until the protein

bands were well resolved. Proteins were then transferred to nitrocellulose membranes (GE) using the NuPAGE system. Protein transfer was confirmed by ponseau-red (Thermo Scientific) staining of the membrane, which was then washed with TBS-Tween (TBS-T: 0.5 mM sodium chloride, 20 mM Tris-HCl (pH7.4) and 0.1% (v/v) Tween-20) and then blocked with 5% non-fat milk protein in TBS-T for 1 hour. The membrane was then incubated with a pre-titrated primary antibody suspended in 1% BSA in TBS-T for 16-18h at 4^{o}C. The membrane was washed three times with TBS-T before being incubated with a pre-titrated secondary antibody suspended in TBS-T with 5% milk for 1 hour at room temperature. The membrane was washed for a final 3 times before being incubated in ECL Western blotting substrate (Thermo Scientific) and exposed to X-ray film (Kodak).

2.12. Purification of neutralizing antibodies

Antibody producing hybridoma cell lines (kindly provided by Drs Renauld and Van Snick, Ludwig Institute for Cancer Research, Belgium) were cultured at 80-90% confluency for 2 weeks and the supernatant was harvested and filter sterilized. A standard purification column was seeded with sepharose beads covered with Protein G, a bacterial antibody binding protein, and washed with 5 column volumes of binding buffer (0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 at pH7). Cell supernatants were then run through the column at 4^{o}C using capillary action. The column was then washed with a further 50ml of PBS and protein was eluted from the column by adding 8ml of elution buffer (0.1 M glycine at pH 2.7) and collected in 15 x 1ml fractions in eppendorf tubes. Each tube contained 500 μl of 1M Tris-HCl at pH 8.5 to bring the eluted proteins back to physiological pH. The concentration of protein in each fraction was measured using a nanodrop and fractions containing >0.1 mg/ml of protein were pooled. Purified protein samples were dialysed overnight in 5 liters of sterile PBS at 4^{o}C. Protein concentration was measured using a nanodrop and the purity of the sample and integrity of the protein was confirmed by protein gel and Coomassie blue staining. Endotoxin was removed from the sample using the Endosafe Kit (Charles River Laboratories, Kent, UK). Endotoxin levels were confirmed to have <0.003 endotoxin units/ml before samples were filter sterilized and stored in concentrated aliquots.

2.13. Statistics

Statistical analysis was preformed on all data sets using the Prism software (GraphPad Software, La Jolla, CA) and statistical significance is shown as *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$. For data sets with two groups: parametric data was analyzed using the one-tailed Student's t-test; for the analysis of non-parametric data, the Mann-Whitney test was used. For data sets with more than 2 groups, the one-way ANOVA with Tukey's post test was used and in experiments where two variables were measured, the two-way ANOVA test was used with the Bonferroni's post test.

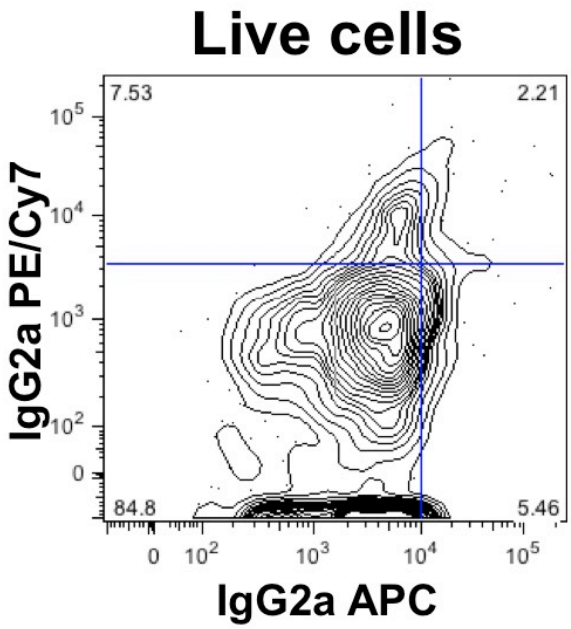
Table 2.1. List of antibodies used in this study.

Antigen	Fluorophores	Clone	Co.	Optimal Concentration (μg/μl)
AA4.1	APC	AA4.1	eB	0.1
B220	E450	RA3-6B2	BL	0.05
CD11b	E450	M1/70	BL	0.05
CD138	PE	281-2	BD	0.05
CD19	AF488	6D5	BL	0.05
CD19	Biotin	ID3	BD	0.05
CD19	PE/Cy7	6D5	BL	0.05
CD1d	PE	1B1	BL	0.05
CD2	Biotin	RM2-5	BL	0.1
CD21	E450	eBio4E3	eB	0.05
CD23	PE/Cy7	B3B4	BL	0.05
CD25	APC	PC61	BL	0.1
CD28	Biotin	37.51	BL	0.1
CD3	AF488	145-2C11	BL	0.05
CD3	PE	145-2C11	BL	0.05
CD4	Biotin	L3T4	BD	0.1
CD4	PE	RM4-5	BD	0.1
CD4	PerCP	RM4-5	BD	0.1
CD43	PE	1B11	BL	0.05
CD43	PE/Cy7	1B11	BL	0.05
CD5	Biotin	53-7.1	BL	0.1
CD8	APC	53-6.7	BL	0.2
CD8	PE/Cy7	53-6.7	BL	0.05
CD9	PE	MZ3	BL	0.05
CTLA-4	Biotin	UC10-4B9	BL	0.1
FoxP3	PE	FJK-165	eB	0.2
GL7	FITC	553666	BD	0.05
HSA	PerCP-Cy5.5	M1/69	BL	0.05
IFNγ	PE	XMG1.2	BL	0.2
IgD	PerCP/Cy5.5	11-26c.2a	BL	0.05
IgM	APC/Cy7	RMM-1	BL	0.05
IL-10	APC	JES5-16E3	eB	0.2
IL-17A	APC	TC11-18H10.1	BL	0.2
IL-22	PE	Poly5164	BL	0.2
IL-4	PE	11B11	BL	0.2
TLR4	APC	67518	R&D	0.2
BAFF-R	FITC	eBio7H22-E16	eB	0.05

Table 2.2. Cell surface markers used to phenotype B cell populations: as defined by Allman and Pillai (351,352,355,356).

Population	Phenotype
T1	$CD19^{+}$ $CD93^{+}$ $CD21^{-}$ $CD23^{-}$ $IgD^{low/-}$ IgM^{hi}
T2	$CD19^{+}$ $CD93^{+}$ $CD21^{int}$ $CD23^{+}$ IgD^{hi} IgM^{hi}
T3	$CD19^{+}$ $CD93^{+}$ $CD21^{int}$ $CD23^{+}$ IgD^{hi} $IgM^{low/-}$
MZP	$CD19^{+}$ $CD93^{-}$ $CD21^{hi}$ $CD23^{+}$ $CD1d^{hi}$ IgD^{hi} IgM^{hi}
MZ	$CD19^{+}$ $CD93^{-}$ $CD21^{hi}$ $CD23^{-}$ $CD1d^{hi}$ IgD^{-} IgM^{hi}
Fo1	$CD19^{+}$ $CD93^{-}$ $CD21^{low/-}$ $CD23^{+}$ IgD^{hi} $IgM^{low/-}$
Fo2	$CD19^{+}$ $CD93^{-}$ $CD21^{low/-}$ $CD23^{+}$ IgD^{hi} IgM^{hi}
GC	$CD19^{+}$ $CD43^{-}$ $CD24^{+}$ $GL7^{+}$
Pre-plasma	$(CD4, CD8, GR1, CD11b, CD11c \& F4/80)^{-}$ $CD138^{+}$ $CD19^{+}$ $B220^{+}$
Plasmablast	$(CD4, CD8, GR1, CD11b, CD11c \& F4/80)^{-}$ $CD138^{+}$ $CD19^{+}$ $B220^{-/low}$
Plasma	$(CD4, CD8, GR1, CD11b, CD11c \& F4/80)^{-}$ $CD138^{+}$ $CD19^{-}$ $B220^{-/low}$
B1a	$CD19^{+}$ $CD43^{+}$ IgM^{hi} $CD5^{+}$
B1b	$CD19^{+}$ $CD43^{+}$ IgM^{hi} $CD5^{-}$

A


Live cells
7.53
2.21
84.8
5.46
IgG2a PE/Cy7
IgG2a APC
0
10^2
10^3
10^4
10^5

B

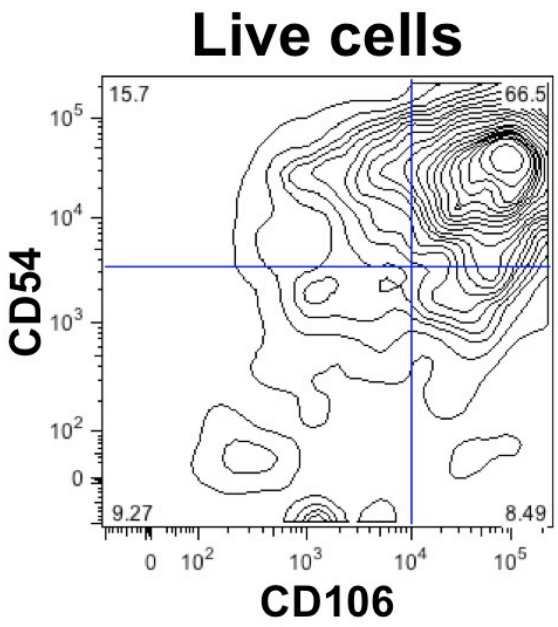

Live cells
15.7
66.5
9.27
8.49
CD54
CD106
0
10^2
10^3
10^4
10^5

Figure 2.1. Gating strategy for the detection of fibroblasts. Fibroblasts were confirmed as being double positive for CD54 (ICAM-1) and CD106 (VCAM-1) (357). Isotype staining is shown in panel (A) and the positive stain is shown in panel (B).

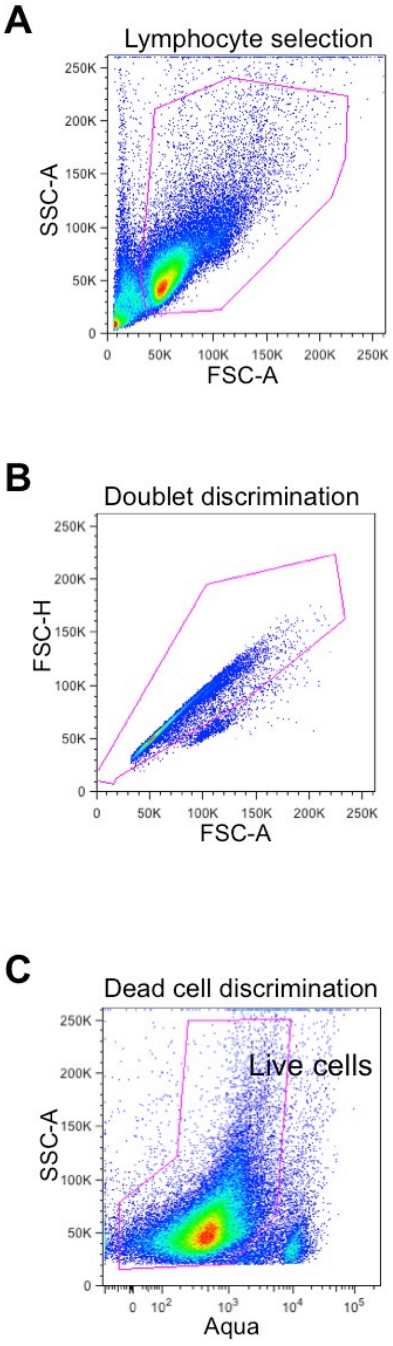

A
Lymphocyte selection
SSC-A
FSC-A
B
Doublet discrimination
FSC-H
FSC-A
C
Dead cell discrimination
Live cells
SSC-A
Aqua

Figure 2.2. Gating strategy for the detection of live cells

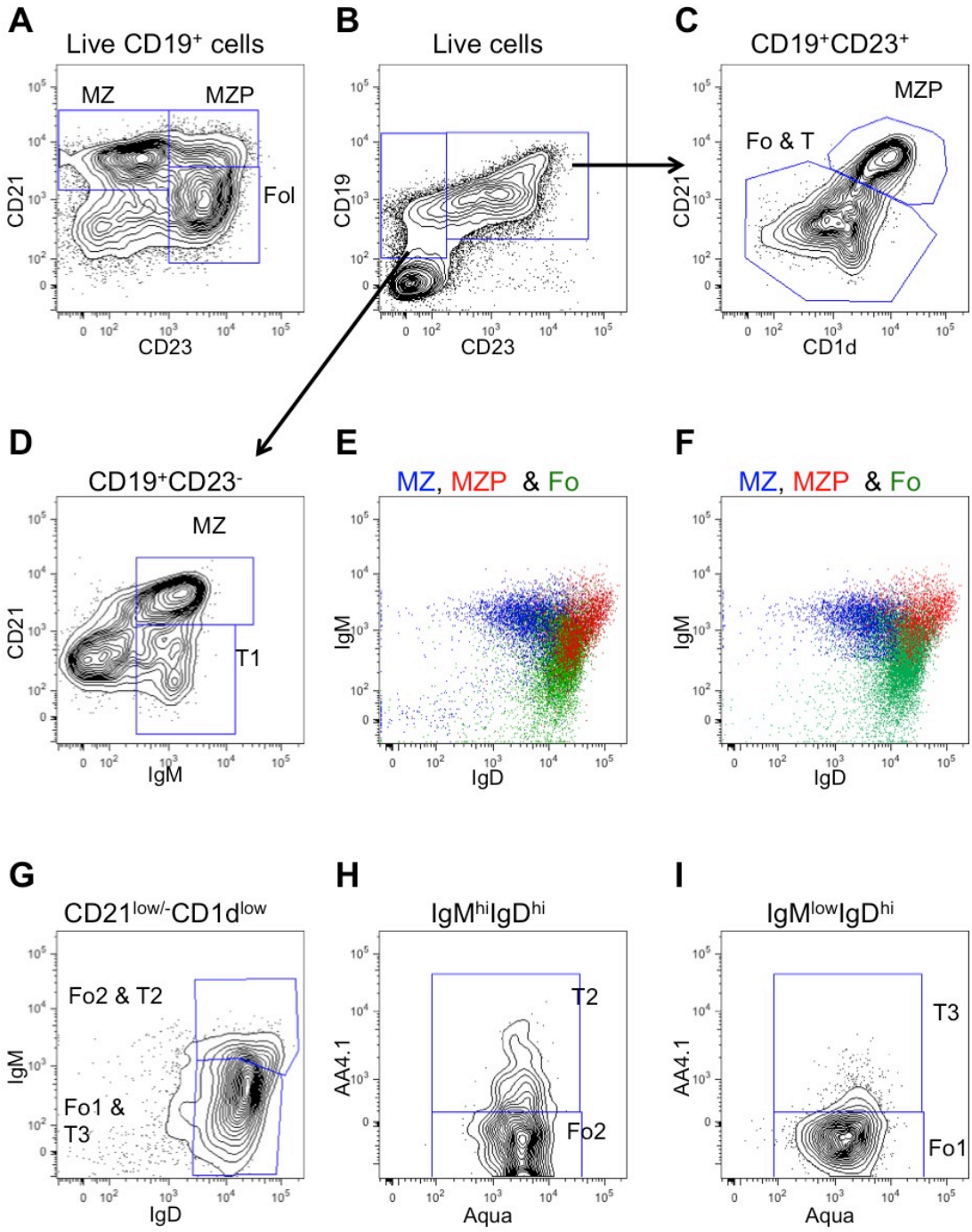

A
Live CD19+ cells
MZ
MZP
Fol
CD21
CD23
B
Live cells
CD19
CD23
C
CD19+CD23+
MZP
Fo & T
CD21
CD1d
D
CD19+CD23-
MZ
T1
CD21
IgM
E
MZ, MZP & Fo
IgM
IgD
F
MZ, MZP & Fo
IgM
IgD
G
CD21low/-CD1dlow
Fo2 & T2
Fo1 & T3
IgM
IgD
H
IgMhiIgDhi
T2
Fo2
AA4.1
Aqua
I
IgMlowIgDhi
T3
Fo1
AA4.1
Aqua

Figure 2.3. Gating strategy for the detection of live transitional, marginal zone precursor, marginal zone and follicular B cells.

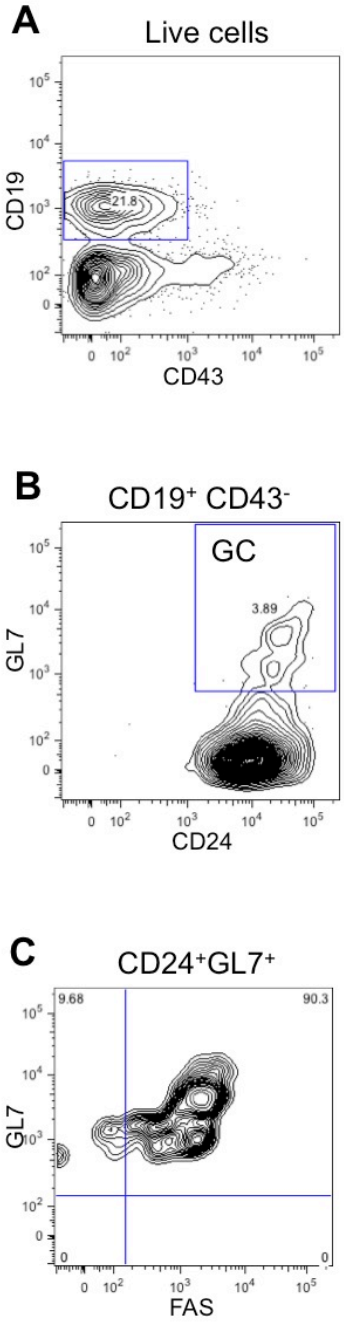

A
Live cells
CD19
CD43
21.8
B
CD19+ CD43-
GC
3.89
GL7
CD24
C
CD24+GL7+
9.68
90.3
GL7
FAS

Figure 2.4. Gating strategy for the detection of germinal centre B cells.

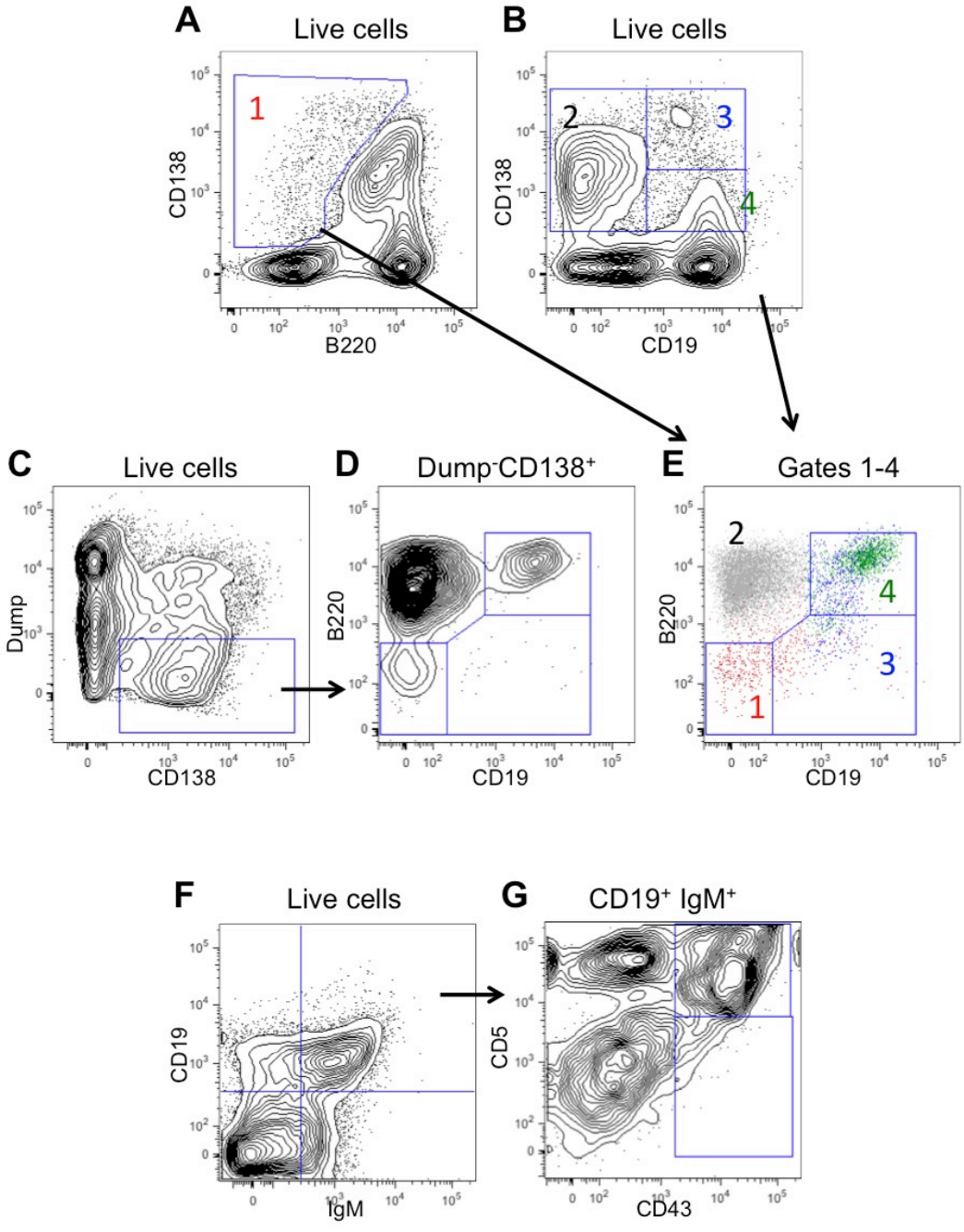
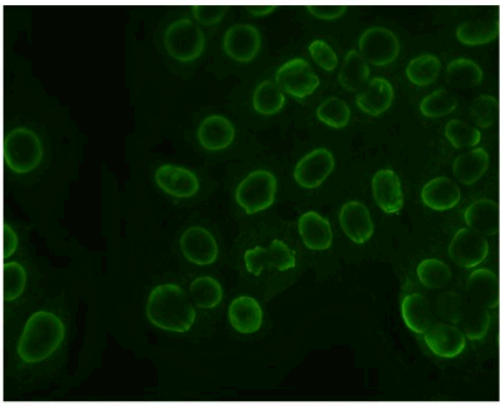

A
Live cells
1
CD138
B220
B
Live cells
2
3
4
CD138
CD19
C
Live cells
Dump
CD138
D
Dump-CD138+
B220
CD19
E
Gates 1-4
2
4
3
1
B220
CD19
F
Live cells
CD19
IgM
G
CD19+ IgM+
CD5
CD43

Figure 2.5. Gating strategy for the detection of antibody producing B cells.

A Negative control

B Positive control



C MRL/*lpr* mouse serum

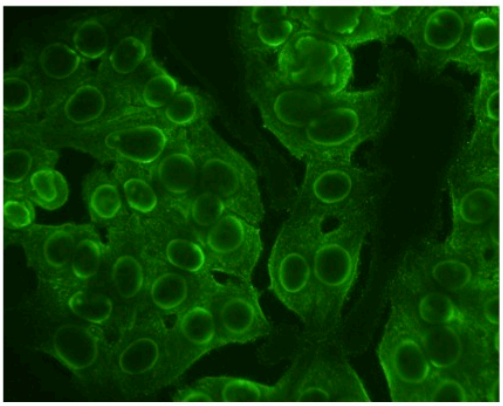


Figure 2.6. Representative images of ANA stained Hep-2 slides. Hep-2 slides (Antibodies incorporated) were stained with 40μl of negative (A) and positive (B) control serum that is provided with the kit. Hep-2 slides were stained with 40μl of diluted (1:40) serum from MRL/*lpr* mice (C).

3. ES-62 prevents development of proteinuria in the MRL/*lpr* mouse by modulation of the IL-17A and IL-22 axis

3.1. Introduction

In recent years, much interest has focused on exploiting the relationship between parasitic worms and the immune system, as helminths can actively regulate immune responses via the production of immunomodulatory molecules. The safe modulation of the immune system by helminth products has developed through the co-evolution of parasite and host, and may provide the basis for the design of novel therapeutics for the treatment of diseases where the immune system is aberrantly hyper-responsive, such as in autoimmune and allergic inflammatory conditions (311). ES-62 is one such helminth-derived immunomodulator, secreted by the rodent filarial nematode *Acanthocheilonema viteae,* that protects against the development of collagen-induced arthritis and ovalbumin-induced airway hypersensitivity in mice via the modulation of Th17/Th1 and Th17/Th2 effector cytokine responses, respectively (305,336,338). In this chapter the therapeutic potential of this molecule in a mouse model of systemic lupus erythematosus (SLE), a disease which exhibits a mixed cytokine phenotype is explored (74,358).

T helper cell responses have been widely studied for their pathogenic role in driving renal pathology, as both Th1 and Th2 phenotypes are characteristically unbalanced in SLE patients (73). More recently, the implication of Th17 cells in driving rheumatoid arthritis, and other autoimmune pathologies, has led to scrutiny of the role of this cell type in SLE (111). Consistent with a role for Th17 cells in pathogenesis, IL-17A levels have been found to be raised in SLE patient serum; furthermore, IL-17A producing cells infiltrate the inflamed kidneys of SLE patients (103,359). Recent findings in mouse models of SLE have further supported a role for IL-17A in renal pathology (112-114) although the importance of other Th17 related cytokines, such as IL-22, which may act in synergy with IL-17A in SLE, remain to be determined (137,138,142). As ES-62 appears to be protective in models of inflammation that are regulated by IL-17, the aims of this chapter were therefore to determine: whether ES-62 is protective in the MRL/*lpr* mouse model of SLE and if so, which parameters of pathology are reduced; whether IL-17, and associated cytokines such as IL-22, play a pathogenic role in SLE; and whether suppression of lupus-associated pathology in the MRL/*lpr* mouse by ES-62 is associated with a modulation of the IL-17A/IL-22 axis.

3.2. Results

3.2.1. ES-62 suppresses the development of proteinuria in the MRL/*lpr* mouse

MRL/MP mice develop a lupus-like disease over 12-18 months that recapitulates most of the immunopathology and serology of SLE patients. However, the loss of FAS signaling in the MRL/MP$^{lpr/lpr}$, herein referred to as MRL/*lpr* mice, results in an accelerated disease where the mice develop high titres of anti-nuclear antigen (ANA) autoantibodies, glomerulonephritis, arthritis-like footpad inflammation, systemic vasculitis, splenomegaly, lymphadenopathy and neurological/behavioral changes within 4 months (Fig. 3.1). Consistent with this, analysis of the intensity and characteristic nuclear staining of Hep-2 cells revealed the presence of autoantibodies with specificities for both cytoplasmic and nuclear antigens in MRL/*lpr* mice; whilst MRL/MP mice also displayed some nuclear staining, this was found at much lower levels (Fig. 3.1A). Likewise, assessment of kidney pathology showed much higher levels of glomeruloproliferation (Fig. 3.1B) and cellular infiltration (Fig. 3.1C) in kidneys from MRL/*lpr*, relative to MRL/MP mice at this time point. Moreover, the MRL/*lpr* mice exhibited clear splenomegaly and lymphadenopathy (Fig. 3.1D) that reflected substantial increases in cell numbers (Fig. 3.1E & F). Glomerulonephritis is the major lupus-associated pathology observed in the MRL/*lpr* mouse and the progression of kidney damage is assessed by twice weekly measurements of proteinuria. MRL/*lpr*, but not MRL/MP, mice develop high levels of proteinuria (>3 mg/ml) within 16 weeks and are culled around 20-22 weeks due to the severity of the renal disease (~20 mg/ml) (Fig. 3.2). Prophylactic treatment of MRL/*lpr* mice with twice-weekly (from week-7) subcutaneous (s.c) injections of ES-62 (2 μg/dose) significantly reduced the concentration of proteinuria (Fig. 3.2). Despite the reduction of proteinuria by ES-62, histological evaluation of renal tissue sections stained using haematoxylin and eosin (H & E) or staining with periodic acid and Schiff's stain (PAS) protocols revealed no clear improvement in glomerular nephritis, cellular infiltration (H & E) or basement membrane thickening (PAS). Furthermore, treatment with ES-62 did not appear to reduce the level of immune complex or complement deposition (Fig. 3.3).

3.2.2. ES-62 and T cell dynamics in the MRL/*lpr* mouse

As exposure to ES-62 did not result in any substantial improvement in renal pathology, it was hypothesized that ES-62 was acting to modulate the autoimmune response; in particular, the composition of the inflammatory cell infiltrate and the cytokines that control inflammation in the kidney. A striking feature of the lupus-like disease that develops in MRL/*lpr* mice is the enlargement of the spleen and lymph nodes (LN), termed splenomegaly and lymphadenopathy respectively. This is largely due to a loss of peripheral FAS-mediated cell death and exacerbated proliferation of lymphocytes (Fig. 3.1D-F). ES-62 did not prevent the development of splenomegaly or lymphadenopathy and may even have exacerbated it, as the spleens of 12- and 20- week old MRL/*lpr* mice treated with ES-62 were found, if anything, to contain more cells, although lower total numbers of renal cells were found at 12-weeks in the kidney (Fig. 3.4A & B).

Dysregulation of T cell responses have been strongly implicated in the pathology of SLE reflecting the ability of T cells to orchestrate immune responses(73). Consistent with this, characterization of the dynamics of $CD3^+CD4^+$ helper T cell, $CD3^+CD8^+$ cytotoxic T cell and $CD3^+CD4^-CD8^-B220^+$ double negative (DN) T cell populations throughout the course of the MRL/*lpr* model (Fig. 3.4C) revealed that whilst the proportion of $CD4^+$ and $CD8^+$ T cells declined throughout the course of disease, despite their numbers remaining relatively constant, the proportion and number of DN T cells, drastically increased from week 13 such that by week 20, DN T cells represented ~80% of the lymphocyte population (Fig. 3.4D & E). This expansion of the DN T cell population was therefore the major cause for the lymphadenopathy and splenomegaly observed in these mice (Fig. 3.1D-F) and the onset of their expansion correlated with the development of proteinuria (Fig. 3.2).

Interestingly, therefore, prior to the onset of disease (at 12 weeks of age) the levels of $CD4^+$, $CD8^+$ (number) and $\gamma\delta$ (proportion and number) T cells were reduced in the pLN of ES-62 treated mice (Fig. 3.5A). The proportion and number of $CD4^+$ and $CD8^+$ T cells were also reduced in the rLN of 12-week MRL/*lpr* mice treated with ES-62 whereas the proportion of DN T cells was enhanced (Fig. 3.5B). None of these effects, however, were observed in either the peripheral lymph nodes (pLN) or renal lymph nodes (rLN) by 21-weeks (Fig. 3.5C & D).

Nevertheless, these data are perhaps suggestive of ES-62 modulating the early initiation of pathogenic T cell responses; interestingly, both $CD4^+$ and $\gamma\delta$ T cells have been proposed to mediate pathogenic IL-17A responses in lupus (58,360,361). Despite significantly increasing the number of DN T cells at 21-weeks, treatment with ES-62 did not appear to affect T cells in the spleen either prior to or during the onset of disease (Fig. 3.6A & B). Moreover, although no differences were apparent at 12-weeks (Fig. 3.6C), exposure to ES-62 tended to decrease the levels of all the T cell populations infiltrating the kidneys at 21-weeks, although this only reached significance in terms of the proportions of DN and gd T cell (Fig. 3.6D). Interestingly, the reduction of the T cell and $B220^+CD3^-$ B cell populations in the kidney at 21-weeks was associated with a tendency to observe increased proportions of $CD4^+$, $CD8^+$ and DN T cells in the blood and bone marrow of ES-62 treated mice, supporting the possibility that the parasite product suppressed the migration of lymphocytes to sites of inflammation (Fig. 3.7). Furthermore, ES-62 appeared to reduce the proportion of $B220^-CD3^+CD8^{low}$ cells, which may either represent a population of activated cytotoxic T cells(362) or a population of MHC class I restricted T cells found in B cell follicles(363). These cells were significantly increased in the spleens of ES-62 treated 21-week MRL/*lpr* mice (mean % of live cells ± SEM: PBS, 0.5 ± 0.07; ES-62, 0.77 ± 0.09; p=0.044) whilst being significantly decreased in the kidneys (mean % of live cells ± SEM: PBS, 0.82 ± 0.23; ES-62, 0.19 ± 0.04; p=0.021).

3.2.3. ES-62 modulates the levels of lineage negative cells in the MRL/*lpr* mouse

There has been increasing recognition in recent years of the importance of innate lymphocytes(364) and this has led to the identification of novel classes of lymphocytes (LTi, ILC1, ILC2, ILC17 and ILC22) delineation of their roles in the immune system(364). During this investigation, in addition to modulating T cell dynamics, it became clear that exposure to ES-62 also modulated cells in the lymphocyte gate that were negative for lineage specific markers ($CD3^-CD19^-CD64^-CD11b^-$) and are likely to represent populations of innate lymphoid cell (ILC). Interestingly, therefore, it has became apparent over the past 4 years that ILC appear to be involved in the regulation of immune-mediated disease (364) and indeed, studies with SLE patients have suggested they may play a protective role

in Lupus (50). The dynamics of 3 potential ILC populations based on their Lin^- status in concert with their expression of CD25 and/or CD127 were characterized in the MRL/*lpr* mice. Two small populations that were either $Lin^-CD25^+CD127^-$ (ILC2-like) or $Lin^-CD25^-CD127^+$ (LTi-like) were detected in the blood of MRL/*lpr* mice but no clear population of $Lin^-CD25^+CD127^+$ (natural helper- or neuocyte-like) cells was found (364,365). Nevertheless, all of these populations were found at reduced levels in the blood of ES-62 treated mice (Fig. 3.8A & B). By contrast, although all three of these populations were also detectable in the bone marrow, their levels did not appear to be modulated by the parasite product (Fig. 3.8C & D).

Likewise, all three Lin^- populations were detectable in the spleen, pLN, rLN and kidneys of 12- and 21-week old MRL/*lpr* mice. Prior to the onset of disease, at 12-weeks), the proportions of Lin^-CD25^+ and Lin^-CD127^+ cells were reduced in the spleen, pLN and rLN of ES-62 treated mice but not in the kidney (Fig. 3.9A-D), perhaps suggesting that they were involved in the initiation of pathogenesis. By contrast, whilst these populations were not significantly changed in the spleen, pLN and rLN of 21 week old mice (Fig. 3.9E-G), in the kidney, the proportions of the Lin^-CD127^+ and $Lin^-CD25^+CD127^+$ populations were significantly reduced in mice exposed to ES-62 (Fig. 3.9H) suggesting that these cells may also play a pathogenic role in the kidney during established disease; reflecting their suppression by ES-62 observed in the spleen and pLN at 12-weeks (albeit the $CD25^+CD127^+$ populations were not significantly reduced) rather than a defect in migration.

3.2.4. ES-62 does not appear to modulate IFNγ production

To address whether the ES-62-mediated modulation of T cell dynamics reflected modulation of immune phenotype, the effects of exposure to ES-62 on cytokine production by MRL/*lpr* mice were assessed. IFNγ producing Th1 cells have traditionally been associated with the pathogenesis of SLE and IL-12p40 appears essential for the development of proteinuria in the MRL/*lpr* mouse (366). However, preliminary data on the dynamics of IFNγ production throughout the MRL/*lpr* model in mice treated with or without ES-62 suggested that the parasite product did not modulate IFNγ production (McGrath and Harnett, unpublished data). To confirm this, the effect of ES-62 on the production of IFNγ in the pLN, rLN and kidneys of

21-week old MRL/*lpr* mice was determined (Fig. 3.10A-D) and indeed, no significant effects of ES-62 were detected (Fig. 3.10B-D).

3.2.5. IL-17A may have a dual role in pathogenesis in the MRL/*lpr* mouse and is modulated by ES-62

Since the study by Kikawada *et al.* which revealed that IL-12p40 was essential for the development of disease in MRL/*lpr* mice, leading to the proposal of a key role for IFNγ in pathogenesis of lupus, it has become apparent that IL-12p40 is also a component of IL-23, a cytokine required for the maintenance of IL-17A producing cells (91,366). Interestingly, therefore, IL-17A-producing cells have recently been implicated in the pathogenesis of SLE (113,367) and can be found in the kidneys of both lupus-prone mice and SLE patients (359). Furthermore, IL-23 stimulated IL-17A producing T cells from $B6^{lpr/lpr}$ mice can transfer lupus-like renal disease to $RAG^{-/-}$ mice (112).

Preliminary data had suggested that ES-62 suppressed a spike in IL-17A prior to the onset of disease and it was hypothesized that this may act to initiate pathogenesis (McGrath & Harnett, unpublished data). This was confirmed as prior to the onset of disease, in 12-week old MRL/*lpr* mice, ES-62 reduced the levels of IL-17A producing cells in the pLN and rLN (Fig. 3.11A & B). At this stage, no IL-17-producing cells were detected in the kidney (data not shown). Surprisingly, however, analysis at 21-weeks revealed that treatment of MRL/*lpr* mice with ES-62 increased the proportion and number of IL-17A producing cells in the pLN (Fig. 3.11C) but not the rLN or kidney (Fig. 3.11D & E). Nevertheless, the concentration of IL-17A was significantly elevated in the renal interstitial fluid of ES-62 treated mice (Fig. 3.12A) and IL-17A was detected in the renal interstitial fluid from MRL/MP mice although this was significantly less that in that of the MRL/*lpr* mice. IL-17E and IL-17F were also detectable in the renal interstitial fluid of 21-week MRL/*lpr* mice however, no difference was observed between mice treated with or without ES-62 (data not shown). Interestingly, the number of $IL\text{-}17^+$ lymphocytes was significantly increased in asymptomatic 21-week MRL/MP mice compared to age-matched MRL/*lpr* mice (Fig. 3.12B); however there was no correlation between proteinuria and IL-17 production (Fig. 3.12C).

Collectively, these data suggested a potential dual role for IL-17A in the MRL/*lpr* mouse, as ES-62 mediated protection against disease was associated with reduced IL-17A production prior to the onset of pathology and elevated IL-17A production during established disease. To explore this theory IL-17A responses were neutralized by treatment with an anti-IL-17A neutralizing antibody from 7-12 weeks of age whilst recombinant IL-17A (rIL-17) was administered from 12-21 weeks of age; replicating the effect of ES-62 on enhancing late IL-17A production. Consistent with a dual role for IL-17A, the administration of rIL-17A from 12-weeks resulted in significantly reduced proteinuria levels whilst although it did not prevent development of proteinuria, the neutralization of early IL17A production slowed the onset and reduced severity of the early phase of disease (Fig. 3.13A). Thus it appeared that IL-17A contributed to pathogenesis prior to onset of proteinuria yet acted to reduce the severity of inflammation during established disease in the MRL/*lpr* mouse (Fig. 3.13A). The inability of anti-IL-17A to fully suppress the development of proteinuria may suggest that IL-17A is not sufficient for pathogenesis and/or reflect the long half-life of the antibody, which as it was administered up until 12 weeks, may also have blocked some of the protective effects resulting from IL-17A produced during the established phase of disease. Nevertheless, treatment with anti-IL-17A or rIL-17A did not appear to substantially affect either renal histology or the production of ANA (Fig. 3.13B & C).

3.2.6. IL-22 is essential for the development of proteinuria in the MRL/*lpr* mouse

These IL-17-related data are therefore somewhat at odds with recent studies by Kyttaris *et al.* showing that early neutralization of IL-23 suppresses disease in the MRL/*lpr* mouse whilst IL-23R deficiency prevents disease development in the $B6^{lpr/lpr}$ model of SLE (368,369). However, IL-23 is not only crucial for the maintenance of IL-17A producing cells, but is also important for production of IL-22 by cells of both the adaptive and innate immune systems (126). Thus, the neutralization of IL-23 that was shown to suppress disease in MRL/*lpr* mice (368,369) may also affect IL-22 production, suggesting that IL-22 may have a pathogenic role in SLE that may be modulated by ES-62 (137,138). Preliminary data from this lab identified that cells produced either IL-17A or IL-22 exclusively, and double producing cell populations were not detected *in vivo* (M^{c}Grath and

Harnett unpublished observations). IL-22 producing cells were therefore analyzed independently of other cytokine production. Prior to the onset of disease, as with IL-17, ES-62 reduced the levels of IL-22-producing cells in the pLN and rLN of MRL/*lpr* mice (Fig. 3.14A & B). However, ES-62 also reduced the proportion of IL-22 positive cells in the pLN of 21-week old mice (Fig. 3.14C). Although the levels of IL-22-producing cells were not significantly reduced in the rLN (Fig. 3.14D), those in the kidneys of MRL/*lpr* mice were significantly reduced in mice treated with ES-62 (Fig. 3.14E). The concentration of IL-22 in the renal interstitial fluid of ES-62 treated mice was significantly reduced and was undetectable in the renal interstitial fluid from MRL/MP mice (Fig. 3.15A). Furthermore, the number of IL-22-producing lymphocytes in the pLN positively correlated with proteinuria in MRL/*lpr* mice whilst IL-22-producing cells were not detectable in age-matched MRL/MP mice (Fig. 3.15B & C).

Taken together, these data suggested that IL-22 was associated with pathogenesis in the MRL/*lpr* mouse. To test this theory recombinant cytokines and neutralizing antibodies were employed: as in preliminary experiments IL-22 production was not detectable in the serum of MRL/*lpr* mice prior to the onset of disease, both anti-IL-22 and rIL-22 were administered from 12-21 weeks of age. Administration of recombinant IL-22 indeed significantly accelerated and exacerbated disease whereas neutralization of this cytokine suppressed the development of proteinuria: collectively, these data are consistent with the proposal that IL-22 is sufficient and essential for the development of renal disease in the MRL/*lpr* mouse (Fig. 3.16A). Despite its profound effects on proteinuria, treatment with neutralizing anti-IL-22 antibodies, or rIL-22, had no significant affect on renal histology or ANA production (Fig. 3.16B& C).

3.2.7. Analysis of the phenotype of the cytokine producing cells

As reported above, ES-62 clearly modulated the capacity of cells to produce various cytokines in MRL/*lpr* mice: however, it was unclear whether this modulation reflected a general suppression of cytokine production or targeting of specific cell populations. Although IFNγ was largely unaffected by ES-62 (Fig. 3.10), the proportion of $IFN\gamma^+$ DN T cells was reduced in pLN and rLN of ES-62 treated mice (Fig. 3.17A-C); whilst in the pLN, the proportion of $IFN\gamma^+$ Lin^- cells

were elevated significantly (Fig. 3.17A & B). Moreover, although only a small component, the proportion of IFNγ-producing $CD4^+$ T cells was significantly decreased in the kidneys of ES-62 treated mice (Fig. 3.17D).

By contrast, prior to the onset of disease, the levels of IL-17-producing cells were significantly decreased in ES-62 treated MRL/*lpr* mice (Fig. 3.11). Whilst the conventional IL-17A-producing populations, $CD4^+$ (Th17) and DN T cells, were elevated in the pLN, and perhaps in the rLN, of these mice; the proportion of B cells, NK cells, γδ T cells and ILC were significantly reduced and likely accounted for the overall decline in IL-17A production (Fig. 3.18A&B). The proportion of IL-17^+ cells in the pLN of PBS treated 21-week MRL/*lpr* mice (mean ± SEM; 1.72 ± 0.27) was significantly reduced ($p=<0.0001$, student's t-test) compared to that of the PBS treated 12-week old mice (mean ± SEM; 20.36 ± 0.96); and surprisingly, the proportion and number of IL-17^+ cells was elevated in the pLN but not the rLN of ES-62 treated mice (Fig. 3.11C&D). This increase in IL-17A-producing cells in the pLN appears to be due to a significant rise in the levels of IL-17^+Lin^- cells (Fig. 3.18C) that was mirrored by a similar increase in the rLN (Fig. 3.18D). However, in the rLN, and consistent with their proposed role in initiating pathogenesis (112,359), the proportion of Th17 cells was decreased (Fig. 3.18D) perhaps suggesting that their elevated levels in the LN of 12-week old mice (Fig. 3.18B & C) represented their sequestration away from the site of inflammation. During established disease IL-17^+ cells were detectable in the kidneys of MRL/*lpr* mice (Fig. 3.11E) and at similar levels to those previously reported by Tsokos and colleagues (112). Treatment with ES-62 did not appear to modulate the overall proportion of IL-17^+ cells in the kidney (Fig. 3.11E), perhaps reflecting that although it did not reach significance, the increase in IL-17-producing DN T cells would likely cancel out the significant decrease in the proportion of IL-17^+Lin^- cells (Fig. 3.18F). Although ILC have been proposed to be protective in SLE, these data could suggest that IL-17^+ILC may be pathogenic and that their reduction in the kidney may reflect their ES-62-mediated retention in the pLN and rLN (Fig. 3.18D & E) to sequester them from the site of inflammation. These data suggest that IL-17^+Lin^- may be a pathogenic source of IL-17A in the MRL/*lpr* mice.

In contrast to what was observed with IL-17, ES-62 suppressed the levels of IL-22-producing cells both prior to the onset of pathology and during established disease. The early suppression of IL-22 production appeared to be due to a significant reduction in the proportion of the ILC populations, IL-22$^+$ Lin$^-$ and IL-22$^+$NK cells, in the pLN, and CD4$^+$ Th22 cells in the rLN (Fig. 3.19A-B): whereas the cells targeted in both pLN and rLN during established disease were DN T cells, which constitute the predominant source of this cytokine at this stage (Fig. 3.19D & E). By contrast, the major phenotype of IL-22$^+$ cells in the kidney was that of Lin$^-$ ILC-like cells: however, ES-62 did not appear to significantly modulate the proportion of any particular IL-22$^+$population, but rather appeared to suppress levels overall (Fig. 3.19F).

The expansion of the DN T cell population correlates with the development of proteinuria in the MRL/*lpr* mouse. These cells have also been identified in SLE patients and found to infiltrate the kidney where they produce IL-17 (359). Interestingly, therefore, exposure to ES-62, albeit not significantly, increased the levels of such IL-17-producing cells in kidney during established disease suggesting, that in keeping with previous reports (112,359), DN could play a protective role in limiting inflammation. However, DN T cells also appear to be a major potential source of IL-22 in MRL/*lpr* mice that is targeted by ES-62 (Fig. 3.19). As DN T cells produce either IL-17A or IL-22, they may comprise a functionally heterogeneous group, a proposal that may go some way to resolving the contradictory evidence about their role in the MRL/Lpr mouse (370). Nevertheless, as the total DN T cell population was elevated in the spleen and blood of ES-62 treated mice but reduced in the kidneys (Fig. 3.6 & 3.7), these data suggest that ES-62 may have been targeting a pathogenic DN T cell population or inducing a switch in their functional phenotype. To address this, DN T cells were adoptively transferred from the pLN of 21-week old PBS or ES-62 treated MRL/*lpr* mice into 7-week old recipient MRL/*lpr* mice: no significant effect of DN T cells from either PBS- or ES-62-treated mice could be observed in terms of proteinuria relative to that observed in mice simply receiving PBS i.v. (Fig. 3.20).

3.3. Discussion

Parasitic helminths can survive within the host for up to 10 years; one strategy that enables them to do so is the secretion of molecules that modulate the immune system. Such immunomodulatory molecules may have therapeutic potential in inflammatory diseases where the hyper-activation of the immune system results in damage to the host, such as allergy and autoimmunity (311). ES-62 is one such immunomodulatory glycoprotein, secreted by the murine filarial nematode *A. viteae,* that has been shown to modulate the immune system via TLR4 and MyD88 dependent mechanisms (322) and can suppress pathogenesis in mouse models of rheumatoid arthritis (335,336) and asthma (228,305,335). ES-62 mediated protection, in both of these pathologies, is associated with modulation of the T helper cell balance by suppression of IL-17A production(336,338). Although SLE exhibits an ambiguous immunological phenotype (63,73), Th17 and γδ T cell-derived IL-17A has been implicated in disease pathogenesis both in mouse models and also in SLE patients (111) and thus, the therapeutic potential of ES-62 was investigated in the MRL/*lpr* mouse model of SLE.

The major pathology in MRL/*lpr* mice is lupus nephritis (14) which is monitored experimentally by measurement of proteinuria. Prophylactic treatment with ES-62 (twice-weekly from 7-weeks of age) was found to significantly reduce the development of proteinuria in MRL/*lpr* mice. Rather surprisingly, therefore, it did not appear to substantially improve renal histology in terms of inflammation, cellular infiltration or the deposition of antibody and complement. Although this was surprising given the drastic effects on proteinuria levels that were observed, it was consistent with other studies in the literature which similarly failed to show global improvements in histology (112,244). Nevertheless, analysis of infiltrating cells extracted from whole kidneys revealed that the migration of certain perhaps pathogenic populations into the kidneys of MRL/*lpr* mice might be prevented by treatment with ES-62. Supporting this blocked migration theory, whilst the proportions of lymphocytes and granulocytes circulating in the blood were elevated in ES-62-treated mice; these cells were reduced in the kidneys. Unfortunately, the effect of ES-62 on the production of chemokines and/or the expression of their receptors was not comprehensively evaluated in these MRL/*lpr* mice: however,

preliminary findings suggested that ES-62 increased CD69 expression (data not shown), which may act as a retention marker for lymphocytes (371); and reduced expression of CD44 on γδ T cells (336), a marker that is associated with lymphocyte migration *in vivo*. Furthermore, the expression of CXCR5 on B cells was reduced in ES-62 treated MRL/*lpr* mice (see chapter 4).

ES-62 suppresses the development of pathogenic IL-17A responses in collagen-induced arthritis and ovalbumin-induced airway hypersensitivity (336,338). Thus, as IL-17A had also been strongly linked to SLE pathogenesis and the development of lupus-like disease in mice, it was hypothesized that the protection afforded by ES-62 might reflect suppression of the pathogenic effects of IL-17A in the MRL/*lpr* mouse. Recent studies using mice deficient in IL-23, a crucial cytokine in the maintenance of Th17 development supported the proposed pathogenic role of such cells in the development of lupus-like disease in the MRL/*lpr* and $B6^{lpr/lpr}$ mice(368,369): however, IL-23 was not consistently detected in the serum or renal interstitial fluid of MRL/*lpr* mice (results not shown) and consequently the effect of ES-62 on IL-23 production or function in the MRL/*lpr* mouse remains unknown. Nevertheless, consistent with these studies, ES-62 suppressed IL-17A and IL-22 responses prior to the onset of disease. By contrast, ES-62 also suppressed IL-22, but not IL-17A responses, during established disease perhaps suggesting that protective effects of IL-23 deficiency were predominantly targeting IL-22 responses.

The role of IL-22 in human SLE patients has not yet been established as whilst some reports conclude that IL-22 is associated with pathogenesis (137-139), others report a negative correlation with disease activity (143,144) or suggest that IL-22 levels may be predictive of specific pathologies, such as tissue involvement, in certain SLE patient groups (142). Interestingly, IL-22 is reduced in SLE patients treated with hydroxychloroquine (140), a report consistent with the finding that IL-22 is required for pathogenesis in the MRL/*lpr* mouse.

Although IL-22 has been proposed to exhibit dual roles in some inflammatory diseases (118,372), this is unlikely to be the case in the MRL/*lpr* mouse as IL-22

production is not detectable in MRL/MP mice and treatment with ES-62 reduced IL-22 production both prior to and following onset of disease.

As DN T cells were the major IL-22-producing population that was targeted by ES-62 during established disease, these cells were adoptively transferred into young MRL/*lpr* mice on the basis that those from PBS-treated mice might accelerate and/or exacerbate onset of pathology whilst those from ES-62 might potentially ameliorate disease when compared to those mice receiving only PBS. Rather surprisingly, no significant differences were detected amongst the 3 groups although it appeared that the onset of proteinuria was slightly slower and less severe in mice receiving either set of DN T cells. Although difficult to interpret, these data, which exhibited a rather remitting and relapsing profile especially with respect to the DN T cells from PBS-treated, presumably reflected the heterogeneous (functional) phenotype of DN T cells in MRL/*lpr* mice. Moreover, and quite surprisingly, it proved difficult to further purify this abundant population of cells with the post-sort cohort of cells from ES-62-treated mice in particular showing substantial B cell contamination, perhaps indicating some DN T cell-B cell interactions in these mice.

At first sight, the finding that IL-17A may potentially exhibit a protective role in the MRL/*lpr* mouse contradicts the general perception of a solely pathogenic role for IL-17A in SLE (103,373,374). However, although levels of IL-17A have been widely shown to be elevated in SLE patients, very few of these studies have concluded that IL-17A positively correlates with SLEDAI (114,375-378). Rather, more studies have concluded that IL-17A levels do not correlate with SLEDAI or indeed, are inversely correlated with disease score (379-386). Consistent with these latter findings, SLE patients treated with corticosteroids exhibit increased IL-17A production (387). Moreover, Szeto and colleagues, not only reported that elevated levels of IL-17A mRNA in the urine of SLE patients inversely correlated with disease severity, but also that such IL-17A mRNA levels declined in the urine of SLE patients in the weeks preceding a lupus flare (388,389). Collectively, these data provide compelling evidence for a protective role for IL-17A in SLE.

Further evidence of a dual role for IL-17A in kidney disease can be found in the field of renal transplant rejection, where it is widely accepted that IL-17A is responsible for acute graft rejection (390) as high levels of IL-17A are associated with end stage renal failure (391-396). Moreover, mouse models of acute renal injury have provided experimental evidence that IL-17A drives renal damage (397-399). For example, in acute renal graft rejection, neutrophils are the major population of IL-17A-producing cells (400) and it appears that these cells are required for the recruitment of IFNγ-producing neutrophils (98) that drive inflammation during the late stage leading to rejection; perhaps counter-intuitively, at this stage IL-17A appears to act to limit and resolve inflammation by suppressing IFNγ production (401,402). These findings may therefore go some way to explaining the proposed dual role of IL-17A in the MRL/*lpr* mouse as the data are consistent with pathogenic IL-17A responses driving initiation of autoimmune responses, perhaps by recruiting and/or inducing IL-22-producing cells, as indicated by the slower kinetics of serum IL-22 kinetics (McGrath & Harnett, unpublished data). Interestingly, whilst 21-week-old MRL/MP mice displayed little or no evidence of IL-22-producing cells or serum IL-22, these mice had high levels of IL-17A producing cells and serum IL-17A (McGrath, Harnett & Harnett, unpublished data).

Another mechanism by which IL-17A may be protective in SLE is through the promotion of germinal center formation and the production of long-lived plasma cells (403) rather than the GC-independent short-lived plasma cells, or plasmablasts, that produce anti-dsDNA antibodies and are associated with flare responses in SLE patients (3,195,226). Consistent with this, the decreased levels of IL-17A mRNA, found in the urine of SLE patients preceding flares, inversely correlate with anti-dsDNA titers (388). Thus, prior to the onset of disease, IL-17A may contribute towards pathogenesis by driving the development of the germinal centre reaction and the generation of self-reactive B cells, whilst during the later stages of disease, IL-17A may act to suppress the extra-follicular development of circulating anti-dsDNA specific memory B cells into short-lived plasma cells, thus preventing the generation of pathogenic anti-dsDNA responses (see chapter 4). As IL-22R is expressed by stromal cells in the germinal centre dark zone (404) IL-17A and IL-22 may cooperate in the initiation of GC responses. Rather surprisingly

therefore, treatment with anti-IL-17, rIL-17, anti-IL-22 or rIL-22 did not appear to have an effect on ANA production.

Nevertheless, and regardless of the relevance of these data to the findings with human disease, at first sight the findings presented here appear to conflict with previous reports on the role of IL-17A in mouse models of SLE. For example, Tsokos and colleagues showed that $Rag^{-/-}$ mice, which received IL-23 stimulated T cells from $B6^{lpr/lpr}$ mice, developed a lupus like disease that was associated with IL-17^{+} cells in the kidney (112). Furthermore, Ro52-deficiency in C57BL/6 mice results in the development of a lupus-like disease that is IL-23/IL-17A dependent (16); and the deletion of Akt1 in $Fc\gamma RIIb^{-/-}$ mice prevented IL-17A signaling and suppressed the development of renal inflammation (113). All of these studies utilized parental strains of mice that do not spontaneously develop a lupus-like disease and have no genetic predisposition to SLE other than the *lpr* mutation or engineered deficiency in FcγRIIb or Ro52. Although IL-17A is elevated in mice that spontaneously develop lupus-like disease, it is yet to be shown conclusively that IL-17A is associated with pathogenesis in these mice. It may be, therefore, that genetic factors influence the role of IL-17A in mouse models of SLE and that those focusing on the effect of IL-23 in lupus-prone mice may have overlooked the role of IL-22 and consequently overstated the importance of IL-17A (112,368,369).

A recent study by Kuchroo and colleagues has comprehensively mapped the genes involved in the activation of Th17 cells (91) and suggested that complete activation of pathogenic Th17 cells required FAS signaling, although it is important to note that these observations were made in an *in vitro* setting and that such incompletely activated cells produced IL-17A as well as IL-10 but not IFNγ. These findings might suggest that pathogenic Th17 can cells not develop into pathogenic Th17 cells in MRL/*lpr* or $B6^{lpr/lpr}$ strains of mice, and that Th17 cells are unlikely to be the cellular source of pathogenic IL-17A in the MRL/*lpr* mouse. Indeed, at 12-weeks of age, ES-62 increases the proportion of Th17 cells despite an overall reduction in the levels of IL-17A-producing cells including B, NK or Lin^{-} cells. Furthermore, despite a clear modulation of IL-17A levels in the renal interstitial fluid by ES-62, there was no apparent decrease in the levels of PMA/iononomycin-stimulated IL-17A-producing cells, perhaps suggesting that the cellular source of

IL-17A in the kidney may be neutrophils, which undergo apoptosis in response to this stimulus and have been reported to be responsible for the IL-17-mediated resolution of inflammation in the late phase of acute renal disease (401,402). Another potential IL-17A-producing population that appears to be modulated by ES-62 is of the Lin^- phenotype that potentially comprises $CD25^+$ (natural helper cells or neuocytes) or $CD127^+$ (LTi) ILC (364,365).

It is important to remember that these cytokine data represent cell populations undergoing pharmacological stimulation *ex vivo* and hence are not necessarily representative of what is occurring *in vivo*. Thus, in conclusion, ES-62 improves renal function in MRL/*lpr* mice and this may be due to a modulation of IL-17A and IL-22 production. These studies have identified that IL-22 is essential for the development of proteinuria in the MRL/*lpr* mouse and have highlighted a potential dual role for IL-17A in this model.

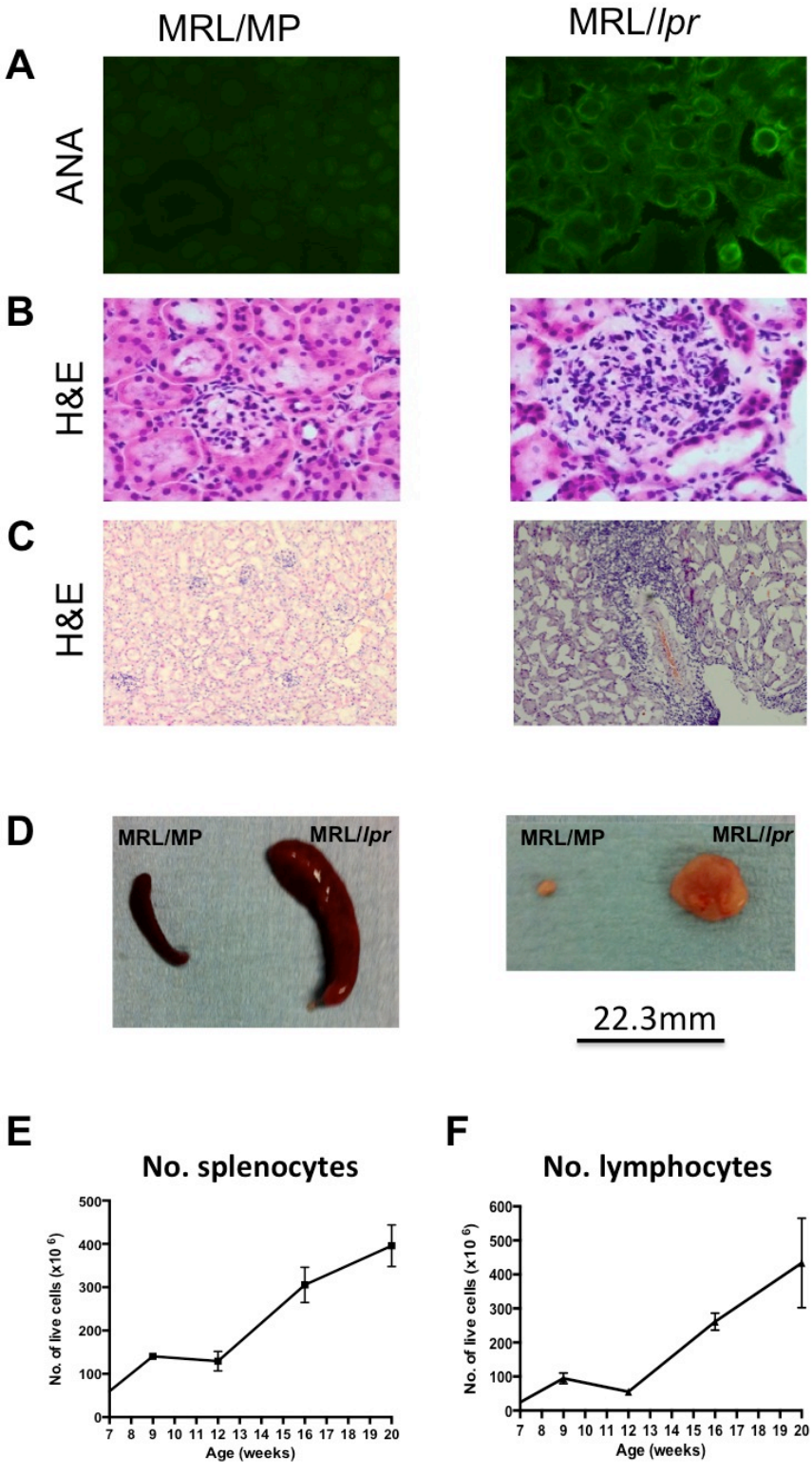

MRL/MP
MRL/lpr
A
ANA
B
H&E
C
H&E
D
MRL/MP
MRL/lpr
MRL/MP
MRL/lpr
22.3mm
E
No. splenocytes
No. of live cells (x10 6)
Age (weeks)
F
No. lymphocytes
No. of live cells (x10 6)
Age (weeks)

Figure 3.1. MRL/*lpr* mice develop a lupus like disease. ANA production in the serum of 21-week old MRL/MP and MRL/*lpr* mice was assessed by analysis of the intensity and pattern of staining of Hep-2 slides (A). Likewise, sections (7 μm) of kidneys from 21-week old MRL/MP and MRL/*lpr* mice were stained with H & E to asses glomerulopathology and cellular infiltration (B & C). Spleens and LN from 21-week old MRL/MP and MRL/*lpr* mice were photographed (D) and the number of splenocytes (E) and lymphocytes (F; from pLN) were counted using a hemocytometer throughout the course of the MRL/*lpr* model.

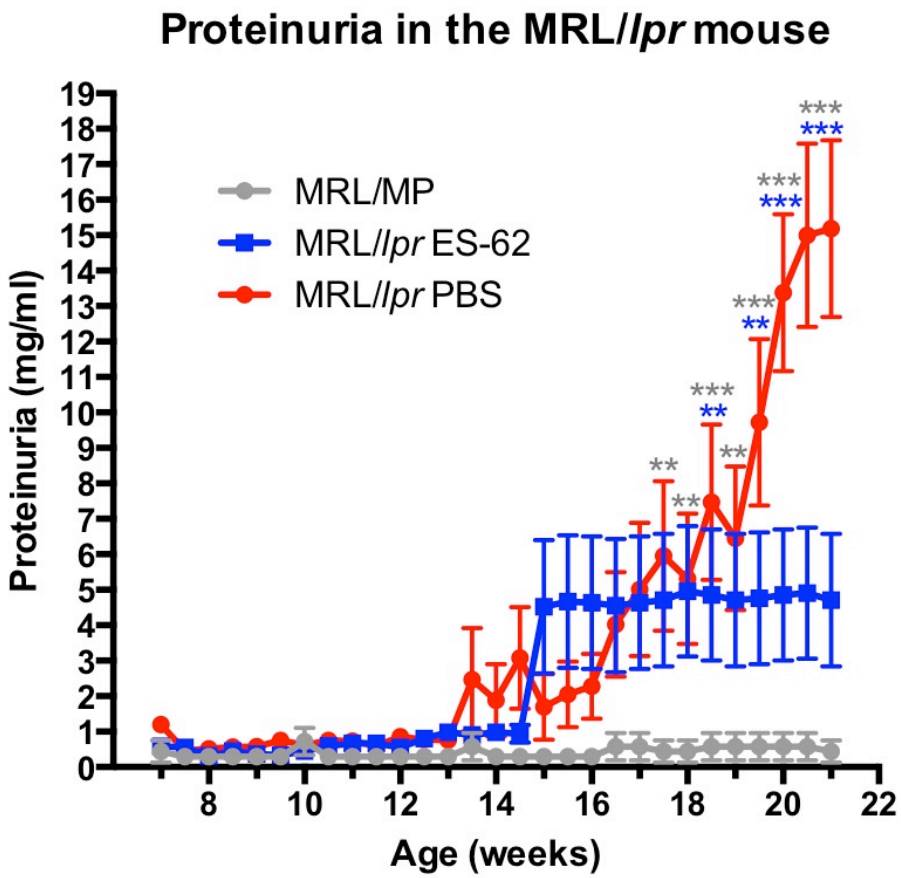

Proteinuria in the MRL/*lpr* mouse
MRL/MP
MRL/*lpr* ES-62
MRL/*lpr* PBS
Proteinuria (mg/ml)
Age (weeks)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
8
10
12
14
16
18
20
22
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Figure 3.2. ES-62 prevents the development of proteinuria in the MRL/*lpr* mouse. MRL/MP (n = 15) or MRL/*lpr* mice were maintained from 7-21 weeks of age. MRL/*lpr* mice were injected subcutaneously twice a week with either PBS (n= 41) or ES-62 (n= 19; 2 μg/mouse). Proteinuria was measured using Siemens Multistix 10 SG. The protective effects of ES-62 are representative of 3 independent experiments collated and the PBS data is representative of 5 independent experiments collated. Statistical significance was determined by 2-way ANOVA using the Tukey post-test analysis. Significance is shown as **=$p<0.001$ and ***=$p<0.0001$ where the colour of the asterisk reflect the group in comparison.

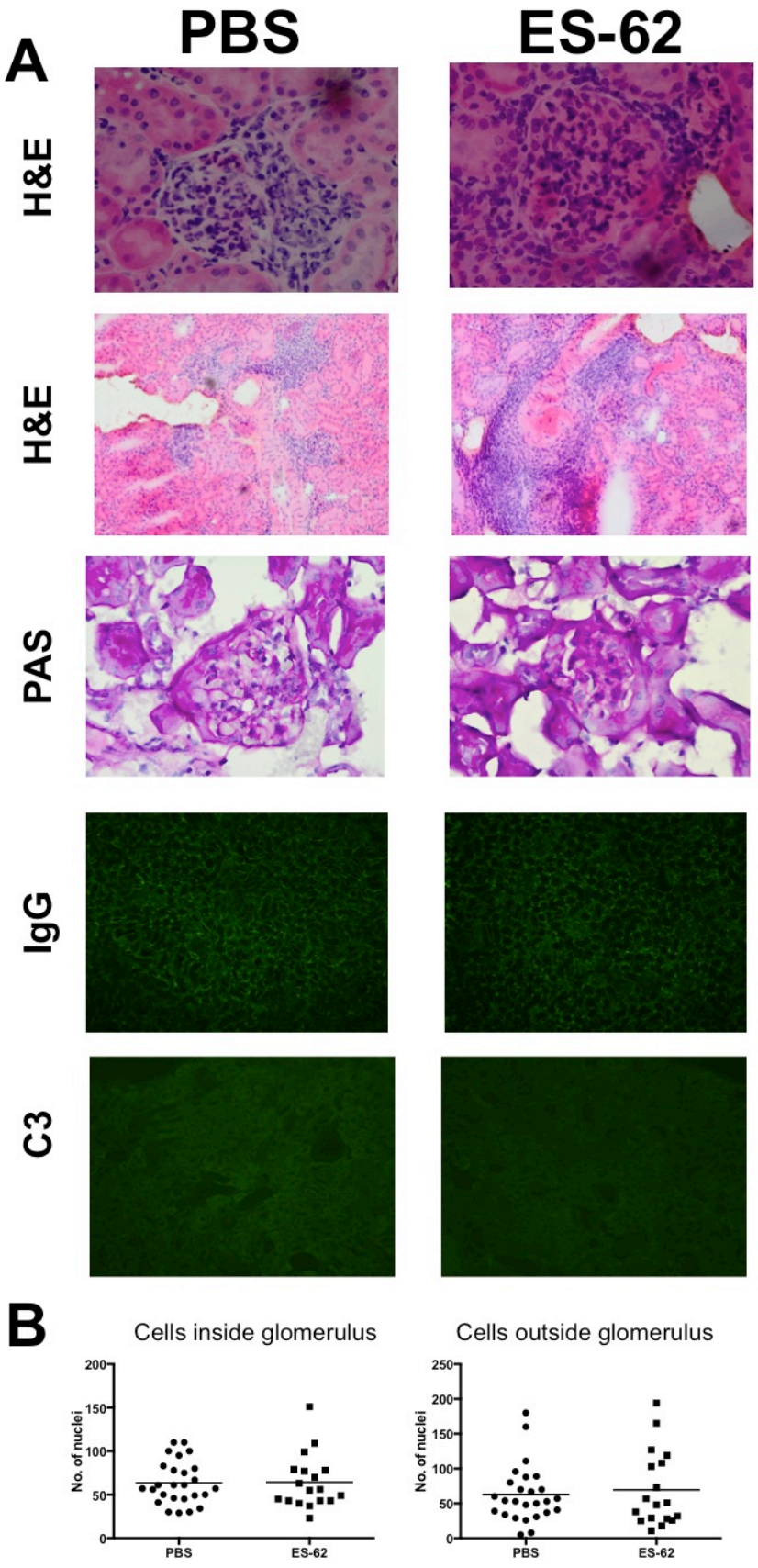
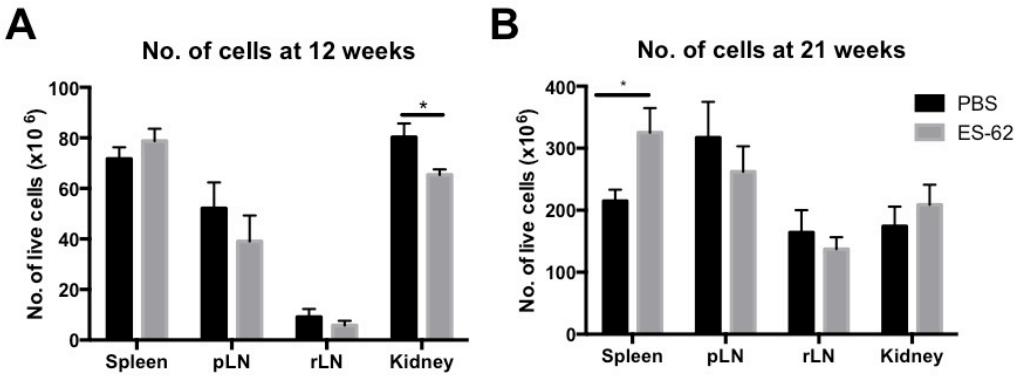
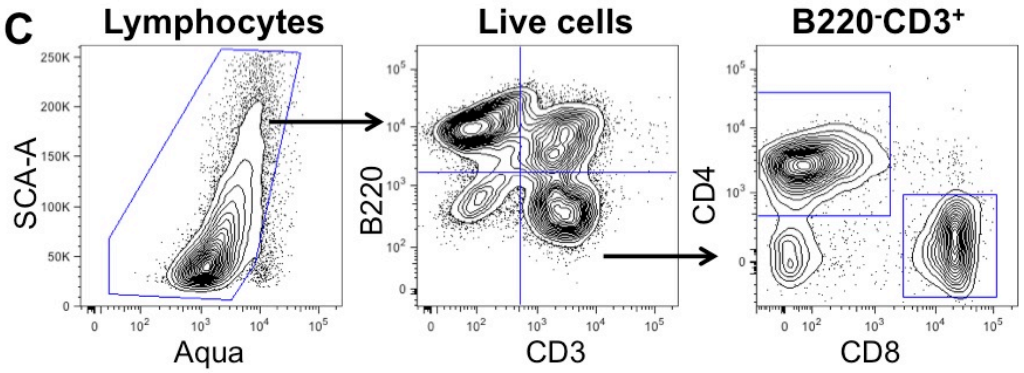

A
PBS
ES-62
H&E
H&E
PAS
IgG
C3
B
Cells inside glomerulus
Cells outside glomerulus
No. of nuclei
200
150
100
50
0
250
PBS
ES-62

Figure 3.3. ES-62 does not improve renal histology in ES-62 treated mice. Kidney sections from 21-week old MRL/*lpr* mice treated with PBS or ES-62 were stained with H & E to determine glomerulonephritis, cellularity and infiltration; PAS to determine basement membrane thickness and FITC conjugated anti-IgG or anti-C3 antibodies to determine immune complex or complement deposition (A). The number of cells inside or outside of the glomeruli from PBS (n=25) or ES-62 (n=18) treated mice were counted (B).


A
No. of cells at 12 weeks
No. of live cells (x10 6)
Spleen
pLN
rLN
Kidney
B
No. of cells at 21 weeks
No. of live cells (x10 6)
PBS
ES-62
Spleen
pLN
rLN
Kidney


C
Lymphocytes
Live cells
B220-CD3+
SCA-A
Aqua
B220
CD3
CD4
CD8

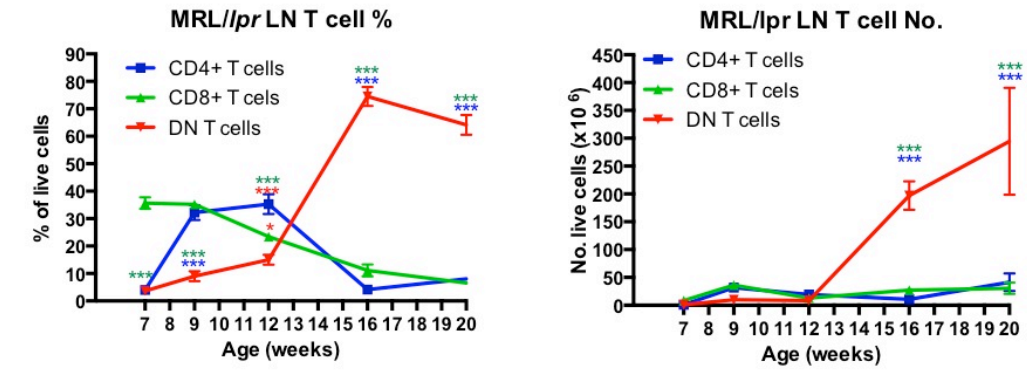

D
MRL/lpr LN T cell %
CD4+ T cells
CD8+ T cels
DN T cells
% of live cells
Age (weeks)
E
MRL/lpr LN T cell No.
CD4+ T cells
CD8+ T cels
DN T cells
No. live cells (x10 6)
Age (weeks)

Figure 3.4. T cell dynamics in MRL/*lpr* mice. The number of cells in the spleens, pLN, rLN or kidneys from MRL/*lpr* mice at 12- (A; PBS n=5, ES-62 n = 5) and 21-weeks (B; PBS, n = 11; ES-62, n = 18) was determined by cell counting using a hemocytometer. The proportions of $CD4^+$, $CD8^+$ and DN T cells were determined by flow cytometry (C + D; n = 5 at each time-point) throughout the time-course of the MRL/*lpr* mouse model. Absolute numbers were determined from total cell counts of the LN (E; n = 5). Statistical significance was determined using the student's t-test (A & B) and two-way ANOVA (D & E): **=$p<0.01$ and ***=$p<0.001$ where the colour of the asterisk reflect the group in comparison.

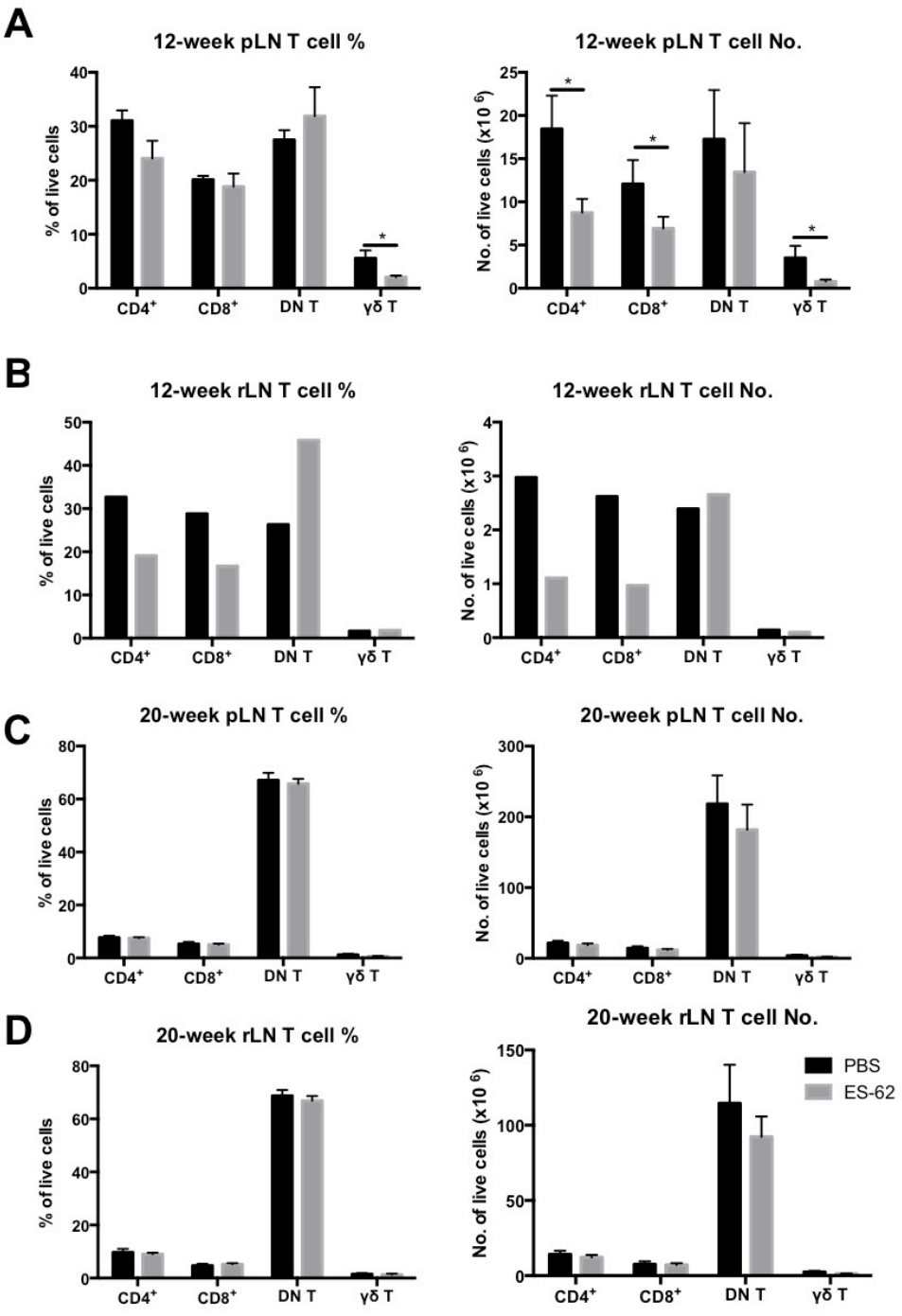

A
12-week pLN T cell %
% of live cells
CD4+
CD8+
DN T
γδ T
12-week pLN T cell No.
No. of live cells (x10 6)
B
12-week rLN T cell %
12-week rLN T cell No.
C
20-week pLN T cell %
20-week pLN T cell No.
D
20-week rLN T cell %
20-week rLN T cell No.
PBS
ES-62

Figure 3.5. ES-62 modulates T cell levels in the LN. The proportion and absolute number of $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, DN T cells and γδ T cells were determined in the pLN (A; 5 mice for each group) and rLN (B; 5 mice pooled for each group) of 12-week old mice; and the pLN (C) and rLN (D) of 21-week mice. Black bars represent PBS treated mice (12-weeks n = 5 & 21-weeks n = 11) and grey bars represent ES-62 treated mice (12-weeks n= 5 & 21-weeks n = 18). 21-week data is pooled from 3 independent experiments whereas the 12-week data is representative of one experiment; statistical significance was determined using the student's t-test (A-D).

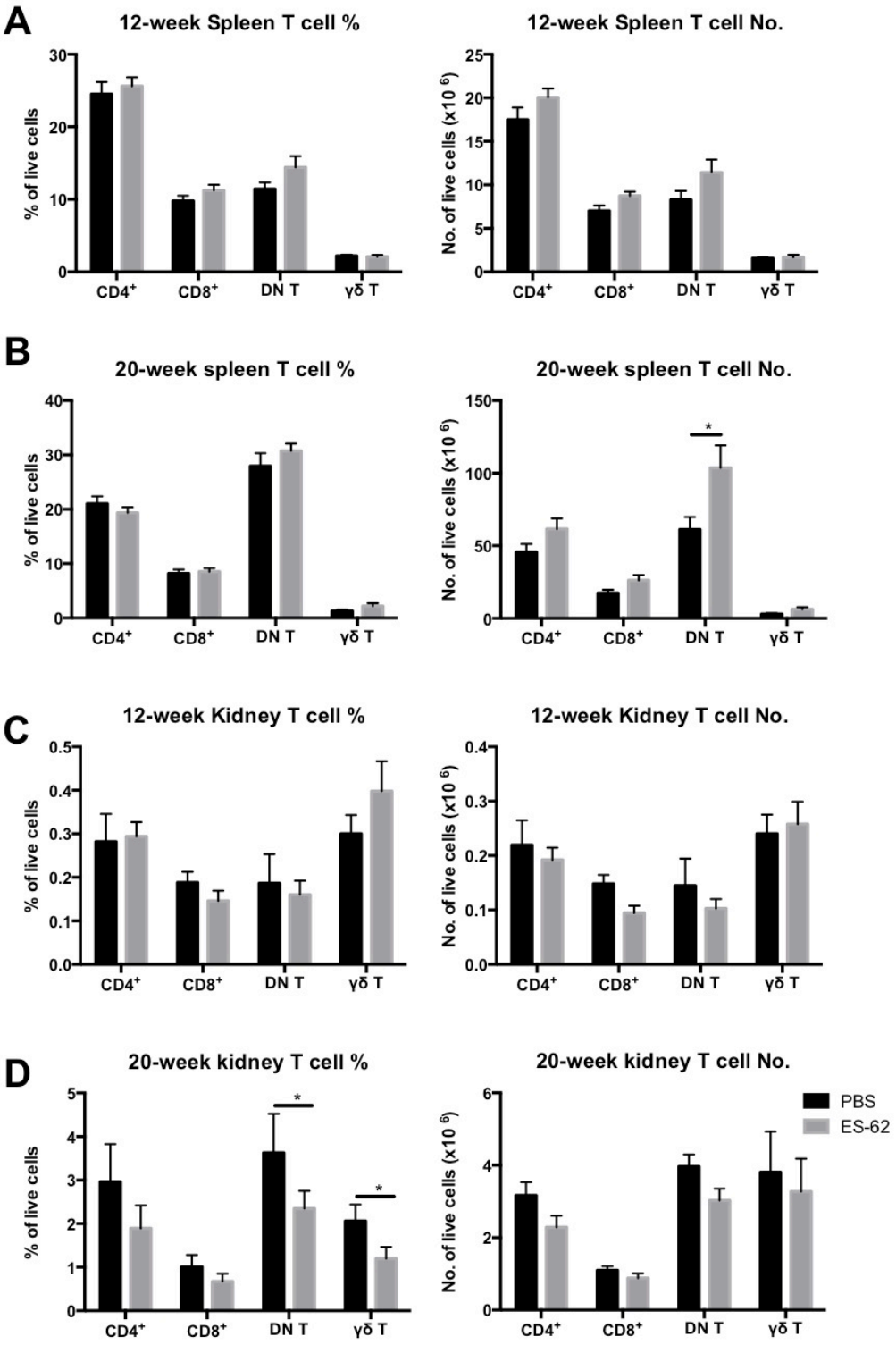

A
12-week Spleen T cell %
% of live cells
CD4+
CD8+
DN T
γδ T
12-week Spleen T cell No.
No. of live cells (x10 6)
B
20-week spleen T cell %
20-week spleen T cell No.
C
12-week Kidney T cell %
12-week Kidney T cell No.
D
20-week kidney T cell %
20-week kidney T cell No.
PBS
ES-62

Figure 3.6. ES-62 modulates T cell levels in the spleen and kidney. The proportion and absolute number of $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, DN T cells and γδ T cells were determined in the spleens of 12-week (A) and 21-week (B) old mice; and the kidneys of 12-week (C) and 21-week (D) old mice. Black bars represent PBS treated mice (12-weeks n = 5 & 21-weeks n = 11) and grey bars represent ES-62 treated mice (12-weeks n= 5 & 21-weeks n = 18). 21-week data is pooled from 3 independent experiments whereas the 12-week data is representative of one experiment; statistical significance was determined using the student's t-test (A-D).

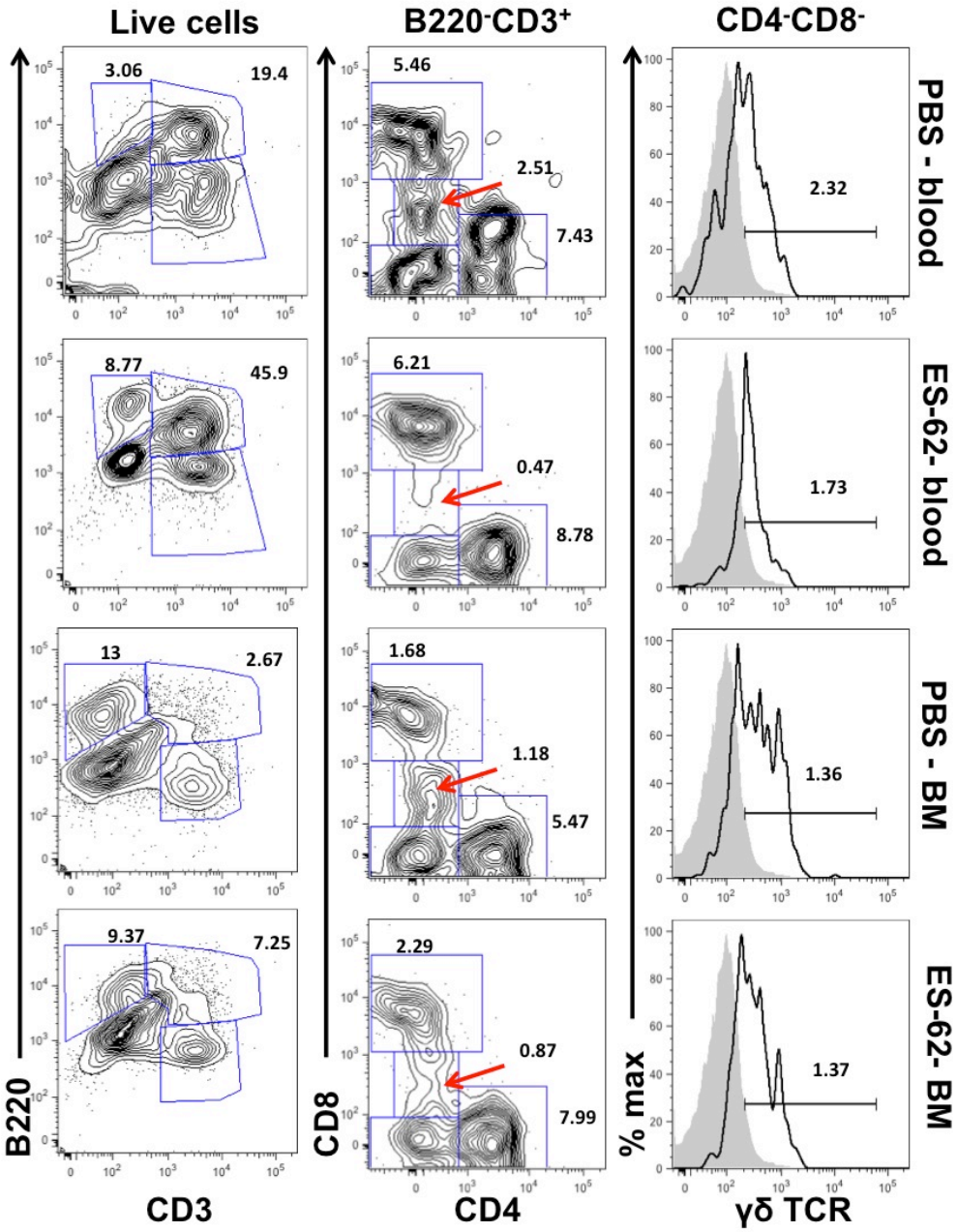

Live cells
B220-CD3+
CD4-CD8-
PBS - blood
ES-62- blood
PBS - BM
ES-62- BM
3.06
19.4
5.46
2.51
7.43
2.32
8.77
45.9
6.21
0.47
8.78
1.73
13
2.67
1.68
1.18
5.47
1.36
9.37
7.25
2.29
0.87
7.99
1.37
B220
CD3
CD8
CD4
% max
γδ TCR

Figure 3.7. T cells are retained in the blood of ES-62 treated mice. The blood and bone marrow (BM) of 4 mice, treated with PBS or ES-62, were pooled before the proportion of $B220^{+}CD3^{-}$ B cells, $CD3^{+}B220^{-}CD4^{+}$ T helper cells, $CD3^{+}B220^{-}CD8^{high}$ and $CD3^{+}B220^{-}CD8^{low}$ cytotoxic T cells, $CD3^{+}B220^{+}$ DN T cells and $CD3^{+}B220^{-}CD4^{-}CD8^{-}\gamma\delta TCR^{+}$ $\gamma\delta$ T cells were determined by flow cytometry. Numbers adjacent to terminal gates refer to the frequency of live cells for each population. Data are representative of two independent experiments.

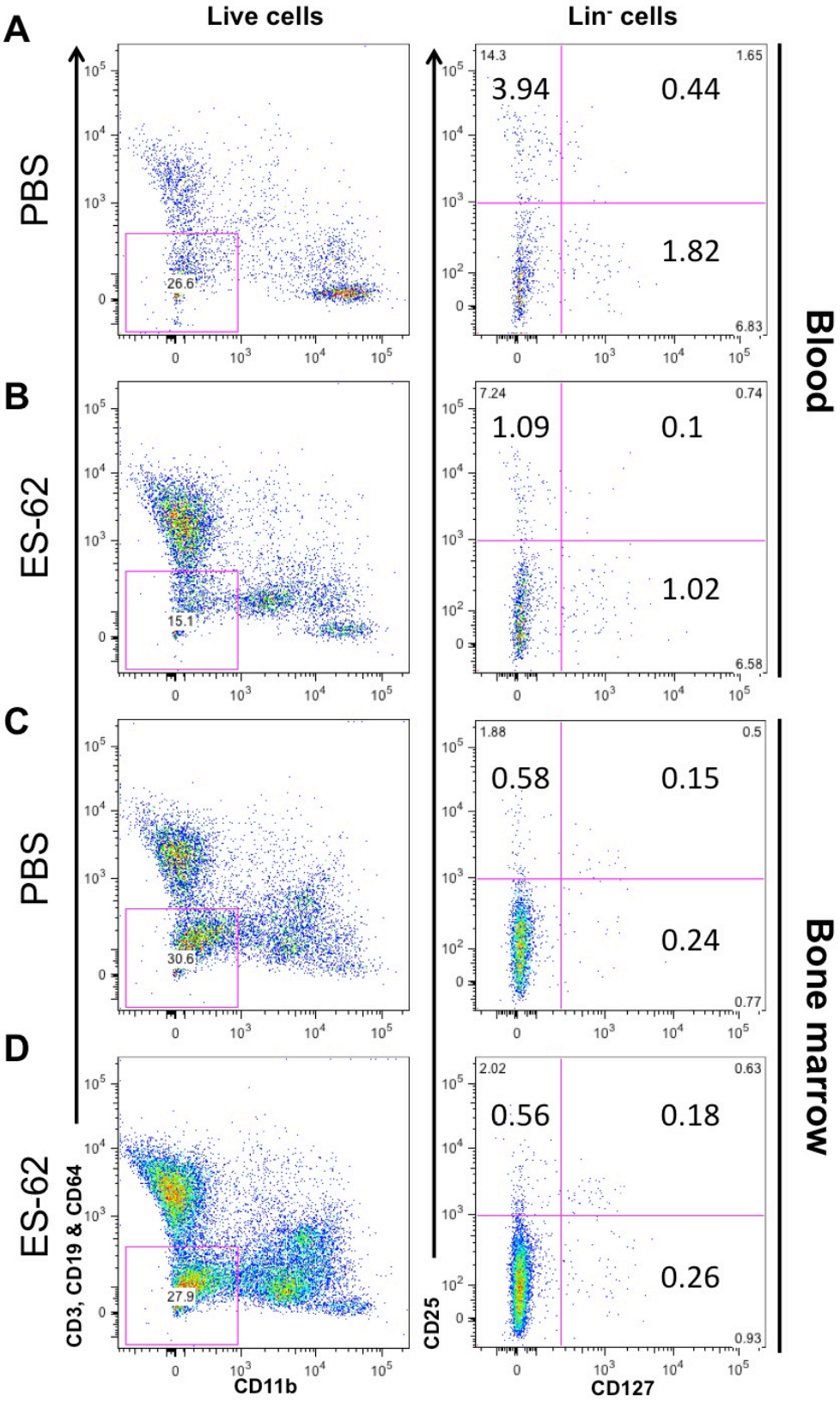

A
B
C
D
Live cells
Lin- cells
PBS
ES-62
PBS
ES-62
Blood
Bone marrow
26.6
15.1
30.6
27.9
14.3
3.94
1.65
0.44
1.82
6.83
7.24
1.09
0.74
0.1
1.02
6.58
1.88
0.58
0.5
0.15
0.24
0.77
2.02
0.56
0.63
0.18
0.26
0.93
CD3, CD19 & CD64
CD11b
CD25
CD127

Figure 3.8. ILC populations in the blood and bone marrow. The proportions of $CD25^+$ and/or $CD127^+$ (Lin; CD3, CD19, CD64 and $CD11b)^-$ cells were determined in the blood (A & B) or bone marrow (C & D) of PBS (A & C) and ES-62 (B & D) treated mice. Blood and bone marrow analysis was performed on cells pooled from 4 mice from each treatment group and the proportion of live cells is indicated in each population gate. Data is representative of 2 independent experiments.

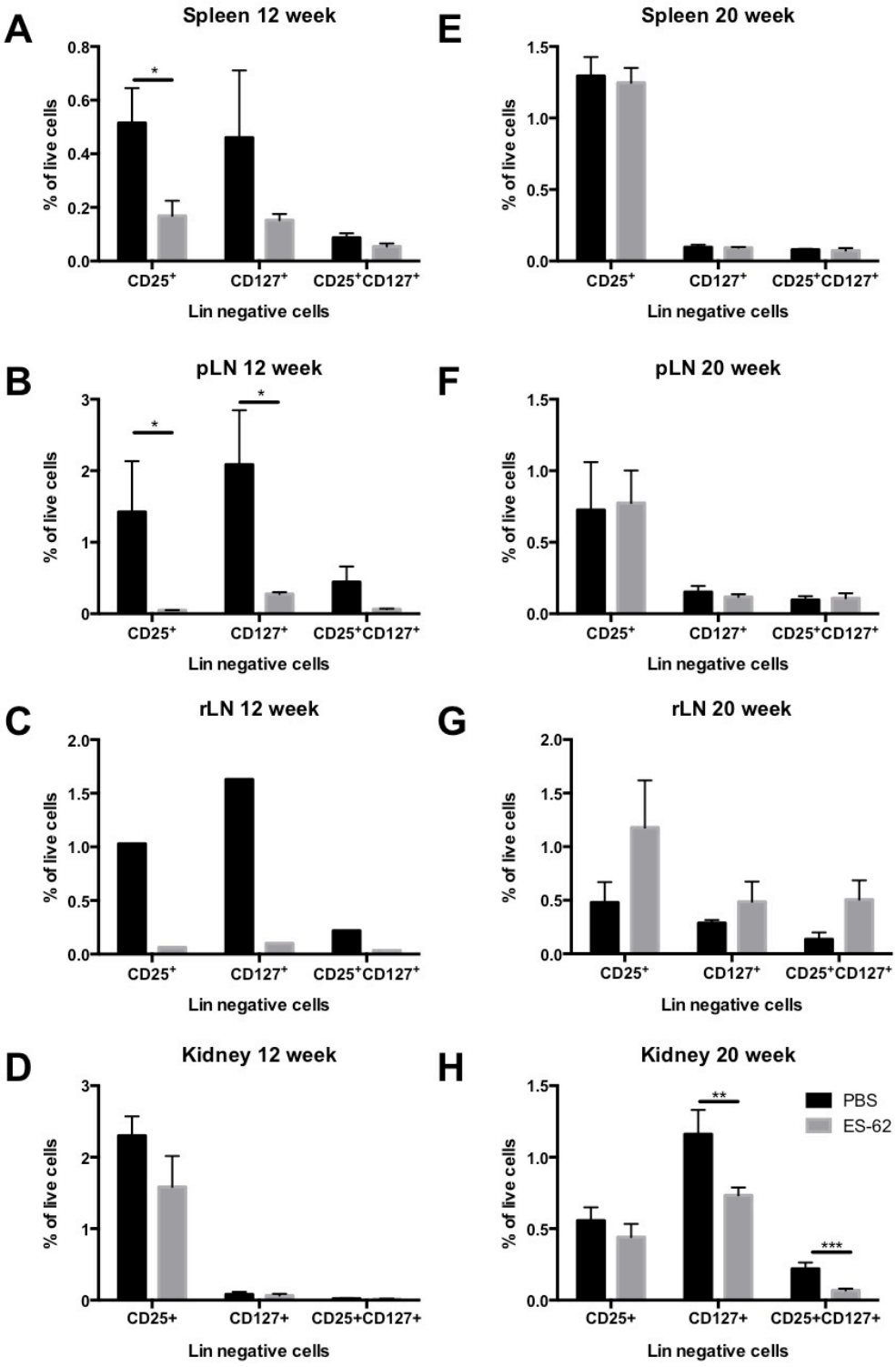

A
Spleen 12 week
% of live cells
0.8
0.6
0.4
0.2
0.0
*
CD25+
CD127+
CD25+CD127+
Lin negative cells
E
Spleen 20 week
% of live cells
1.5
1.0
0.5
0.0
CD25+
CD127+
CD25+CD127+
Lin negative cells
B
pLN 12 week
% of live cells
3
2
1
0
*
*
CD25+
CD127+
CD25+CD127+
Lin negative cells
F
pLN 20 week
% of live cells
1.5
1.0
0.5
0.0
CD25+
CD127+
CD25+CD127+
Lin negative cells
C
rLN 12 week
% of live cells
2.0
1.5
1.0
0.5
0.0
CD25+
CD127+
CD25+CD127+
Lin negative cells
G
rLN 20 week
% of live cells
2.0
1.5
1.0
0.5
0.0
CD25+
CD127+
CD25+CD127+
Lin negative cells
D
Kidney 12 week
% of live cells
3
2
1
0
CD25+
CD127+
CD25+CD127+
Lin negative cells
H
Kidney 20 week
% of live cells
1.5
1.0
0.5
0.0
**
***
PBS
ES-62
CD25+
CD127+
CD25+CD127+
Lin negative cells

Figure 3.9. ES-62 modulates the proportion of ILC found in lymphoid organs and the kidney in the MRL/*lpr* mouse. The proportions of $CD25^+$ and/or $CD127^+$ Lin^- cells (CD3, CD19, CD64 and $CD11b)^-$ were determined in the spleen (A), pLN (B), rLN (C, 5 mice pooled) and kidneys (D) of 12-week MRL/*lpr* mice treated with PBS (black bar; n = 5) or ES-62 (grey bar; n = 5); and in the spleen (E), pLN (F), rLN (G) and kidneys (H) of 21-week MRL/*lpr* mice treated with PBS (black bar; n = 7) and ES-62 (grey bar; n = 11). 21-week data is pooled from 3 independent experiments whereas the 12-week data is representative of one experiment; statistical significance was determined using the student's t-test (A-H).

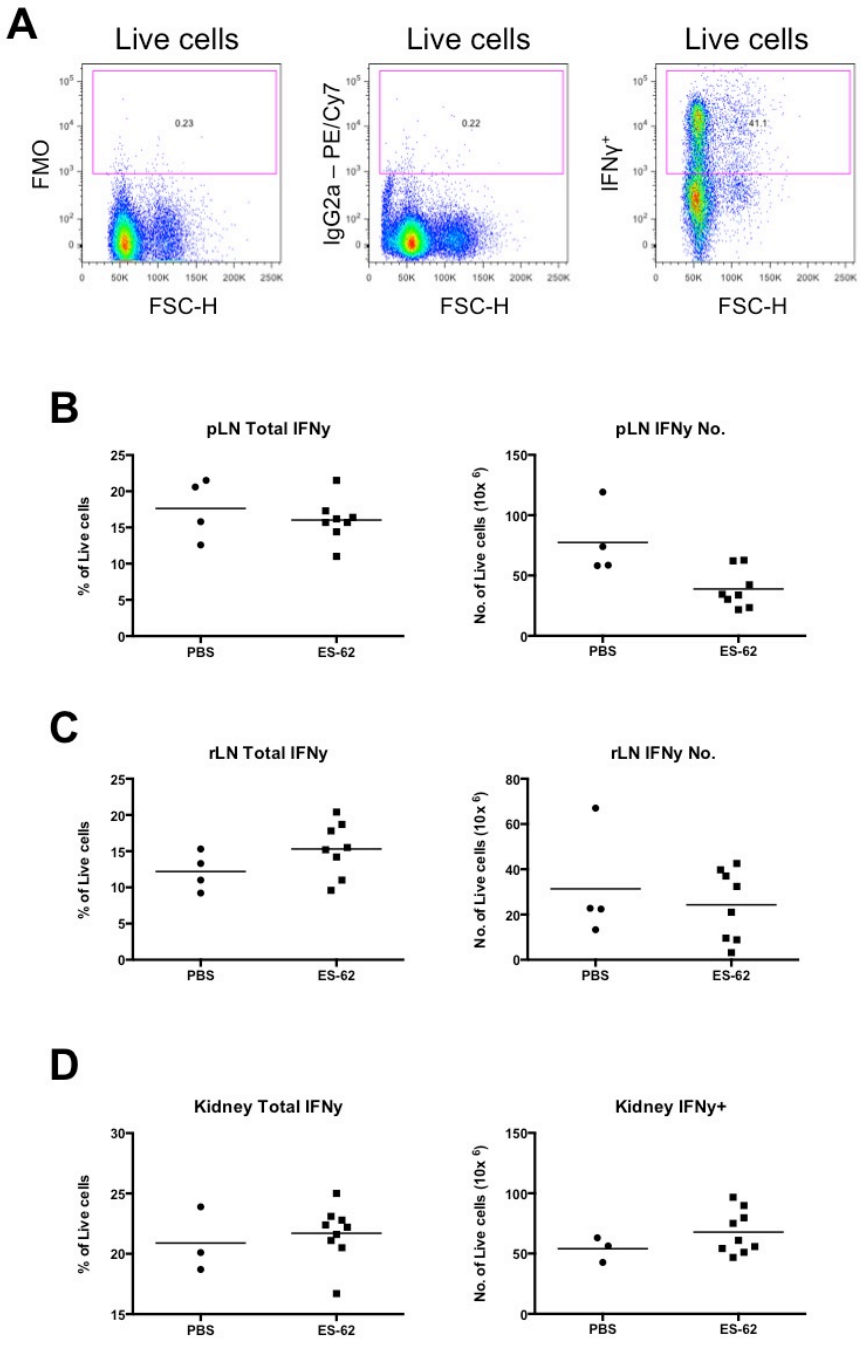

A
Live cells
Live cells
Live cells
FMO
IgG2a – PE/Cy7
IFNγ+
0.23
0.22
41.1
FSC-H
FSC-H
FSC-H
B
pLN Total IFNy
pLN IFNy No.
% of Live cells
No. of Live cells (10x 6)
PBS
ES-62
C
rLN Total IFNy
rLN IFNy No.
D
Kidney Total IFNy
Kidney IFNy+

Figure 3.10. ES-62 does not modulate IFNγ production. The proportion of IFNγ producing cells were determined in LN cells stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes. Cells were washed and stained for viability and surface markers before being analyzed by flow cytometry (A). The proportion and total number of $IFN\gamma^+$ cells were determined in the pLN (B), rLN (C) and kidneys (D) of 21-week MRL/*lpr* mice treated with PBS (n = 4) or ES-62 (n = 8).

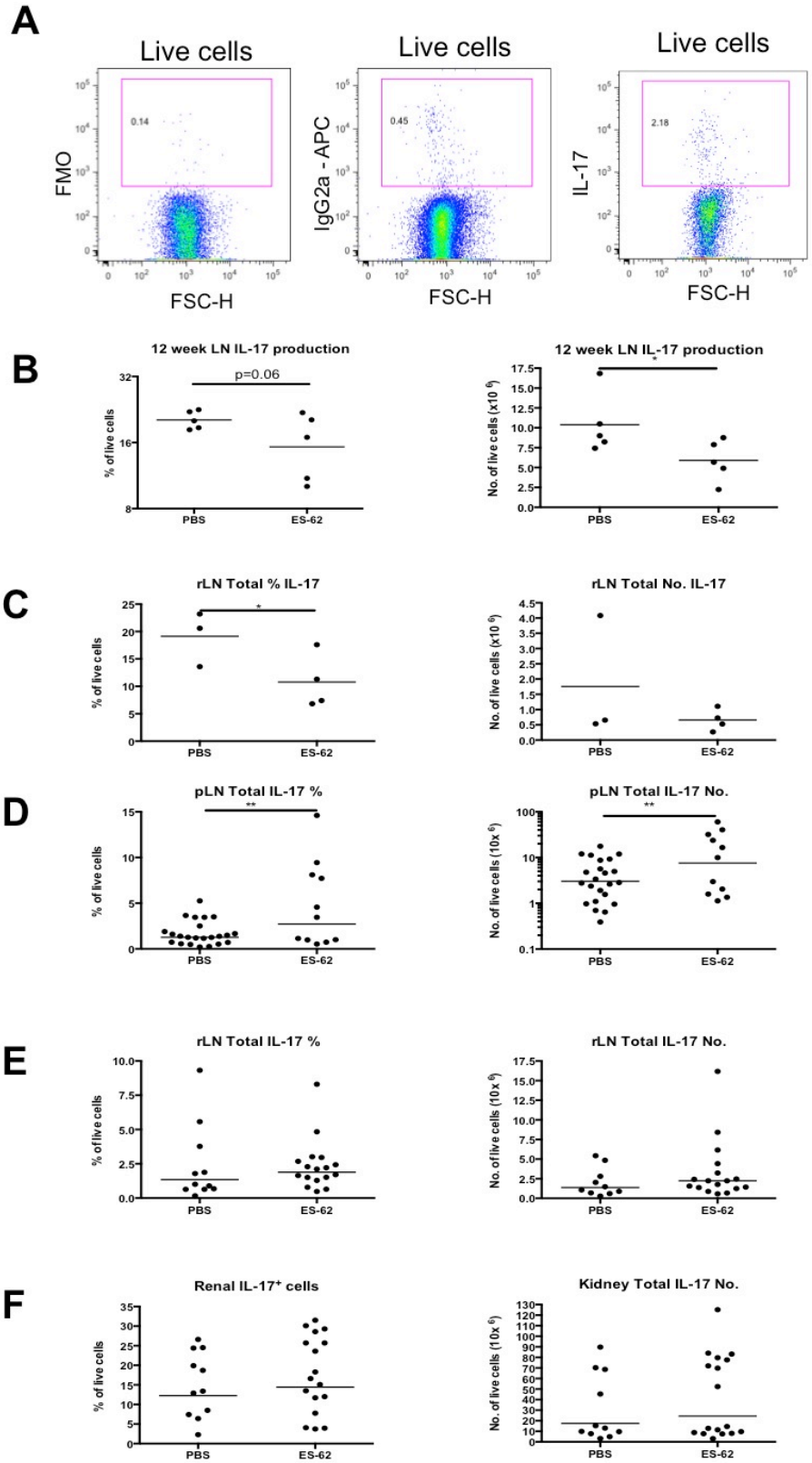

A
Live cells
Live cells
Live cells
FMO
IgG2a - APC
IL-17
0.14
0.45
2.18
FSC-H
FSC-H
FSC-H
B
12 week LN IL-17 production
p=0.06
% of live cells
PBS
ES-62
12 week LN IL-17 production
No. of live cells (x10 6)
PBS
ES-62
C
rLN Total % IL-17
% of live cells
PBS
ES-62
rLN Total No. IL-17
No. of live cells (x10 6)
PBS
ES-62
D
pLN Total IL-17 %
% of live cells
PBS
ES-62
pLN Total IL-17 No.
No. of live cells (10x 6)
PBS
ES-62
E
rLN Total IL-17 %
% of live cells
PBS
ES-62
rLN Total IL-17 No.
No. of live cells (10x 6)
PBS
ES-62
F
Renal IL-17+ cells
% of live cells
PBS
ES-62
Kidney Total IL-17 No.
No. of live cells (10x 6)
PBS
ES-62

Figure 3.11. ES-62 suppresses early but enhances late IL-17A production. Cells were stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes. Cells were washed and stained for viability and surface markers before being analyzed by flow cytometry; IL-17^{+} gates was set based on fluorescent minus 1 (FMO) and isotype staining (A). The proportion and number of IL-17A producing cells were determined in pLN (B) and rLN (C) of 12-week mice treated with PBS (n=5) or ES-62 (n=5); and the pLN (D), rLN (E) and kidneys (F) of 21-week old MRL/*lpr* mice treated with PBS (n = 11) or ES-62 (n = 18). 21-week data is pooled from 3 independent experiments whereas the 12-week data is representative of one experiment; statistical significance was determined using the student's t-test (B-F).

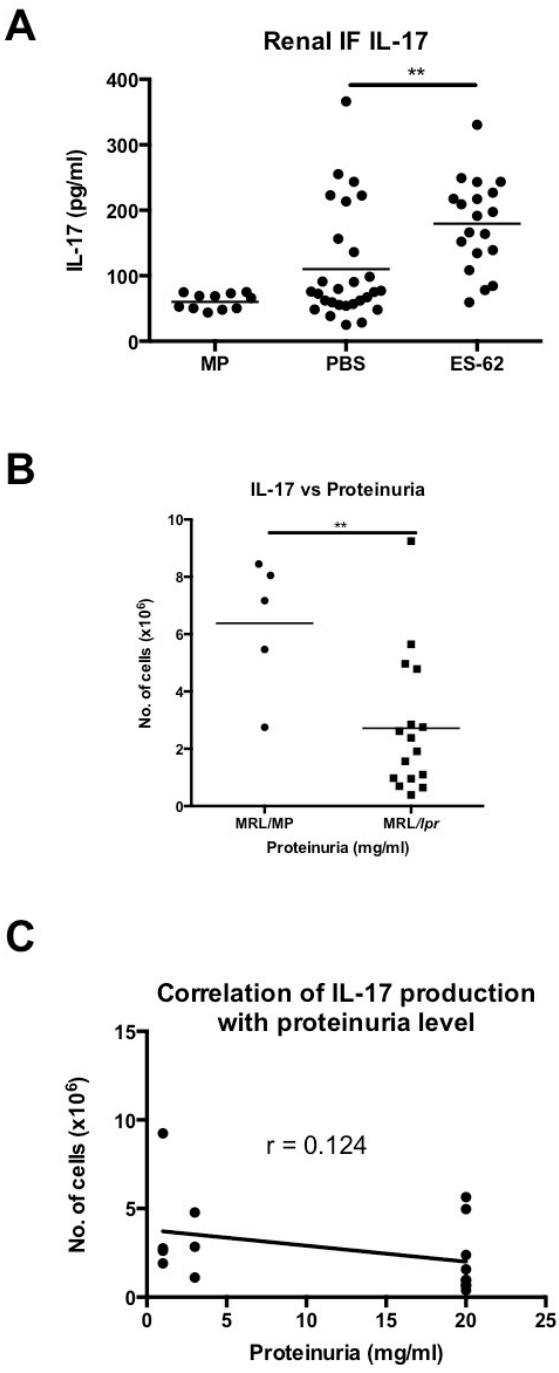

A
Renal IF IL-17
**
IL-17 (pg/ml)
400
300
200
100
0
MP
PBS
ES-62
B
IL-17 vs Proteinuria
**
No. of cells (x10^6)
10
8
6
4
2
0
MRL/MP
MRL/lpr
Proteinuria (mg/ml)
C
Correlation of IL-17 production with proteinuria level
No. of cells (x10^6)
15
10
5
0
r = 0.124
0
5
10
15
20
25
Proteinuria (mg/ml)

Figure 3.12. IL-17A may be protective in the MRL/*lpr* mouse. The concentration of IL-17A in the renal interstitial fluid from PBS (n = 28) or ES-62 (n = 18) treated mice, and 7-(n=5), 12-(n=5) and 21-week (n=5) MRL/MP mice, was measured by ELISA (A). The absolute number of IL-17A$^+$ cells in the pLN of 21-week old MRL/MP or MRL/*lpr* mice were determined by flow cytometry (B) and data from MRL/*lpr* mice was plotted against proteinuria level (C). Statistical significance was tested using the student's t-test.

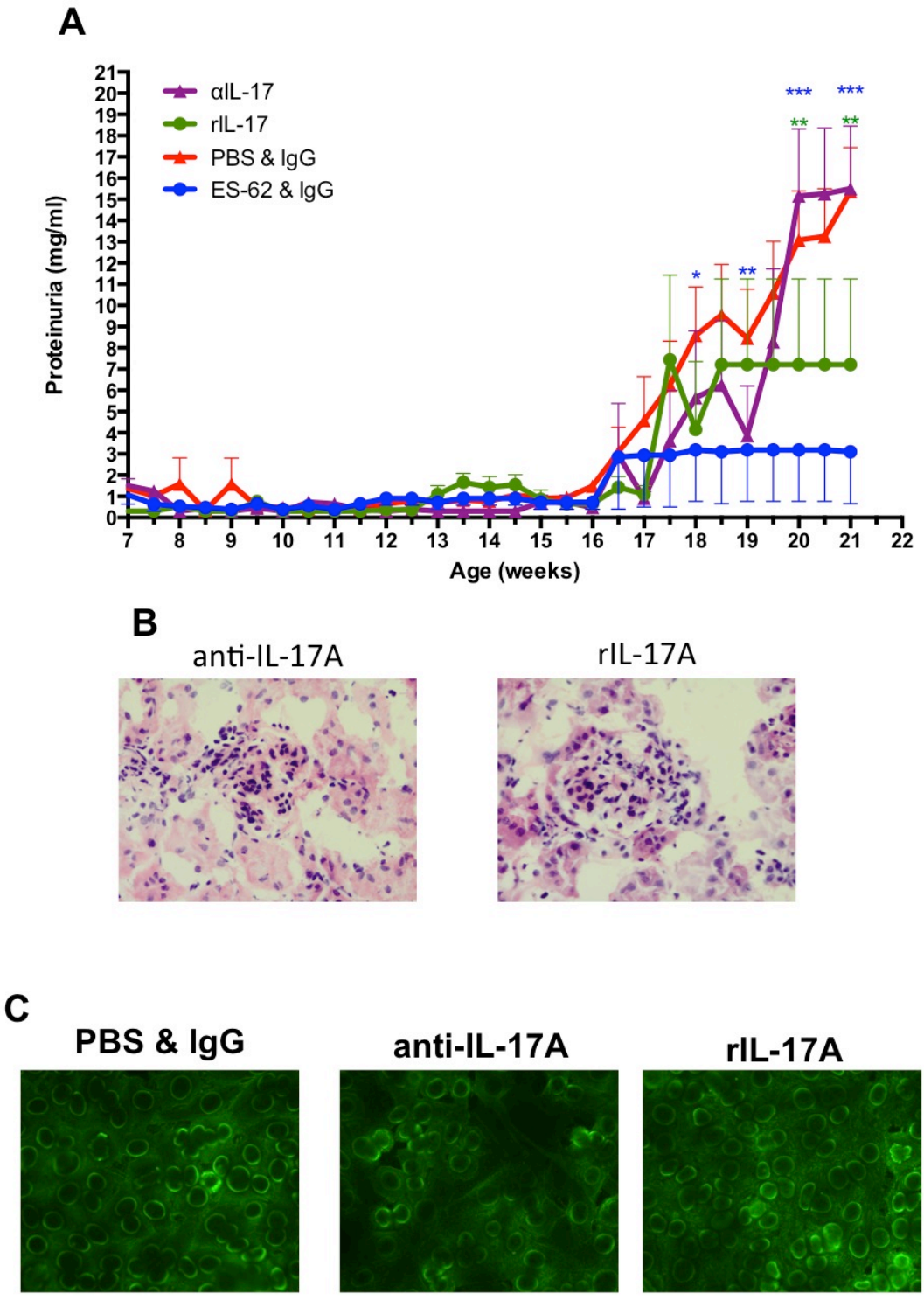

A
αIL-17
rIL-17
PBS & IgG
ES-62 & IgG
Proteinuria (mg/ml)
Age (weeks)
B
anti-IL-17A
rIL-17A
C
PBS & IgG
anti-IL-17A
rIL-17A

Figure 3.13. IL-17A may be protective in the MRL/*lpr* mouse. MRL/*lpr* mice received twice weekly injections of murine IgG (i.p.; 100 μg in 100 μl of PBS; until 12 weeks of age), anti-IL-17A (i.p.; 100 μg in 100 μl of PBS; until 12 weeks of age), PBS (s.c.; 100 μl) or ES-62 (s.c.; 2 μg in 100 μl of PBS) from 7 weeks of age or rIL-17A (i.p.; 1 μg in 100 μl of PBS) from 12-21-weeks of age. Proteinuria was measured twice weekly. Kidneys from mice that received anti-IL-17A or rIL-17A were sectioned and stained with H & E (D) and serum from mice that received PBS, anti-IL-17A or rIL-17A was used to stain Hep-2 slides to assess ANA levels (E). Data is pooled from 3 independent experiments and statistical significance was tested by and two-way ANOVA.

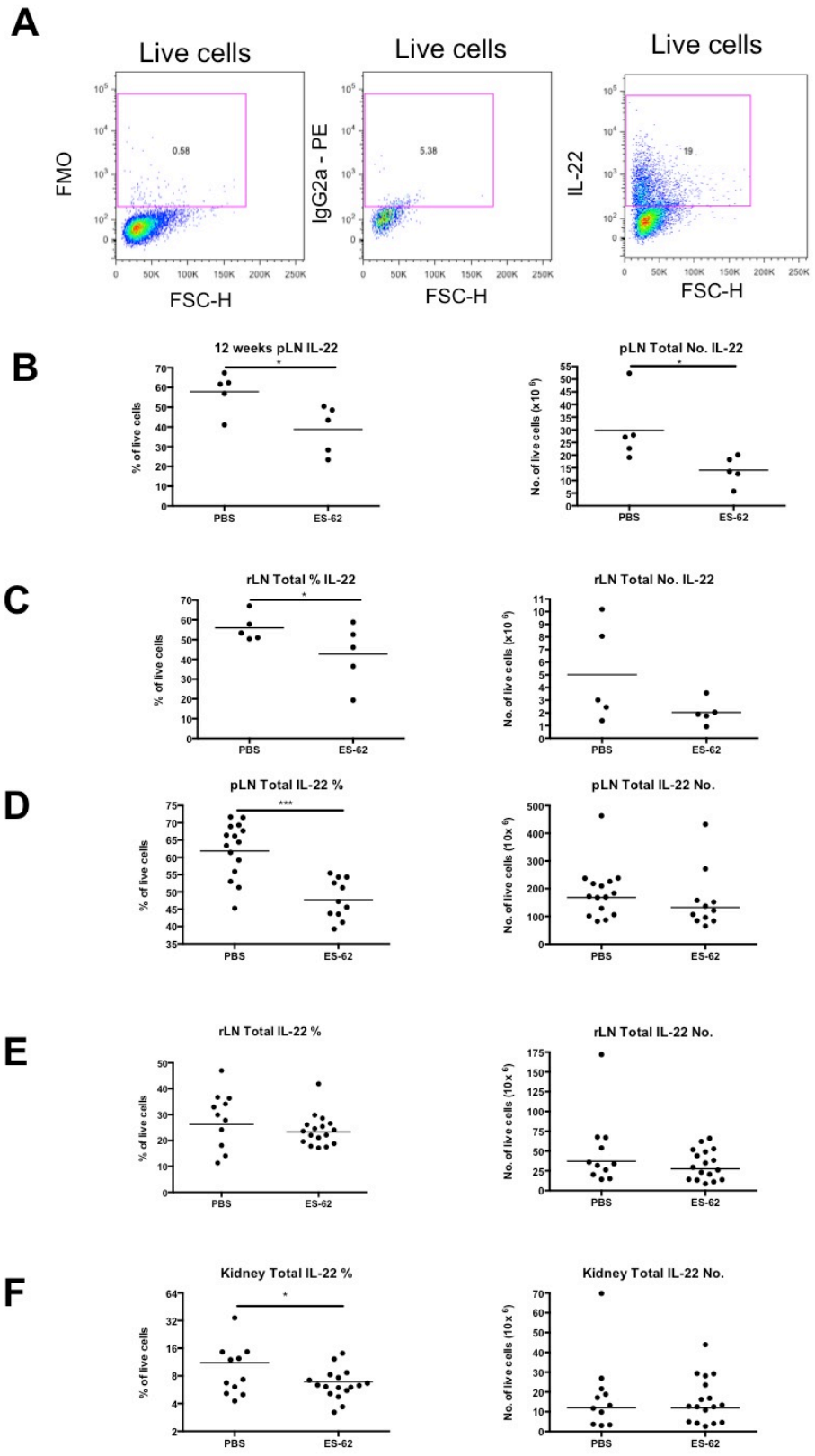
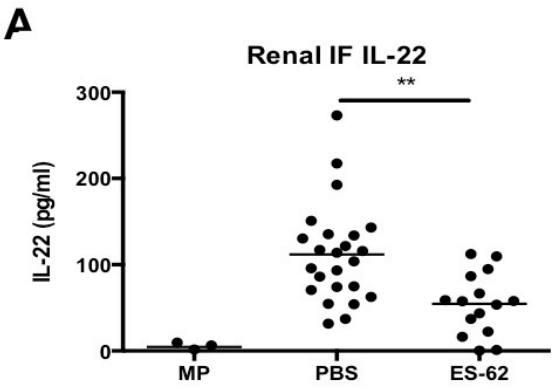
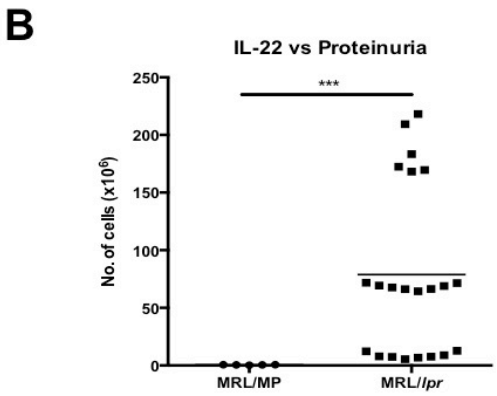

A
Live cells
Live cells
Live cells
FMO
IgG2a - PE
IL-22
0.58
5.38
19
FSC-H
FSC-H
FSC-H
B
12 weeks pLN IL-22
pLN Total No. IL-22
% of live cells
No. of live cells (x10 6)
PBS
ES-62
C
rLN Total % IL-22
rLN Total No. IL-22
D
pLN Total IL-22 %
pLN Total IL-22 No.
No. of live cells (10x 6)
E
rLN Total IL-22 %
rLN Total IL-22 No.
F
Kidney Total IL-22 %
Kidney Total IL-22 No.

Figure 3.14. ES-62 suppresses early and late IL-22 production. Cells were stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes. Cells were washed and stained for viability and surface markers before being analyzed by flow cytometry (A). The proportion of IL-22 producing cells were determined in pLN (A) and rLN (B) of 12-week mice treated with PBS (n=5) or ES-62 (n=5); and the pLN (C), rLN (D) and kidneys (E) of 21-week old MRL/*lpr* mice treated with PBS (n = 11) or ES-62 (n = 18). 21-week data is pooled from 3 independent experiments whereas the 12-week data is representative of one experiment; statistical significance was determined using the student's t-test (A-E).


A
Renal IF IL-22
**
IL-22 (pg/ml)
300
200
100
0
MP
PBS
ES-62


B
IL-22 vs Proteinuria
***
No. of cells (x10^6)
250
200
150
100
50
0
MRL/MP
MRL/lpr

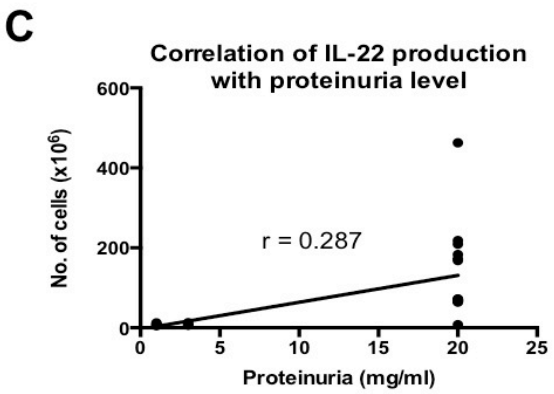
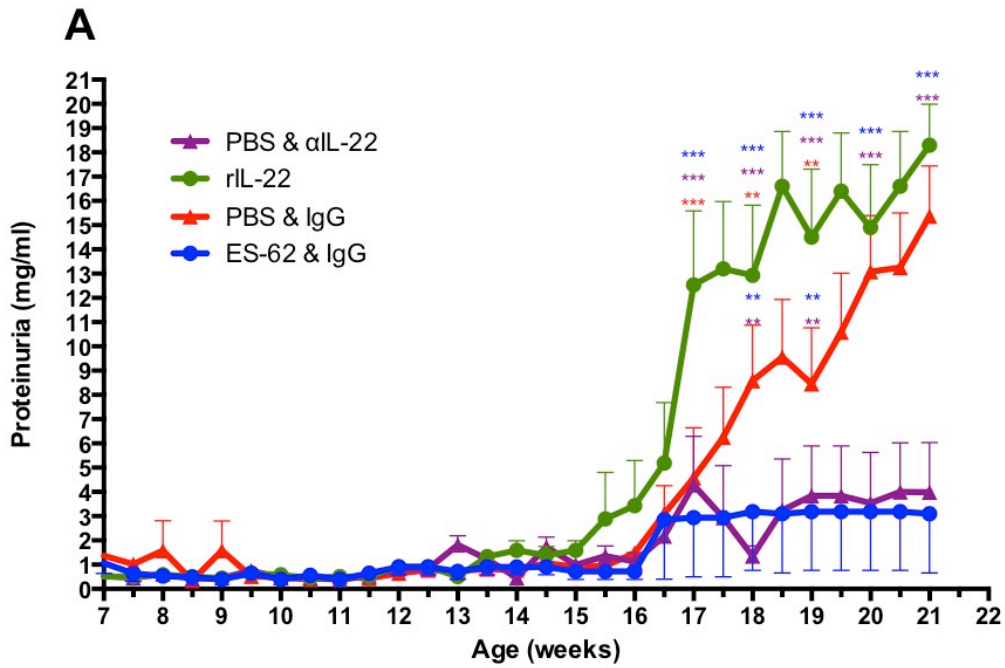
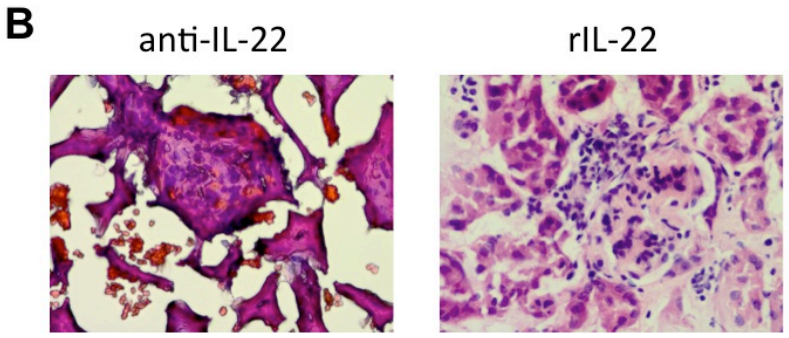

C
Correlation of IL-22 production
with proteinuria level
No. of cells (x10^6)
600
400
200
0
r = 0.287
0
5
10
15
20
25
Proteinuria (mg/ml)

Figure 3.15. IL-22 is pathogenic in the MRL/*lpr* mouse. The concentration of IL-22 in the renal interstitial fluid from PBS (n = 28) or ES-62 (n = 18) treated MRL/*lpr* mice, and 7-(n=5), 12-(n=5) and 21-week (n=5) MRL/MP mice, was measured by ELISA (A). The absolute number of IL-22$^+$ cells in the pLN of 21-week old MRL/MP or MRL/*lpr* mice were determined by flow cytometry (B) and the MRL/*lpr* data was plotted against proteinuria level (C). Statistical significance was tested using the student's t-test.


A
Proteinuria (mg/ml)
Age (weeks)
PBS & αIL-22
rIL-22
PBS & IgG
ES-62 & IgG


B
anti-IL-22
rIL-22

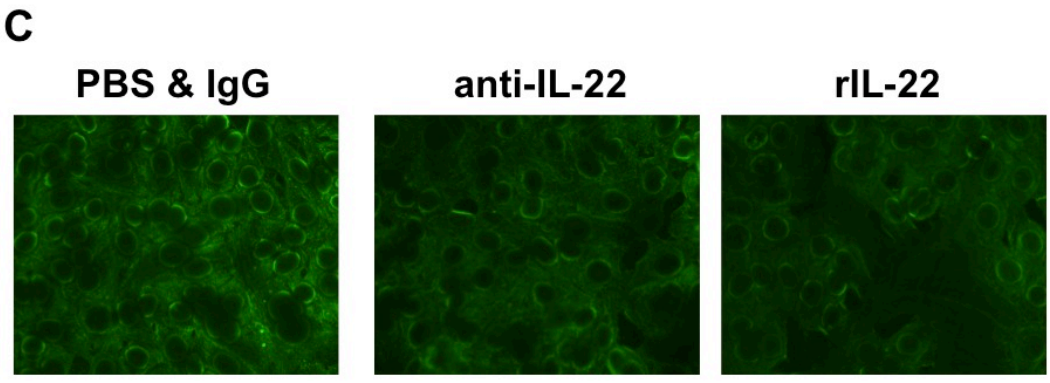
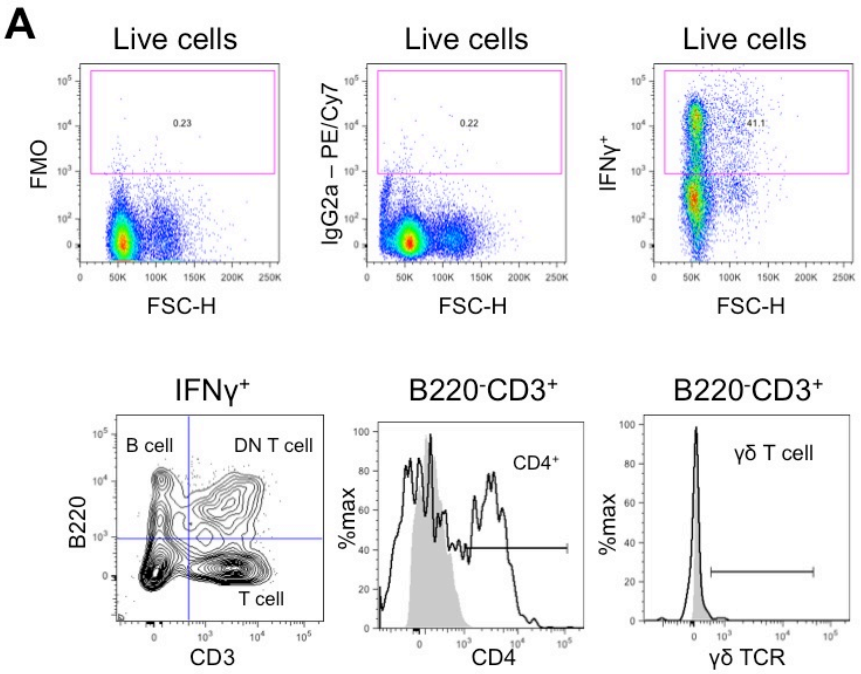
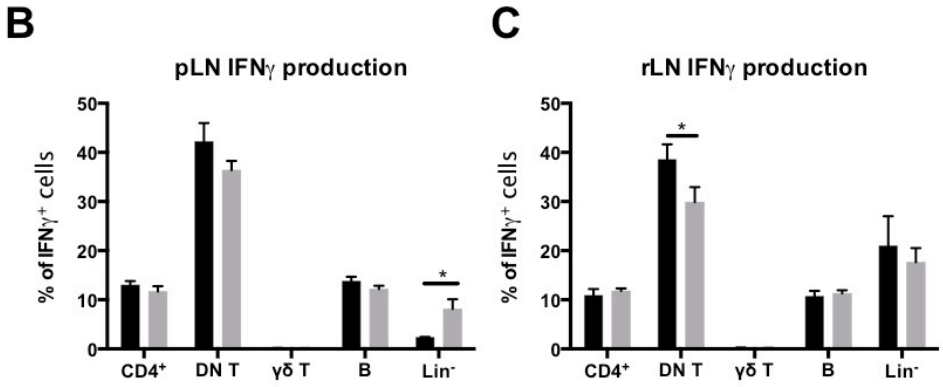

C
PBS & IgG
anti-IL-22
rIL-22

Figure 3.16. IL-22 is pathogenic in the MRL/*lpr* mouse. MRL/*lpr* mice received twice weekly injections of murine IgG (i.p.; 100 μg in 100 μl of PBS; from 12-21-weeks of age), anti-IL-22 (i.p.; 100 μg in 100 μl of PBS; from 12-21-weeks of age), PBS (s.c.; 100 μl) or ES-62 (s.c.; 2 μg in 100 μl of PBS) from 7 weeks of age or rIL-22 (i.p.; 1 μg in 100 μl of PBS) from 12-21-weeks of age. Proteinuria was measured twice weekly. Kidneys from mice that received anti-IL-22 or rIL-22 were sectioned and stained with H & E (D) and serum from mice that received PBS, anti-IL-22 or rIL-22 was used to stain Hep-2 slides to assess ANA levels (E). Statistical significance was tested by two-way ANOVA (C).


A
Live cells
Live cells
Live cells
FMO
IgG2a – PE/Cy7
IFNγ+
0.23
0.22
41.1
FSC-H
FSC-H
FSC-H
IFNγ+
B220-CD3+
B220-CD3+
B cell
DN T cell
T cell
B220
CD3
CD4+
%max
CD4
γδ T cell
%max
γδ TCR


B
pLN IFNγ production
% of IFNγ+ cells
CD4+
DN T
γδ T
B
Lin-
C
rLN IFNγ production
% of IFNγ+ cells
CD4+
DN T
γδ T
B
Lin-

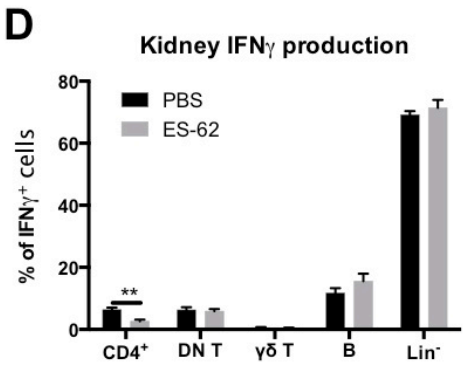

D
Kidney IFNγ production
PBS
ES-62
% of IFNγ+ cells
CD4+
DN T
γδ T
B
Lin-

Figure 3.17. ES-62 modulation of cells producing IFNγ. Cells were stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes. Cells were washed and stained for viability and surface markers before being analyzed by flow cytometry (A). The proportion of IFNγ^+ CD4$^+$ T, DN T, γδ T, B and Lin$^-$ cells were determined in pLN (B), rLN (C) and kidney (D) of PBS (black bars; n = 4) or ES-62 (grey bars; n = 8) treated MRL/*lpr* mice at 21-weeks. Statistical significance was tested using the student's t-test (B-D).

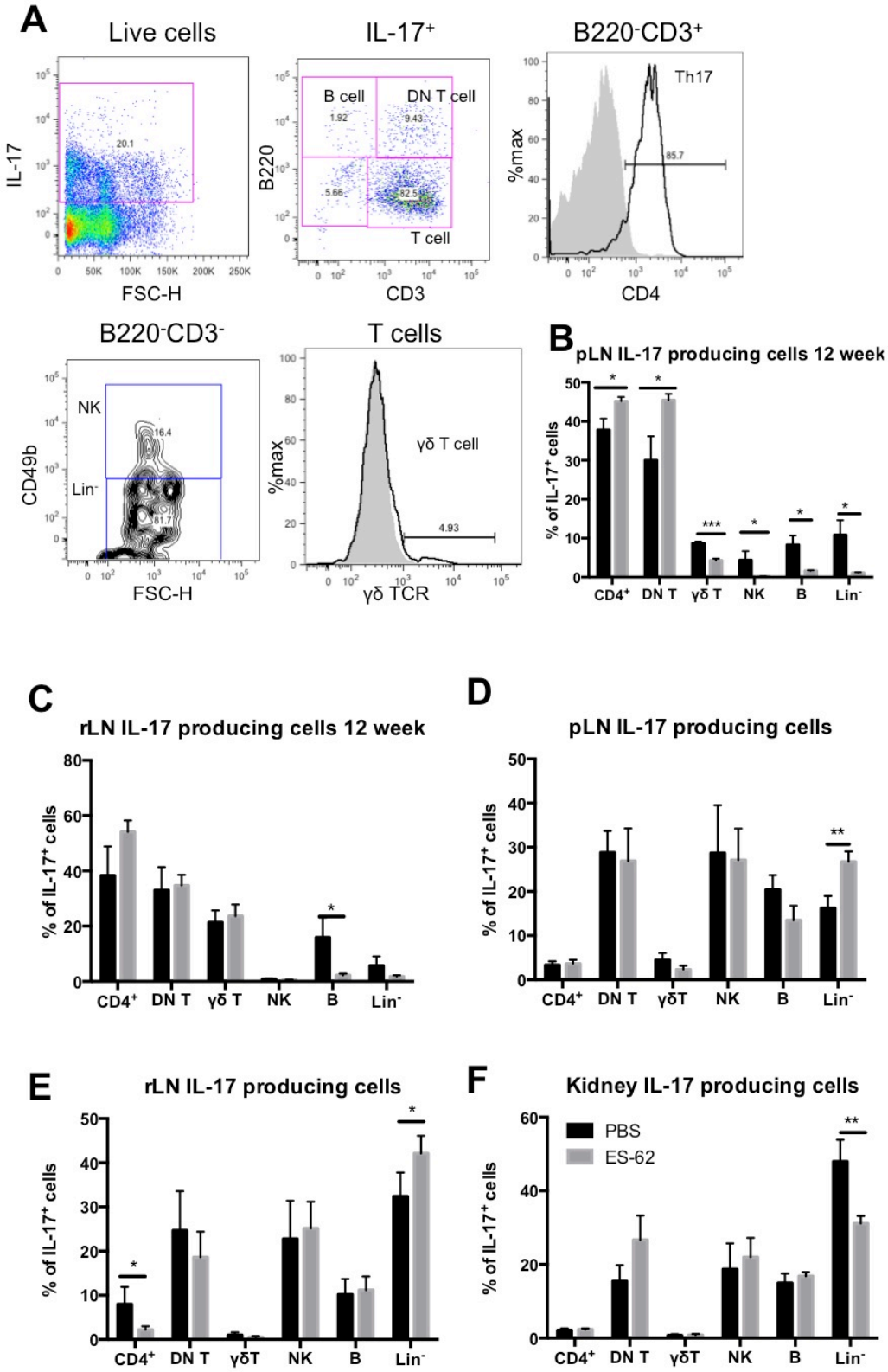

A
Live cells
IL-17+
B220-CD3+
IL-17
FSC-H
B220
CD3
B cell
DN T cell
T cell
Th17
%max
CD4
B220-CD3-
T cells
NK
Lin-
CD49b
FSC-H
γδ T cell
γδ TCR
B
pLN IL-17 producing cells 12 week
% of IL-17+ cells
CD4+ DN T γδ T NK B Lin-
C
rLN IL-17 producing cells 12 week
D
pLN IL-17 producing cells
E
rLN IL-17 producing cells
F
Kidney IL-17 producing cells
PBS
ES-62

Figure 3.18. ES-62 modulates the proportion of cells producing IL-17A. Cells were stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes. Cells were washed and stained for viability and surface markers before being analyzed by flow cytometry where the proportion of IL-17A$^+$ CD4$^+$ Th17, DN T, γδ T, B and Lin$^-$ cells (A) were determined in the pLN (B) & rLN (C) of 12-week MRL/*lpr* mice treated with PBS (black bars; n = 5) or ES-62 (grey bars; n = 5) (B); and the pLN (D), rLN (E) and kidney (F) of PBS (black bars; n = 11) or ES-62 (grey bars; n = 18) treated MRL/*lpr* mice at 21-weeks. 12-week data are representative of 1 experiment whereas 21-weel data are representative of 3 independent experiments; statistical significance was tested using the student's t-test (B-F).

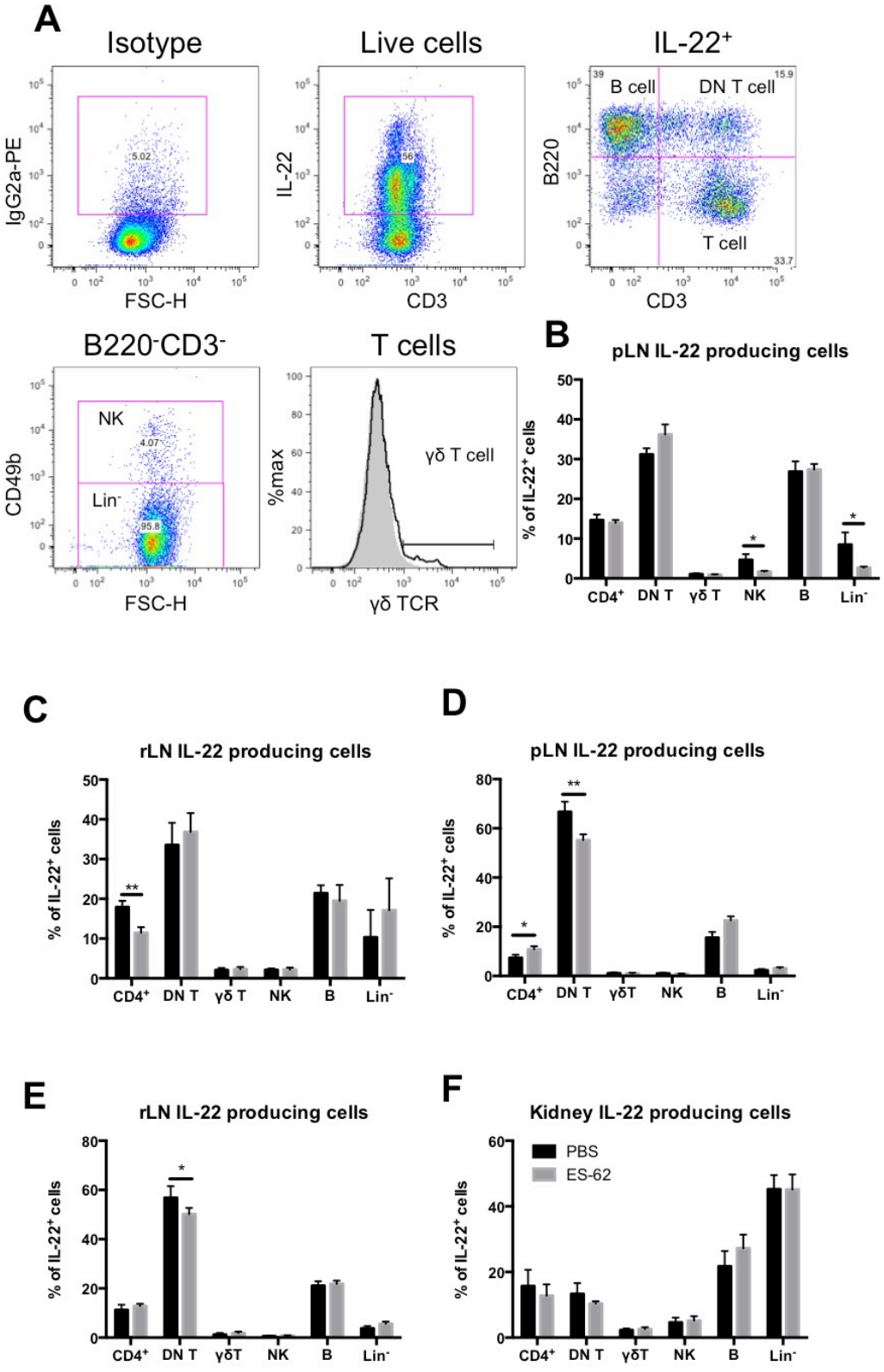
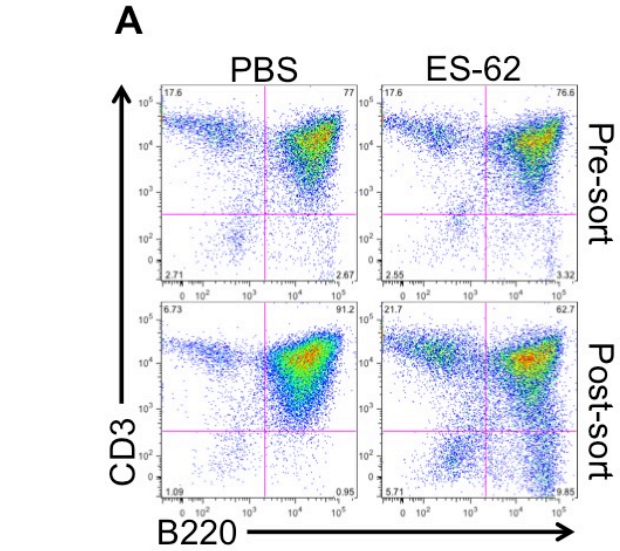

A
Isotype
Live cells
IL-22+
IgG2a-PE
FSC-H
IL-22
CD3
B220
B cell
DN T cell
T cell
B220-CD3-
T cells
NK
Lin-
CD49b
%max
γδ T cell
γδ TCR
B
pLN IL-22 producing cells
% of IL-22+ cells
CD4+ DN T γδ T NK B Lin-
C
rLN IL-22 producing cells
D
pLN IL-22 producing cells
E
rLN IL-22 producing cells
F
Kidney IL-22 producing cells
PBS
ES-62

Figure 3.19. ES-62 modulates the proportion of cells producing IL-22. Cells were stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes. Cells were washed and stained for viability and surface markers before being analyzed by flow cytometry where the proportion of IL-22$^+$ CD4$^+$ Th17, DN T, γδ T, B and Lin$^-$ cells were determined (A) in pLN (B) and rLN (C) of 12-week MRL/*lpr* mice treated with PBS (black bar; n = 5) or ES-62 (grey bar; n = 5); and the pLN (D), rLN (E) and kidney (F) of PBS (black bars; n = 11) or ES-62 (grey bars; n = 18) treated MRL/*lpr* mice at 21-weeks. 12-week data are representative of 1 experiment whereas 21-week data are representative of 3 independent experiments; statistical significance was tested using the student's t-test (B-F).


A
PBS
ES-62
Pre-sort
Post-sort
CD3
B220

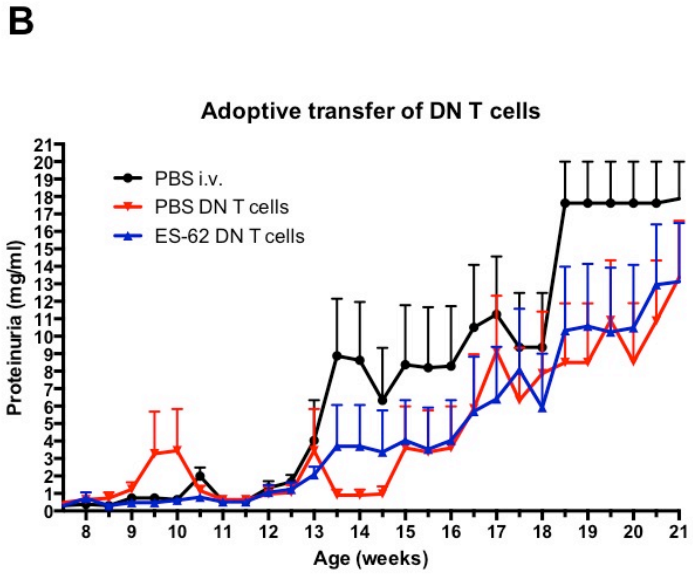

B
Adoptive transfer of DN T cells
PBS i.v.
PBS DN T cells
ES-62 DN T cells
Proteinuria (mg/ml)
Age (weeks)

Figure 3.20. Adoptive transfer of DN T cells. In two independent experiments, DN T cells were purified from the pLN of 21-week old PBS (n = 4/3) or ES-62 (8/3) treated MRL/*lpr* mice were purified by depletion of $CD19^+$, $CD4^+$ and $CD8^+$ cells using antibodies conjugated to magnetic beads (Miltenyi). Cell purity was checked by flow cytometry but found to be very inconsistent (A). 5×10^6 purified DN T cells were injected into the tail vein of 7-week old MRL/*lpr* mice which were maintained until 21-weeks of age.

4. ES-62 suppresses the generation of pathogenic B cell responses and promotes the development of IL-10-producing B cells, in the MRL/*lpr* mouse

4.1. Introduction

Systemic Lupus Erythematosus (SLE) is characterized by high titers of auto-antibodies, against nuclear antigens (ANA), resulting in immune-complex mediated inflammation in the kidney, skin and joints as well as cardiovascular pathologies [5]. B lymphocytes are central to the pathogenesis of this disease, not only in terms of their production of autoantibodies, but also with respect to cytokine production and cellular interactions [6, 7][8]. Reflecting this, several B cell-specific genes have been associated with the development of SLE [9]. Moreover, whilst overexpression of the B cell growth factor, BAFF, promotes lupus-like pathology in mouse models [10], depletion of B cells ameliorates disease [6, 11]. The success of the B-cell-depleting monoclonal antibody, rituximab, as a therapy in a number of autoimmune conditions, as well as open-label studies in SLE, indicate that B cell depletion therapies are a rationale choice for treatment of Lupus. Despite this, Rituximab proved disappointing in clinical trials [12, 13] and Belimumab (anti-BAFF) [14-16], the first SLE-specific treatment to be granted FDA approval in 50 years, is successful in only 14% of patients. Nevertheless, associated mechanistic studies are suggestive of the future utility of such B cell-targeting therapies in more stratified lupus patient subsets.

The ability of B-cell targeting therapies such as Rituximab to reset the imbalance between effector and regulatory B cells, which contributes to the pathology of autoimmune disease, [10, 17] has not only highlighted the important role of IL-10-producing regulatory B cells (Bregs) in protecting against the development of autoimmunity, but has also identified their therapeutic potential in such disorders, including SLE [18-20]. It is therefore of interest to note that Breg populations are increased in patients with multiple sclerosis who are infected with parasitic worms [21], and that infection with *Schistosoma mansoni* prevents and reverses lung remodelling in a mouse model of asthma by induction of Bregs [22]. Moreover, ES-62 exhibits therapeutic potential in both allergic [23, 24] and autoimmune [25-27] inflammatory disease, conditions in which it can induce hyporesponsive effector B cell responses [2, 25, 26, 28] and modulate antibody production (335) whilst stimulating IL-10 production by splenocytes [26, 29], responses consistent with the parasite product resetting the effector: regulatory B cell balance.

As ES-62 can protect against the development of proteinuria in the MRL/*lpr* mouse, it was the core aim of this chapter to determine whether protection is associated with a resetting of the balance of effector and regulatory B cell responses. Specifically, the aims of this section were to determine the effect of ES-62 on the generation of pathogenic and regulatory B cell responses. Moreover, if ES-62 induced a regulatory population of B cells, it was also planned to determine whether transfer of such cells was sufficient to confer the protection afforded by ES-62.

4.2. Results

4.2.1. ES-62 suppression of proteinuria is associated with suppressed effector B cell responses

One of the striking features of the MRL/*lpr* mouse is the development of splenomegaly (Fig. 4.1A) due to the accumulation of DN T cells in MRL/*lpr*, but not MRL/MP, mice (Fig.3.1 & 3.4). In the parental MRL/MP strain the proportion of splenic $CD19^+$ B cells remains constant, whereas, although the proportions of B cells decrease in MRL/*lpr* mice over time, the numbers of splenic $CD19^+$ B cells actually increase around the time of onset of disease (Fig. 4.1B & C). This rise in the numbers of $CD19^+$ B cells was reflected by increased levels of transitional type 1, marginal zone (MZ), follicular (Fo) and germinal center (GC) B cell populations in the spleens of MRL/*lpr*, but not MRL/MP mice, with kinetics that correlated with onset and progression of disease (Fig. 4.1D-G). By contrast, although the levels are generally lower than those in MRL/MP mice, following an initial rise, there is a decrease in the levels of marginal zone precursor (MZP) B cells in the spleens of MRL/*lpr* mice between 12-16 weeks (Fig. 4.1H), kinetics that may reflect their proposed role as IL-10-producing Bregs that can protect against development of disease in the MRL/*lpr* mouse [18, 19]. Interestingly, given that they also develop proteinuria after about 12 months, the levels of MZP cells in the spleens of MRL/MP mice drop dramatically to those of MRL/*lpr* mice by the time they reach 5 months of age, perhaps indicating the initiation of pathology.

Prior to the onset of disease, at 12-weeks of age, the proportion and number of B cells in the spleen and rLN were unchanged in ES-62 treated mice however they were significantly decreased in the pLN and significantly elevated in the kidneys (Fig. 4.2A and data not shown). The reduction in B cells in the pLN was due to reduced levels, both proportions and numbers of Fo1 and plasma B cells; (Fig. 4.2B & F and data not shown) whilst the proportion and number of GC, memory B cells, plasmablasts, T1-3, MZP, MZ and B1 B cells were unaltered in pLN and rLN of ES-62 treated mice (Fig. 4.2 data not shown); however, the proportions and numbers of plasmablasts and plasma cells were both reduced in the rLN whilst the proportion and number of memory B cells was considerably increased at this site (Fig. 4.2D-F and data not shown). Moreover, the levels of plasmablasts and

plasma cells were significantly decreased in the kidneys. Taken together, these data suggest a decrease in the development of antibody producing cells in ES-62 treated 12-week MRL/*lpr* mice.

During established disease, at 21-weeks of age, ES-62-mediated protection was not reflected by suppression of the splenomegaly observed in MRL/*lpr* mice, indeed, the number of splenocytes was significantly increased in mice treated with the parasite product and this reflected a significant increase in both splenic $CD19^+$ B cells (Fig. 4.3A & B) and DN T cells (Fig. 3.6B). The expansion of the B cell compartment was not due to a modulation of the T1-3 B cell (Fig. 4.3C-E), MZ or GC B cell (Fig. 4.4B & E) populations, but rather, the MZP and Fo B cell populations (Fig. 4.4A, C & D). Exposure to ES-62 did not modulate the levels of any of these populations in the pLN or rLN (Fig. 4.3 & 4.4). Although, several of these populations were not detected in the kidney, cells of the MZ phenotype were found at increased levels at this site in ES-62 treated mice (Fig. 4.4B). Likewise, whilst ES-62 had no significant effects on $CD138^+B220^+CD19^+$ pre-plasma cells(354) or $CD138^+B220^{low/-}CD19^-$ plasma cell(352) at any of these sites, the proportions of $CD138^+B220^{low/-}CD19^+$ plasmablasts were reduced in the kidneys of mice exposed to ES-62. Moreover, the proportions of plasmablasts and plasma cells were also reduced in the blood of ES-62-treated mice (Fig. 4.5D).

The differential modulation of plasmablasts and plasma cells may reflect the ES-62-mediated increase in IL-17A during established disease (see chapter 3), as this cytokine has recently been reported to drive the germinal centre reaction (403). Consistent with this, Szeto *et al.* recently reported that IL-17A mRNA levels in the urine of SLE patients inversely correlate with titers of anti-dsDNA antibodies (388), which are thought to be produced by plasmablasts (3,195). Thus, the potentially antagonistic roles reported for IL-17A and IL-22 in the pathogenesis of MRL/*lpr* mice in the previous chapter might suggest that IL-22 could also modulate the development of plasmablasts versus plasma cells. Indeed, this proposal was supported by preliminary data showing that treatment of MRL/*lpr* mice with neutralizing anti-IL-22 antibodies reduced the levels of plasmablasts, but not plasma cells, in the rLN and kidney (Fig. 4.6). Moreover, complementary data from mice treated with rIL-22 indicated that whilst rIL-22 also slightly suppressed the

levels of plasmablasts, but not plasma cells, in the kidney, it increased the levels of both plasmablasts and plasma cells in the rLN (Fig. 4.6).

Finally, Despite being reduced in the pLN, the proportion and number of innate B1 B cells were largely unaltered in ES-62 treated mice (Fig. 4.7).

Consistent with proposals that B cells play both antibody-dependent and independent roles in the pathogenesis of lupus, increased signaling via B cell-associated receptors (BAFF-R [32] and TLR4 [33]) and co-stimulatory molecules (CD40[34], CD80 and CD86 [35]) as well as loss of their immune complex-mediated negative feedback regulatory mechanism (FcgRIIb [CD32] [35]) have been implicated as factors driving autoimmunity in this disease. Interestingly, therefore, whilst exposure to ES-62 downregulated expression of TLR4, CD40, CD80 and CD86, it upregulated that of FcγRIIB and BAFF-R, both in terms of the levels of expression and also the proportion of positive B cells circulating in the blood (Fig. 4.8A-F). Moreover, the levels of CD80 expression by B cells in the spleen, pLN, rLN and kidneys were similarly reduced (Fig. 4.8G) whilst those of BAFF-R expression were generally increased following treatment of MRL/*lpr* mice with ES-62 (Fig. 4.8H). Expression of FcγRIIb (CD32) by B cells was significantly increased in the spleens of ES-62 treated mice but unaltered elsewhere (Fig. 3.8I) whilst perhaps surprisingly, the level of expression of TLR4 was elevated on the B cells infiltrating the kidneys of ES-62 treated mice. By contrast, CD86 and CD40 expression by B cells in the spleen, pLN, rLN and kidney was not modulated by ES-62 (Fig. 4.8J-L). The expression of CXCR5, the receptor for the B cell-attracting chemokine CXCL13, that is a marker for renal disease in patients with SLE [44], is down regulated on B cells in the spleens and pLN yet elevated in the kidneys of MRL/lpr mice treated with ES-62 suggesting that any reduction in the B cells infiltrating the kidneys of ES-62 treated mice was not due to their reduced expression of CXCR5, although the increased expression of this receptor on kidney B cells may indicate desensitization of its signaling in ES-62-treated mice (Fig. 4.8M). Similarly, although overexpression of BAFF has been proposed to play a pathogenic role in lupus by promoting B cell responses, the levels of this B cell survival factor are not decreased in the serum or kidney interstitial fluid of ES-62-treated mice (Fig. 4.8N & O): thus the surprising increase in BAFF-R

expression by B cells in ES-62 treated mice may possibly suggest desensitization of this receptor (Fig. 4.8F & H) or alternatively, that such hyporesponsive B cells act as a sink for BAFF that would otherwise promote autoimmune B cell responses. Consistent with these findings, ES-62 has previously been shown to induce hyporesponsive signaling in B cells via uncoupling of the BCR to the ERK MAP kinase pathway via the recruitment of SHP-1 and Pac-1 (325). It was therefore hypothesized that ES-62 may alter BCR expression or signaling in the B cells of MRL/*lpr* mice. Although the responsiveness of B cells from ES-62 treated mice to BCR stimulation was not tested in this study, the surface expression of IgM and IgD was found to be unaltered by the parasite product (Fig. 4.9).

Collectively, these data suggest that whilst ES-62 does not act to prevent the maturation or migration of all B cells, it induces a more hypo-responsive phenotype of effector B cells and renders them more susceptible to negative feedback inhibition by immune complexes and this is perhaps reflected in the reduction of pathogenic plasmablasts.

4.2.2. ES-62 modulates effector B cell responses

As ES-62-mediated protection was associated with modulation of the levels and responsiveness of effector B cells the ability of the parasite product to modulate autoantibody responses was investigated. Rather surprisingly, ES-62 did not modulate the levels of total IgM, IgG1 or IgG2a in the serum of MRL/*lpr* mice (Fig. 4.10A-C). However, and consistent with the earlier observation that ES-62 reduced the proportion of plasmablasts, which are responsible for the production of pathogenic anti-dsDNA antibodies(3,195), ES-62 significantly suppressed the production of anti-nuclear antibodies (ANA) in the serum of 12- and 20-week MRL/*lpr* mice (Fig. 4.10A & B) and in the renal interstitial fluid from 20-week mice (Fig. 4.11C). As expected, ANA were not detectable at the same intensity in the serum or renal interstitial fluid of age-matched MRL/MP mice, however, by 20-weeks of age, nuclear ANA staining was detectable in some mice (Fig. 4.10A-C). The modulation of ANA production by ES-62 was evidenced not only in terms of intensity of staining, but also in the pattern of reactivity. For example, whilst serum from PBS strongly reacted with antigens in the nucleus and cytoplasm of Hep-2 cells, serum from ES-62 treated mice appeared to recognise the cell membranes

of the Hep-2 cells. Although, the reasons for this altered specificity have not been defined, it is possible that it reflects the anti-phosphorylcholine antibody responses induced by the PC-moieties on ES-62; both IgM and IgG antibodies against PC were significantly elevated in the serum of ES-62 treated mice and it is likely that these recognise phosphatidylcholine in plasma membranes (Fig. 4.11D & E). Although the high titers of these anti-PC antibodies may also have masked the effects of ES-62 on total IgM and IgG (auto) antibody production, they further indicate that the parasite product does not act to suppress all antibody production but rather, acts selectively to target pathogenic ANA and cytoplasmic specificities.

B cell production of IL-6 has recently been shown to be required for Th17 mediated autoimmunity in mice (235) and consistent with its ability to suppress polarization of Th17 responses in mice with CIA, ES-62 is known to reduce IL-6 production by macrophages and DC (see chapter 5) (331,336). It was surprising therefore to find that the number of IL-6 producing cells was elevated in the spleens and rLN, but not the pLN and kidney, of ES-62 treated mice (Fig 4.12A-E). However, the proportion of B cells that were producing IL-6 was significantly reduced in the spleen and pLN although they were significantly increased in the rLN. This latter finding may indicate a modulation of the migration of pathogenic cell types from the renal LN into the kidney.

4.2.3. ES-62 induces IL-10-producing Bregs with a MZP phenotype

The reduction of the levels of IL-6-producing B cells as well as the elevated levels of MZP B cells in the spleen induced by treatment with ES-62 (Fig. 4.4A) could suggest that this subset contains representatives of the Breg population previously shown to have the capacity to suppress development of pathology in the MRL/*lpr* mouse and patients with SLE [18, 19], especially as the kinetics of the decline of this population in MRL/*lpr* mice correlates with disease development (Fig. 4.1H). Supporting this, the proportion (and number) of IL-10 producing B cells was significantly increased in the spleen, rLN and kidneys, but not pLN, of ES-62-treated MRL/*lpr* mice (Fig. 4.13A-D). Moreover, these IL-10-producing B cells predominantly comprised the MZP phenotype that was increased in the spleen (Fig. 4.4A); interestingly, this population was also elevated in the blood of mice treated with ES-62 *in vivo* (Fig 4.13E). Reflecting this induction of IL-10-producing

B cells, exposure to ES-62 also increased the levels of IL-10 found in the interstitial fluid of the kidneys from MRL/*lpr* mice (Fig. 4.13F).

Follicular T helper (Tfh) cells have recently been shown to promote the development of IL-10-producing regulatory B cells via the provision of IL-21 (405). This role is perhaps surprising as Tfh cells are also required for the development of pathogenesis in lupus prone mice (406), suggesting that it is the precise functional phenotype of Tfh cells that is important. Nevertheless, it was interesting to observe that the number of splenic Tfh cells was significantly increased in ES-62 treated mice and correlated with the increased levels of IL-10 producing B cells in the spleens of ES-62 treated mice. By contrast the levels of Tfh cells in the pLN, rLN or kidneys of MRL/*lpr* mice were not modulated by exposure to ES-62 (Fig. 4.14A & B). Tfh cells are also found in the blood of SLE patients and may be linked to the survival and function of antibody secreting cells(407); these cells were present in the blood of MRL/*lpr* mice but were unaltered in ES-62 treated mice (Fig. 4.14C). Interestingly, the proportion and number of Tfh cells were not modulated by treatment with ES-62 prior to the onset of disease; this corresponded with no effect on the levels of IL-10 producing B cells and perhaps suggests that this putative Breg population cells are sufficient at suppressing the immune response prior to the onset of disease (data not shown).

IL-10-producing Bregs have been proposed to mediate their effects, at least in part, via the induction of regulatory T cells, particularly IL-10-producing $CD4^+$ T cells (Tr1 cells), in MRL/*lpr* mice [18, 19]: however, the Breg-dependent induction of either Tr1 or Foxp3 Tregs has also been reported depending on the model of rheumatoid arthritis [37, 38] and asthma (353). Consistent with the reported role for Tregs in protecting against development of lupus (408,409), preliminary data showed that the proportion of $Foxp3^+CD4^+$ Tregs in the LNs of MRL/*lpr*, but not MRL/MP, mice declined with age and with kinetics that correlated with the initiation and progression of proteinuria (McGrath, Harnett & Harnett, unpublished data). However, treatment with ES-62 did not reverse this trend and did not induce enhanced levels of $CD4^+CD25^+Foxp3^+$ T cells (Fig. 4.15A & B) or IL-10-producing Tr1 cells (Fig. 4.15A & C) in the spleens, LNs or kidneys of MRL/*lpr* mice; indeed, at some sites ES-62 reduced the proportion of T cells with a regulatory phenotype.

Collectively, therefore, these data suggest that if ES-62-mediated protection is associated with the induction of Bregs, they do not mediate their effects via the induction of regulatory T cells.

IL-10-producing regulatory B cells have been reported to modulate T helper cell responses and in particular, suppress the development of pathogenic Th17/Th1 responses in mouse models of rheumatoid arthritis (345). Although such suppression was reported to be dependent on the induction of regulatory T cells, it was decided to investigate whether the increase in IL-10 producing B cells in the pLN, rLN and kidneys of ES-62 treated mice correlated with the modulation of IL-17A and IL-22 production in the spleen as in the LN and kidney (Fig. 4.16; see chapter 3). This analysis revealed that the increase in IL-10-producing B cells in the spleen indeed correlated with a reduction in the IL-22-producing and an increase in the IL-17-producing population of splenocytes during the established phase of disease in spleens of ES-62 treated mice; although there was a significant increase in the levels of lin^- cells producing IL-22, this differential modulation of IL-17A and IL-22 production appeared to reflect a general effect on cytokine production rather than targeting of a specific phenotype (Fig. 4.16).

4.2.4. Adoptive transfer of splenic B cells from ES-62-treated MRL/*lpr* mice mimics the ability of ES-62 to suppress development of proteinuria in recipient MRL/*lpr* mice

In order to directly test whether protection afforded by exposure to ES-62 reflected resetting of the effector: regulatory B cell balance, in particular by inducing regulatory B cells, we examined the effect of adoptively transferring purified splenic B cells (~83.55% ± 4.17), harvested from 21-week-old MRL/*lpr* mice, treated with either PBS or ES-62, into recipient 7-week old MRL/*lpr* mice. Analysis of the composition of the transferred B cells (Table 4.1) revealed that the B cell population from ES-62-treated mice was enriched in MZP B cells and reduced in plasmablasts/plasma cells relative to that of PBS-treated mice, albeit these differences did not reach significance. Strikingly, the transfer of B cells from ES-62, but not PBS, treated MRL/*lpr* mice was sufficient to provide significant protection against the development of proteinuria in the recipient mice (Fig. 4.17A & B).

Interestingly, although they did not confer protection, transfer of the B cells from the PBS-treated mice resulted in a profile of proteinuria that was of a somewhat remitting and relapsing nature perhaps reflecting the existence of some "regulatory B cells" that were induced in the donor mice in attempt to control inflammation during the established phase of pathology (Fig. 4.17B). As with treatment with ES-62, there was no substantial improvement in kidney histology (Fig. 4.18).

Consistent with their ability to mimic the action of ES-62, transfer of B cells from ES-62-treated mice similarly resulted in suppressed levels of pathogenic ANA in recipient mice, as indicated by the reduction in nuclear and cytoplasmic staining relative to antibodies from "control" MRL/*lpr* mice (PBS i.v.) or MRL/*lpr* mice receiving B cells from PBS-treated mice (Fig. 4.19A): indeed, serum from mice that received B cells from PBS-treated donor mice contained higher levels of ANA as indicated by elevated staining intensity. Likewise, the reduction in nuclear and cytoplasmic staining by the serum from mice receiving B cells from ES-62 treated mice was associated with strong plasma membrane staining which presumably reflected the ES-62-mediated switch to anti-PC responses.

The transfer of B cells from ES-62 treated mice resulted in an reduction of splenocytes and lymphocytes, although there were more cells found in the kidneys (Fig. 4.20A). Consistent with a role for Il-10-producing regulatory B cells, protection, as with ES-62 treatment, was associated with increased levels of IL-10-producing B cells of the MZP phenotype in the rLN, but not the spleen, albeit these did not reach statistical significance (Fig. 4.20B-E). As in mice treated with ES-62, the transfer of B cells from ES-62 treated donor mice appeared to have no effect on the proportion or number of regulatory T cells in the spleen or rLN (Fig. 4.20F & G).

Intriguingly, preliminary data suggest the transfer of B cells from ES-62 treated donor mice appeared to increase the proportion of cells producing IL-17A in the spleen and rLN, albeit not significantly in the latter case whilst decreasing the numbers of IL-22-producing cells in the spleen and rLN. As with treatment with ES-62, no clear IL-17A-producing population was modulated by ES-62, although the proportion of IL-17A producing B cells in the spleen was significantly elevated in

the mice that received B cells from ES-62 treated donors; however, whilst the proportions of CD4+ T cells producing IL-22 were rather surprisingly elevated, the proportion of IL-22 producing DN T cells was significantly reduced in the rLN of mice that received B cells from ES-62 treated donors, a finding reminiscent of mice treated with ES-62 (Fig. 4.21).

4.3. Discussion

In the search for novel, safe and effective treatments for autoimmune disease, much research has focused on the ability of parasitic helminths to suppress the mammalian immune system via the production of immunomodulatory molecules. One such molecule, ES-62, suppresses disease is mouse models of autoimmune arthritis (335,336) and allergic airway hyper-responsiveness (228,338) via modulation of IL-17A responses. ES-62 has now also been shown to suppress the development of proteinuria in the MRL/*lpr* mouse model of SLE via modulation of the IL-17A/IL-22 axis (Chapter 3). In this chapter, the impact of ES-62 on effector and regulatory B cell populations in the MRL/*lpr* mouse was also investigated. ES-62 was found to promote the expansion of IL-10-producing B cells whilst suppressing the development of pathogenic plasmablast populations and associated ANA production. Furthermore, the protective effect of ES-62 was mimicked by adoptive transfer of $CD43^-$ B2 B cells from ES-62-treated donor MLR/*lpr* mice (21-week old) to recipient (7-week old) MRL/*lpr* mice.

The MRL/*lpr* mouse model of SLE portrays several pathologies associated with the human disease and as in other models of SLE, B cells are essential for the development of disease (162,410). In support of this, the proportions and numbers of several mature B cell populations, mainly the marginal zone and follicular B cells, expand in a manner that correlates with disease. As ES-62 modulates BCR and NFκB signaling, both of which being important in the peripheral development and activation of B cells in the spleen (174) it was hypothesized that the parasite product exerted its protective effects, at least in part, by modulating the levels and/or responsiveness of peripheral B cell subsets. Treatment with ES-62 was indeed found to modulate the proportions and numbers of MZP, Fo1 and Tfh cells in the spleen. The increase in Fo1 cells suggested that ES-62 may prevent these cells from entering the germinal center reaction, however, the level of GC B cells or downstream antibody producing populations, in the spleen, were not reduced by ES-62.

Although the proportion of $CD138^+B220^-CD19^-$ long-lived plasma cells (352) were slightly reduced in the blood of mice treated with ES-62, their levels were

maintained in the spleen, pLN and rLN and, if anything, were slightly elevated in the kidneys of treated mice. Similarly, ES-62 did not modulate the levels of $CD138^{+}B220^{+}CD19^{+}$ pre-plasma cells, which have been reported to secrete autoantibodies and break tolerance in the absence of control by DC or macrophages (356). By contrast, the proportions of $CD138^{+}B220^{low}CD19^{+}$ B cells were reduced in the blood, kidney and renal draining LN of ES-62 treated mice. These cells, which are reported to have plasmablast-like qualities (355,411) but may also represent short-lived plasma cells (352), are associated with disease flares in SLE patients and are thought to be the source of pathogenic anti-dsDNA antibodies (3,195,226). Interestingly, therefore, their reduced levels were reflected by a reduction in the levels of ANA in both the serum and renal interstitial fluid of the ES-62-treated mice.

Despite reducing ANA production, ES-62 had no effect on total antibody levels, perhaps reflecting its lack of modulation of the plasma cell population. However, the parasite product has been shown in the past to induce anti-PC responses in mice and ES-62 treated MRL/*lpr* mice exhibited significantly elevated levels of anti-PC IgG and IgM antibodies, which may mask the loss of some specificities produced in PBS-treated mice. Moreover, the high concentration of anti-PC antibodies possibly explains the atypical cell membrane staining of Hep-2 ANA slides by the serum from ES-62 treated mice. Interestingly, anti-PC responses have been proposed to be protective in SLE, particularly with respect to atherosclerotic complications, by inhibiting inflammatory phospholipids such as PAF(412), which have been implicated in the pathogenesis (413) and promoting the clearance of oxidised lipids and apoptotic cells that is defective in SLE. Such oxidized lipids may be involved in the activation of Th1 cells (414) and have been shown to promote monocyte recruitment to sites of inflammation via the activation of MCP-1 production (415). Anti-PC antibodies may also promote the clearance of apoptotic cell debris by binding to phosphatidylserine; they are therefore believed to be beneficial in SLE as well as other autoimmune diseases (412). In addition, ES-62 has previously been shown to induce high levels of IgG1, but not IgG2a, antibodies to non-PC-epitopes of the parasite molecule in naive wild type BALB/c mice and such antibodies may also mask a decrease in total immunoglobulin responses to lupus-relevant antigens. Interestingly, given that these current

studies show that ES-62 can induce IL-10-producing B cells that appear to contribute to the protective effects of ES-62 in the MRL/*lpr* mouse, in IL-10$^{-/-}$ Balb/c mice, ES-62 induces both IgG1 and IgG2a antibodies, suggesting that IL-10 plays an important role in the suppression of IgG2a specificities by ES-62 (327).

There is increasing evidence that protection against immune mediated disease by some helminth products may be, at least in part, via the activation of regulatory B cell function. Indeed, multiple sclerosis patients infected with helminths exhibit less severe disease and this appears to be associated with elevated levels of IL-10 producing B cells. Support for this has also been provided by experimental studies where, for example in a mouse model of asthma, IL-10 producing B cells induced by the helminth *S. mansoni* can suppress disease (353,416,417). Interestingly, therefore, whilst it has previously been reported that ES-62 stimulates production of IL-10 by peritoneal B cells, suppression of pathogenic antibody production, by the parasite product, in both the collagen induced model of arthritis and the ovalbumin induced model of asthma is associated with elevated levels of IL-10 producing B cells (chapter 6 and Rodgers, Coltherd Harnett & Harnett, unpublished data) (326,335,338). Thus, taken together with the results of this study in MRL/*lpr* mice, there is increasing evidence that ES-62 may protect against harmful inflammation by promoting IL-10-producing (regulatory) B cells.

CD19^{+}CD21^{+}CD23^{+} T2-MZP B cells, have previously been shown to be protective in the MRL/*lpr* mouse and *in vitro* or *in vivo* stimulation of these cells by anti-CD40 can enhance the suppressive qualities of this population (244). This present study has shown that this population declined from 12-weeks of age, the dynamics of which inversely correlated with the rise in proteinuria, providing a cellular rationale for the report by Shlomchik and colleagues that Bregs in the MRL/*lpr* mouse are ineffective (418). Furthermore, treatment with ES-62 significantly increased the proportion of such MZP B cells in the spleen and blood of MRL/*lpr* mice and consistent with these cells having regulatory capabilities, the majority of IL-10-producing B cells in ES-62-treated mice had this phenotype. It has been reported that Bregs act upon regulatory T cells (Treg or Tr1 cells) (345,419), however, ES-62 had no effect on any of the regulatory T cell phenotypes evaluated and this may

suggest that in response to ES-62, Bregs can disseminate around the body to act directly at the site of inflammation.

Consistent with the proposed protective roles for Bregs, IL-10 is an important regulatory cytokine in SLE: indeed, disease is exacerbated in IL-$10^{-/-}$ MRL/*lpr* mice [51]. However, IL-10 is not always protective in lupus due to its ability to promote B cell antibody production. Thus, the precise effect of IL-10 (and other cytokines) is likely determined by the immunological context in which the cytokine is released, for example the immunological receptors engaged in cell-cell interactions at the site of inflammation. It is interesting to note therefore that ES-62 reduces the surface expression of co-stimulatory molecules that are likely to promote autoimmune responses whilst increasing that of negative feedback receptors, such as FcγRIIb, on B cells circulating in the blood, spleen and kidney. The upregulation of FcγRIIb expression is particularly interesting as polymorphisms of this negative feedback antibody receptor (15) are associated with the development of lupus in patients (420); indeed, the loss of this receptor in C57BL/6 and MRL/MP mice causes a lupus-like phenotype that is characterized by the development of anti-dsDNA responses (15,229,421). Thus, by increasing the expression of FcγRIIb on B cells in the spleen and circulating in the blood, ES-62 may promote tolerance by controlling the expansion of autoreactive B cells. The down regulation of CD80 may also be interesting as ligation of CD80 has been shown to reverse the suppressive action of human $CD19^{+}CD24^{hi}CD38^{hi}$ regulatory B cells [19] and to be important in the regulation of Tfh development, consequent GC B cell survival and plasma cell production[36]. Thus, collectively, the increase in BAFF-R by B cells, along with the decrease in pro-inflammatory receptors, may indicate that ES-62 promotes the survival of hypo-responsive B cells which may act as ‘sinks’ for circulating BAFF and hence indirectly suppress autoimmunity promoted by the elevated levels of BAFF found in MRL/*lpr* mice.

Recently it has been proposed that follicular helper T cells promote the development of regulatory B cells via the production of IL-21 (405): it is interesting therefore that this population in the spleen was expanded by ES-62. Although it is not clear whether the remainder were enriched in IL-21-producing cells, that can promote Breg induction, it is tempting to speculate therefore that ES-62 may

modulate B cell hypo-responsiveness via such T cells. However, such hypo-responsiveness could also be promoted by DC or macrophages, as ES-62 suppresses pro-inflammatory responses by these cells, which have also been reported to be involved in the regulation of B cell activation (173).

To determine whether the protective effects of ES-62 in the MRL/*lpr* mouse were predominantly B cell mediated, $CD43^-$ B2 B cells were adoptively transferred from PBS or ES-62 treated 21-week old MRL/*lpr* mice into recipient 7-week old MRL/*lpr* mice: such B cells were sufficient to almost completely abrogate development of proteinuria in recipient mice. This protection, rather surprisingly given that by the cull date these B cells had not been exposed to the parasite product for 3 months, essentially mimicked that afforded by treatment with ES-62 as indicated by decreased ANA and atypical plasma membrane staining of Hep2 cells, as well as an increased proportion of IL-10-producing B cells and modulation of IL-17A- and IL-22-producing splenocytes. Although it is tempting to attribute the success of this treatment to IL-10-producing (regulatory) B cells that were enriched in the population derived from ES-62-treated mice, it should be noted that plasmablasts/plasma cells were depleted from this population relative to that derived from PBS-treated mice. Thus, although it is possible that the transfer of IL-10 producing B cells protected the recipient mice, as the ANA staining from mice that received B cells from ES-62 treated donors replicated that of the ES-62-treated donor mice, it is also possible that protection reflects, at least in part, the transfer of anti-PC plasmablasts/plasma cells or MZ, Fo1 or GC B cells with differential specificities and/or hyporesponsive phenotype that compete with pathogenic specificities for survival factors such as BAFF.

B cell depletion therapy is protective in some SLE patients; however, the therapeutic promise of B cell depletion in SLE patients has not materialized as Rituximab (anti-CD20) failed to meet its primary outcomes in phase III clinical trials and Belimumab (anti-BAFF) is only effective in approximately 14% of patients (38). An explanation for the ineffectiveness of these pan-B cell depletion strategies in SLE may be that they target protective regulatory B cells as well as pathogenic populations. However, as regulatory B cells appear to be ineffective in SLE patients and mouse models, this may not be the case (247,418). Nevertheless,

studies in mouse models of autoimmunity have revealed that the effect of B cell depletion depends upon timing and the status of disease (54) as indicated in lupus prone mice where B cell depletion in 4 week old NZB/W mice exacerbates disease (422), perhaps suggesting that Bregs antagonising inflammation prior to onset of pathology are being targeted in this case: this suggestion resonates with data presented here, that show that the highest levels of MZP cells, which are the candidate IL-10 producing population induced by ES-62, are found in MRL/*lpr* mice at 12-weeks, immediately prior to onset of pathology. Moreover, as B cells produce the pathogenic autoantibodies associated with SLE, they remain a valid therapeutic target for further development (244,423), perhaps especially if they incorporate information exploiting mechanisms utilised by helminths to promote enhanced levels of IL-10-producing (regulatory) B cells and/or targeting antibody-producing plasma cells.

Table 4.1: cellular composition of adoptively transferred B cells. The proportions ± SEM of T1-3, transitional 1-3; MZP, marginal zone precursor; MZ, marginal zone; Fo, follicular; GC, germinal center; pPc, pre-plasma cell; Pb, plasmablast and P, plasma cells over two independent experiments. Statistical significance was shown using the student's t-test.

	PBS	ES-62	P value
T1	2.40 ± 0.44	1.97 ± 0.28	0.21
T2	1.07 ± 0.16	0.79 ± 0.11	0.086
T3	6.03 ± 0.60	4.53 ± 0.65	0.058
MZP	5.37 ± 1.71	6.45 ± 1.46	0.31
MZ	1.95 ± 0.73	1.42 ± 0.39	0.26
Fo1	4.16 ± 0.61	4.76 ± 0.54	0.23
Fo2	0.29 ± 0.07	0.42 ± 0.07	0.1
GC	0.83 ± 0.14	0.90 ± 0.25	0.4
Pb	0.44 ± 0.05	0.33 ± 0.05	0.07
P	2.14 ± 0.78	1.68 ± 0.30	0.29

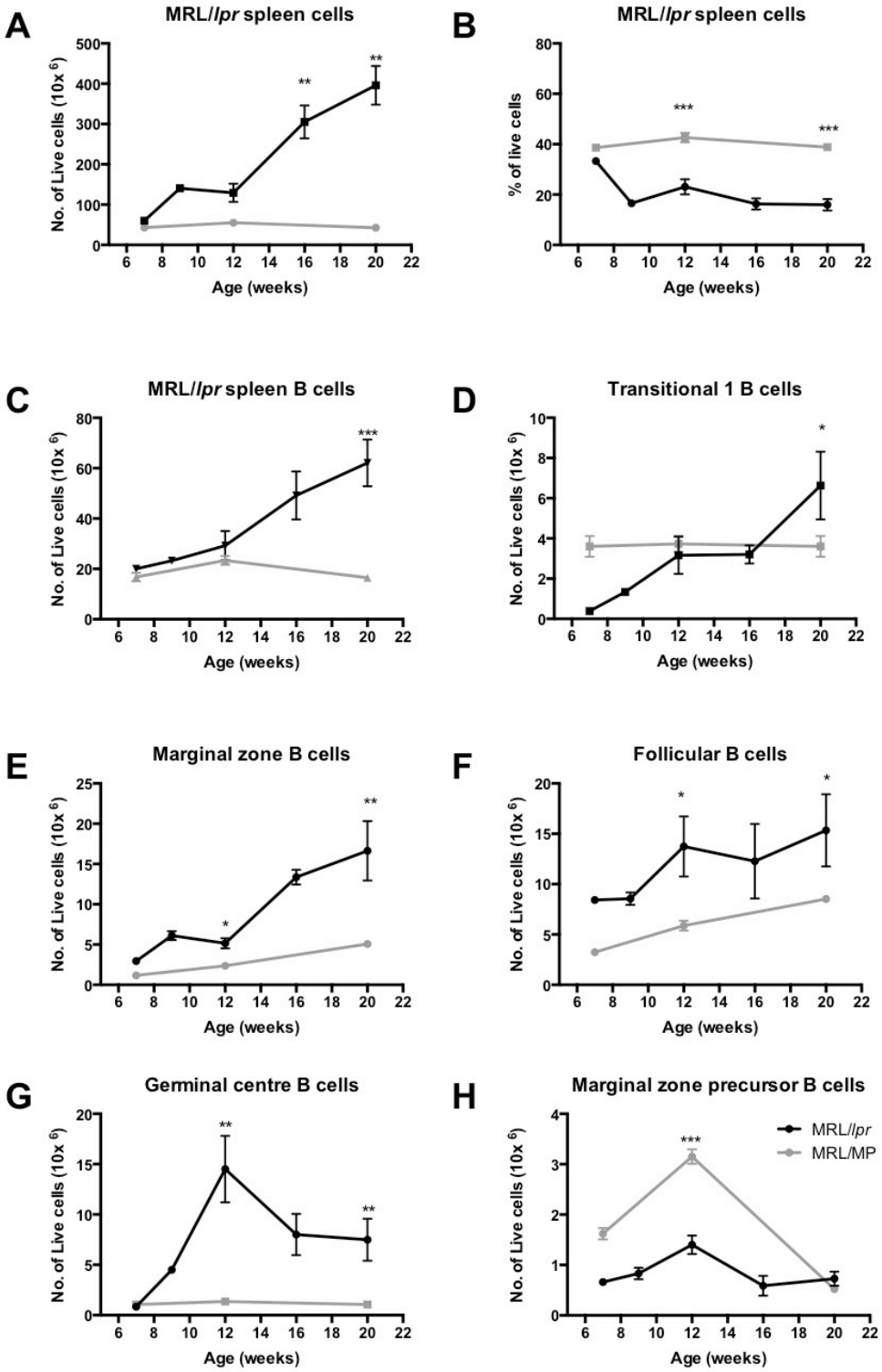

A
MRL/lpr spleen cells
No. of Live cells (10x 6)
Age (weeks)
B
MRL/lpr spleen cells
% of live cells
Age (weeks)
C
MRL/lpr spleen B cells
No. of Live cells (10x 6)
Age (weeks)
D
Transitional 1 B cells
No. of Live cells (10x 6)
Age (weeks)
E
Marginal zone B cells
No. of Live cells (10x 6)
Age (weeks)
F
Follicular B cells
No. of Live cells (10x 6)
Age (weeks)
G
Germinal centre B cells
No. of Live cells (10x 6)
Age (weeks)
H
Marginal zone precursor B cells
No. of Live cells (10x 6)
Age (weeks)
MRL/lpr
MRL/MP

Figure 4.1. Splenic B cell dynamics in the MRL/*lpr* mouse. The number of cells from the spleens of MRL/*lpr* mice (n=5 for each time point) were counted (A) and the proportion (B) and the number (C) of $CD19^+$ B cells were determined. The number of Transitional 1 (T1; D; $CD19^+CD23^-CD21^-IgM^+IgD^-$), marginal zone (MZ; E; $CD19^+CD23^-CD21^+IgM^+IgD^-$), follicular (Fo; F; $CD19^+CD23^+CD21^{low/-}IgM^{+/-}IgD^+$), germinal center (GC; G; $CD19^+CD43^-CD24^+GL7^+$) and marginal zone precursor (MZP; H; $CD19^+CD23^+CD21^+IgM^+IgD^+$) B cells were determined by multicolor flow cytometry. The data are representative of one experiment and the student's t-test was used at each time point to determine statistical significance; *=$p<0.05$, **=p,0.01 and ***=$p<0.001$.

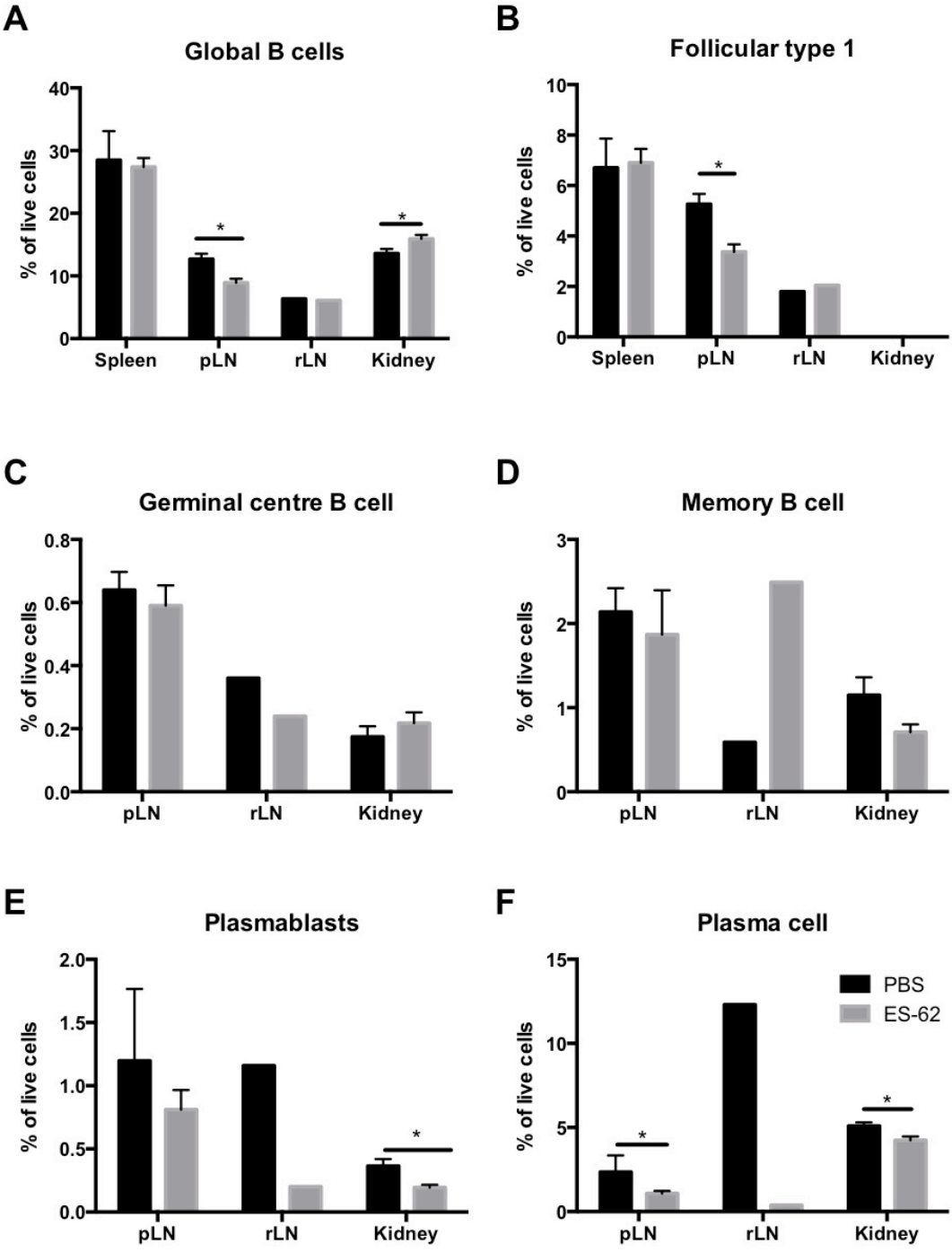

A
Global B cells
% of live cells
40
30
20
10
0
Spleen
pLN
rLN
Kidney
*
*
B
Follicular type 1
% of live cells
10
8
6
4
2
0
Spleen
pLN
rLN
Kidney
*
C
Germinal centre B cell
% of live cells
0.8
0.6
0.4
0.2
0.0
pLN
rLN
Kidney
D
Memory B cell
% of live cells
3
2
1
0
pLN
rLN
Kidney
E
Plasmablasts
% of live cells
2.0
1.5
1.0
0.5
0.0
pLN
rLN
Kidney
*
F
Plasma cell
% of live cells
15
10
5
0
pLN
rLN
Kidney
*
*
PBS
ES-62

Figure 4.2. The effect of ES-62 on peripheral B cell populations, prior to the onset of disease. The number of $CD19^+$ B cells from the spleen, pLN, rLN and kidney of 12-week MRL/*lpr* mice treated with PBS (black bar, n=5) or ES-62 (grey bar, n=5) was determined by flow cytometry (A). The proportion of follicular type 1 (B; $CD19^+CD23^+CD21^{low/-}IgM^-IgD^+$), germinal centre (C; $CD19^+CD43^-CD24^+GL7^+$), memory (D; $CD19^+B220^+IgM^-IgD^-IgG^+$), plasmablast (E; $Dump^-B220^-CD19^+$) and plasma (F; $Dump^-B220^-CD19^-$) cellslwas determined by flow cytometry; where the dump channel contained antibodies against CD3, CD4, CD8, CD11c, CD11b, F4/80 and GR1. The data are presented as the mean values of individual mice ± SEM, except for the rLN, which are the values for cells pooled from the 5 mice due to limitations of cell numbers at this time point. The data are representative of three independent experiments and presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

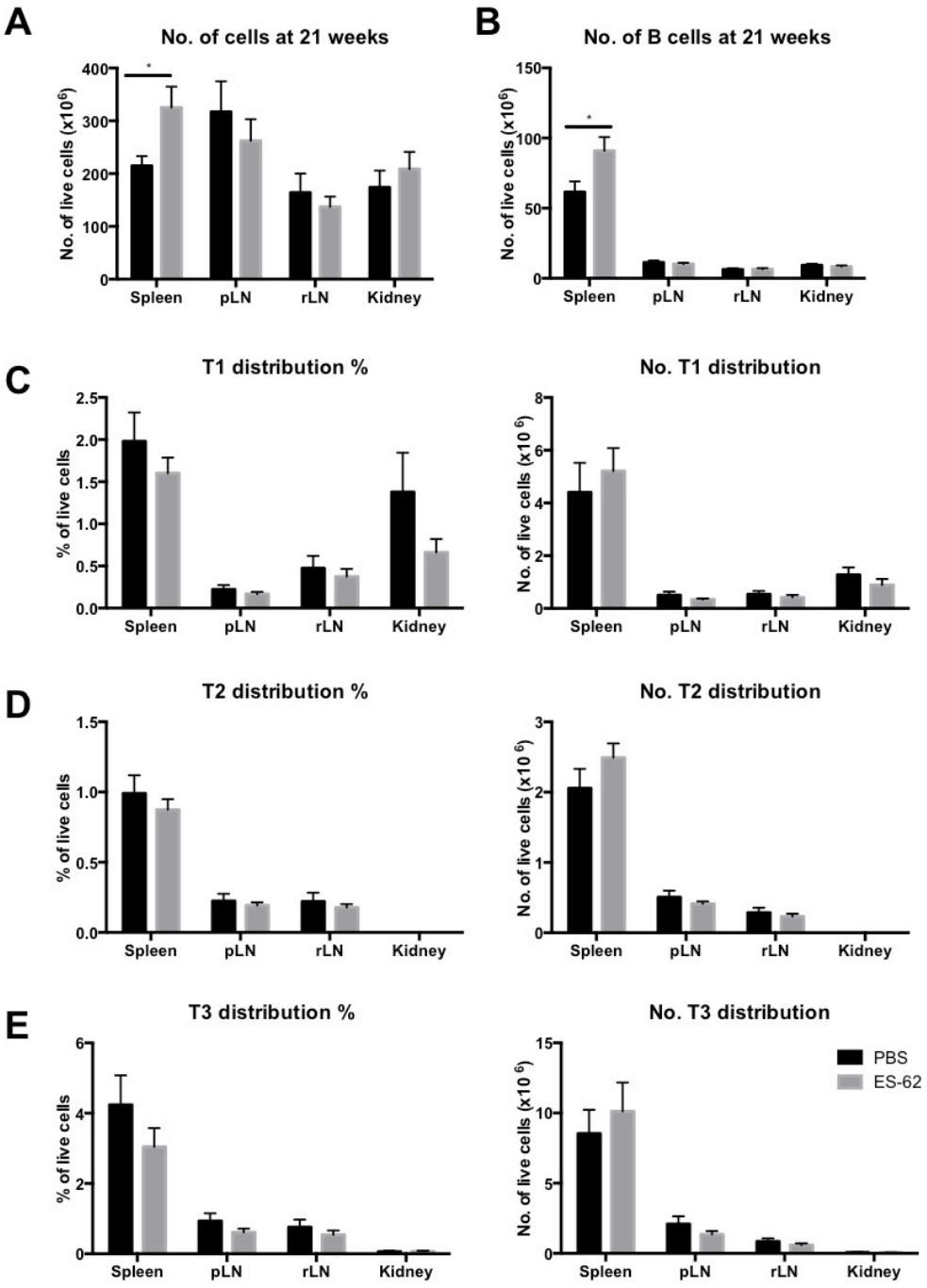

A
No. of cells at 21 weeks
No. of live cells (x10^6)
400
300
200
100
0
*
Spleen
pLN
rLN
Kidney
B
No. of B cells at 21 weeks
No. of live cells (x10^6)
150
100
50
0
*
Spleen
pLN
rLN
Kidney
C
T1 distribution %
% of live cells
2.5
2.0
1.5
1.0
0.5
0.0
Spleen
pLN
rLN
Kidney
No. T1 distribution
No. of live cells (x10^6)
8
6
4
2
0
Spleen
pLN
rLN
Kidney
D
T2 distribution %
% of live cells
1.5
1.0
0.5
0.0
Spleen
pLN
rLN
Kidney
No. T2 distribution
No. of live cells (x10^6)
3
2
1
0
Spleen
pLN
rLN
Kidney
E
T3 distribution %
% of live cells
6
4
2
0
Spleen
pLN
rLN
Kidney
No. T3 distribution
No. of live cells (x10^6)
15
10
5
0
Spleen
pLN
rLN
Kidney
PBS
ES-62

Figure 4.3. The effect of ES-62 on transitional B cell populations during established disease. The number of total cells (A) and $CD19^+$ B cells (B) from the spleen, pLN, rLN and kidney of 21-week old MRL/*lpr* mice treated with PBS (black bar, n=11) or ES-62 (grey bar, n=18) was determined by flow cytometry. The proportion and number of T1 (C; $CD19^+CD23^-CD21^-IgM^+IgD^-AA4.1^+$), T2 (D; $CD19^+CD23^+CD21^-IgM^+IgD^+AA4.1^+$) and T3 (E; $CD19^+CD23^+CD21^-IgM^{low/-}IgD^+AA4.1^+$), was determined by flow cytometry. The data are representative of three independent experiments and presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

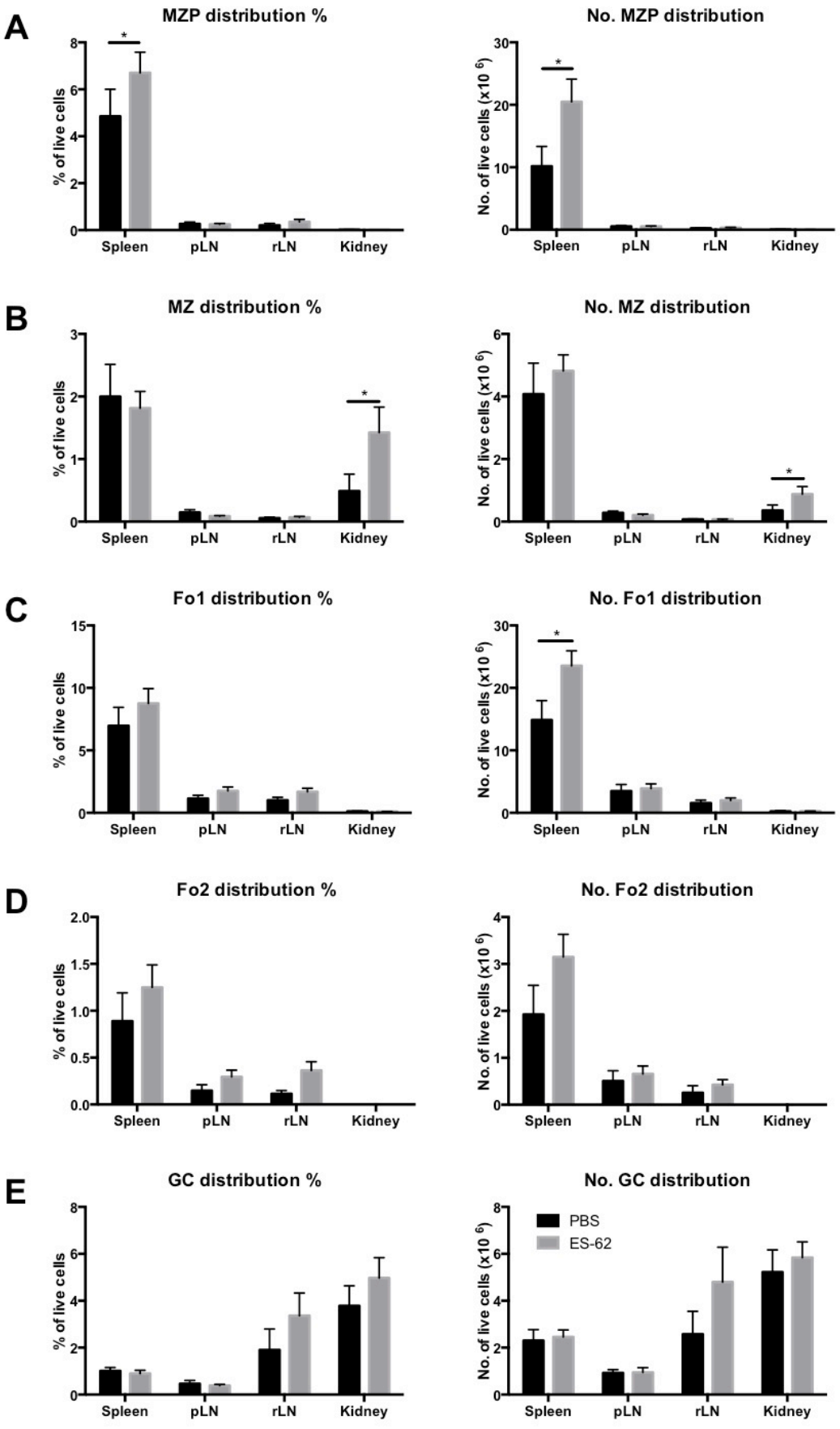

A
MZP distribution %
% of live cells
Spleen
pLN
rLN
Kidney
No. MZP distribution
No. of live cells (x10 6)
B
MZ distribution %
No. MZ distribution
C
Fo1 distribution %
No. Fo1 distribution
D
Fo2 distribution %
No. Fo2 distribution
E
GC distribution %
No. GC distribution
PBS
ES-62

Figure 4.4. The effect of ES-62 on marginal zone precursor, marginal zone, follicular and germinal centre B cell populations during established disease. The proportion and number of MZP (A; $CD19^+CD23^+CD21^+IgM^+IgD^+$), MZ (B; $CD19^+CD23^-CD21^+IgM^+IgD^-$), Fo1 (C; $CD19^+CD23^+CD21^{low/-}IgM^-IgD^+$), Fo2 (D; $CD19^+CD23^+CD21^{low/-}IgM^+IgD^+$) and germinal center (E; $CD19^+CD43^-CD24^+GL7^+$) was determined by flow cytometry in the spleen, pLN, rLN and kidney of 21-week old MRL/*lpr* mice treated with PBS (black bar, n=11) or ES-62 (grey bar, n=18). The data are representative of three independent experiments and presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

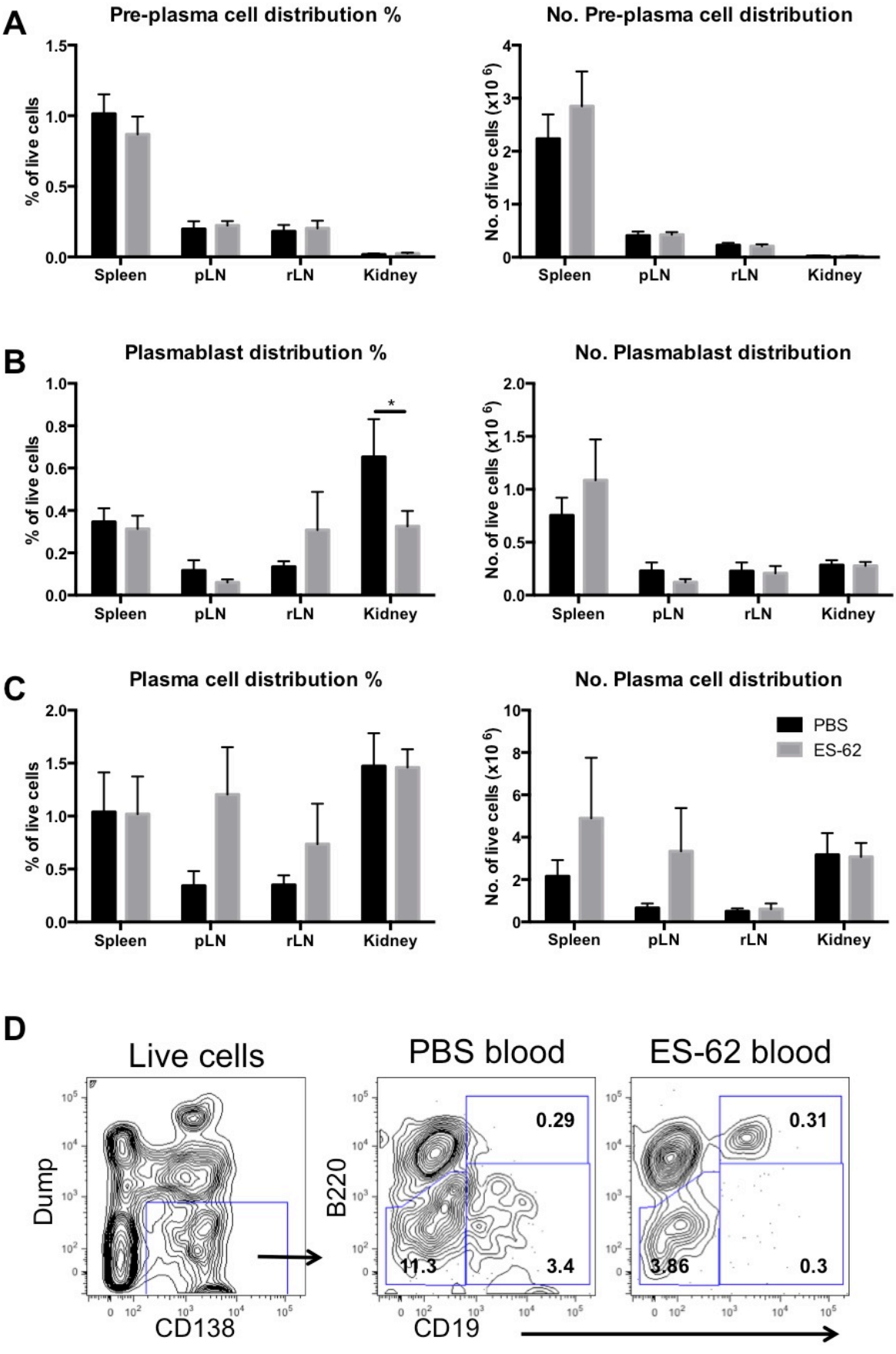

A
Pre-plasma cell distribution %
% of live cells
Spleen
pLN
rLN
Kidney
No. Pre-plasma cell distribution
No. of live cells (x10 6)
B
Plasmablast distribution %
*
No. Plasmablast distribution
C
Plasma cell distribution %
No. Plasma cell distribution
PBS
ES-62
D
Live cells
PBS blood
ES-62 blood
Dump
CD138
B220
CD19
0.29
11.3
3.4
0.31
3.86
0.3

Figure 4.5. The effect of ES-62 on antibody-producing B cell populations during established disease. The proportion and number of pre-plasma (A; $Dump^{-}CD138^{+}B220^{+}CD19^{+}$), plasmablast (B; $Dump^{-}CD138^{+}B220^{-}CD19^{+}$) and plasma cells (C; $Dump^{-}CD138^{+}B220^{-}CD19$) in the spleen, pLN, rLN, kidney (A-C) and blood (D; data representative of two independent experiments) of 21-week old MRL/*lpr* mice treated with PBS (black bar, n=11) or ES-62 (grey bar, n=18) was determined by flow cytometry. The dump channel contained antibodies against CD3, CD4, CD8, CD11c, CD11b, F4/80 and GR1. The data are representative of three independent experiments, unless otherwise stated, and presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

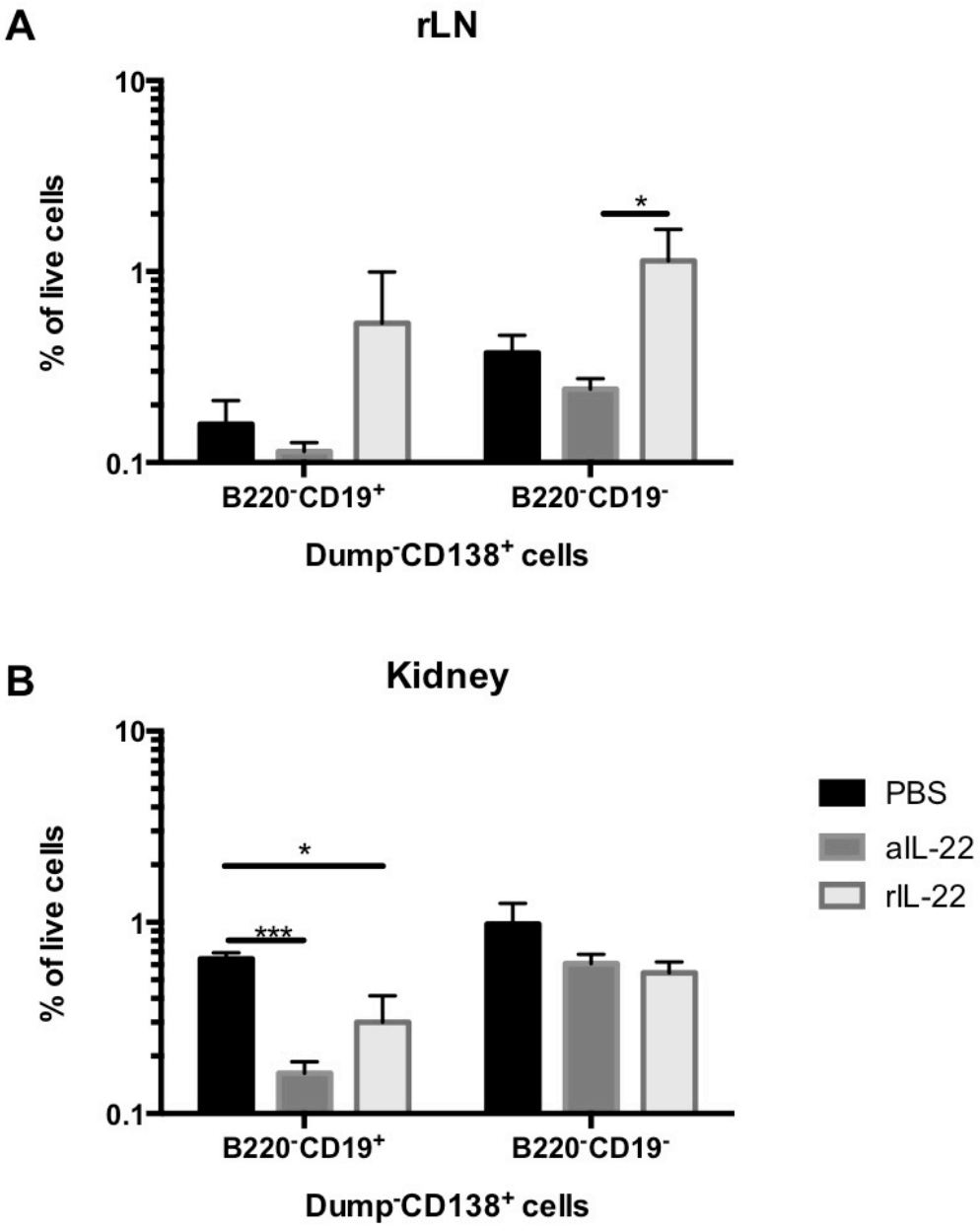

A
rLN
10
1
0.1
% of live cells
*
B220-CD19+
B220-CD19-
Dump-CD138+ cells
B
Kidney
10
1
0.1
% of live cells
*
***
B220-CD19+
B220-CD19-
Dump-CD138+ cells
PBS
aIL-22
rIL-22

Figure 4.6. Treatment of MRL/*lpr* mice with anti-IL-22 suppresses the level of plasmablasts in rLN and the kidney. The proportion $Dump^{-}CD138^{+}B220^{-}CD19^{+}$ plasmablasts and $Dump^{-}CD138^{+}B220^{-}CD19^{-}$ plasma cells was determined, by flow cytometry, in the rLN (A) and kidneys (B) of 21-week MRL/*lpr* mice treated with PBS (black bar, n=4), anti-IL-22 (dark grey bar, n=4) or rIL-22 (light grey bar, n=4). The data are representative of one experiment and presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$ and ***=$p<0.0001$.

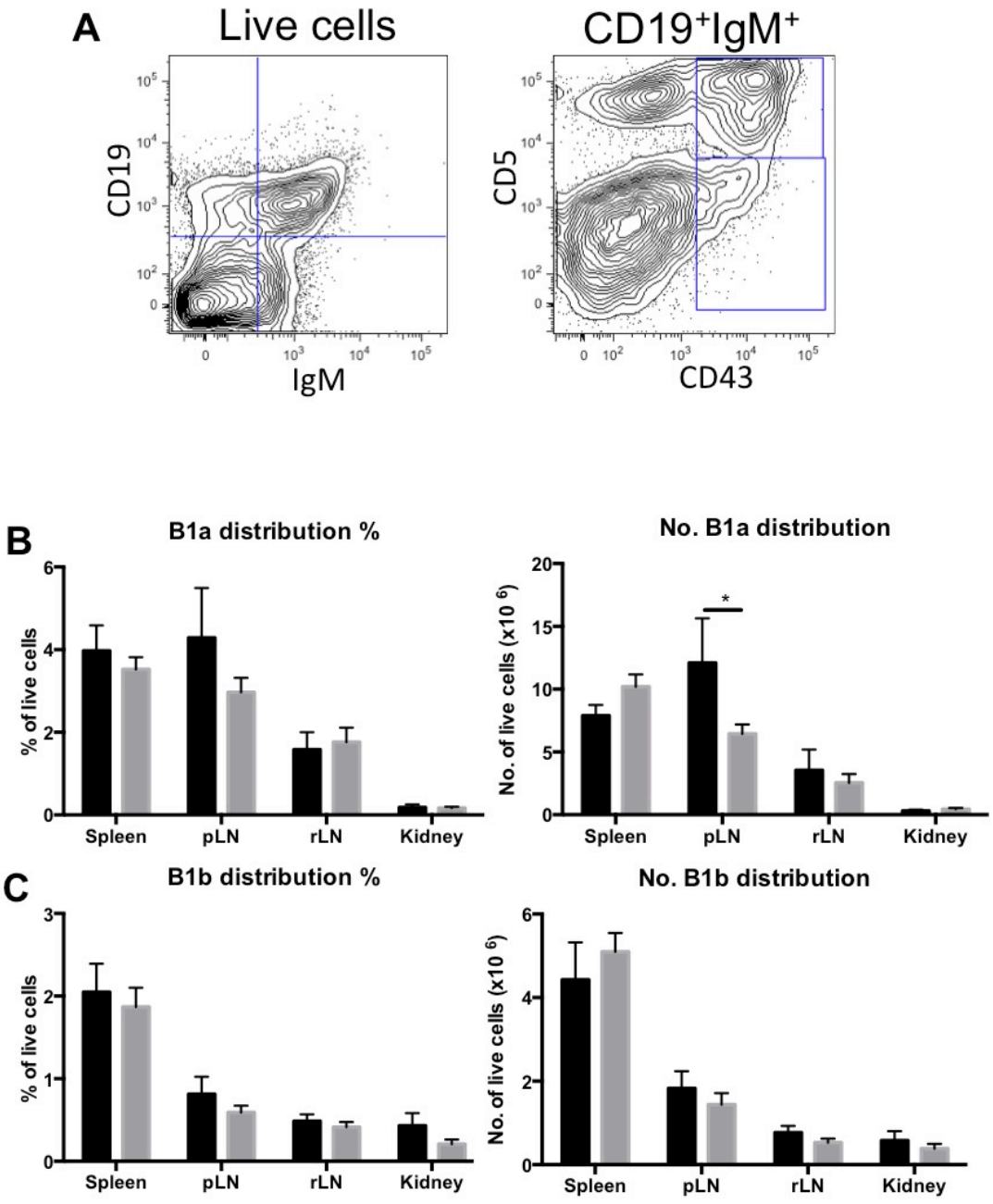

A
Live cells
CD19
IgM
CD19+IgM+
CD5
CD43
B
B1a distribution %
% of live cells
Spleen
pLN
rLN
Kidney
No. B1a distribution
No. of live cells (x10 6)
*
C
B1b distribution %
No. B1b distribution

Figure 4.7. The effect of ES-62 on B1 B cell populations during established disease. The proportion and number of $CD19^+IgM^+CD43^+CD5^+$ B1a (B) and $CD19^+IgM^+CD43^+CD5^-$ B1b (C) B cells in the spleen, pLN, rLN and kidney of 21-week old MRL/*lpr* mice treated with (grey bar, n=18) or without (black bar, n=11) ES-62 was determined by flow cytometry (A). The data were representative of 3 independent experiments and are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

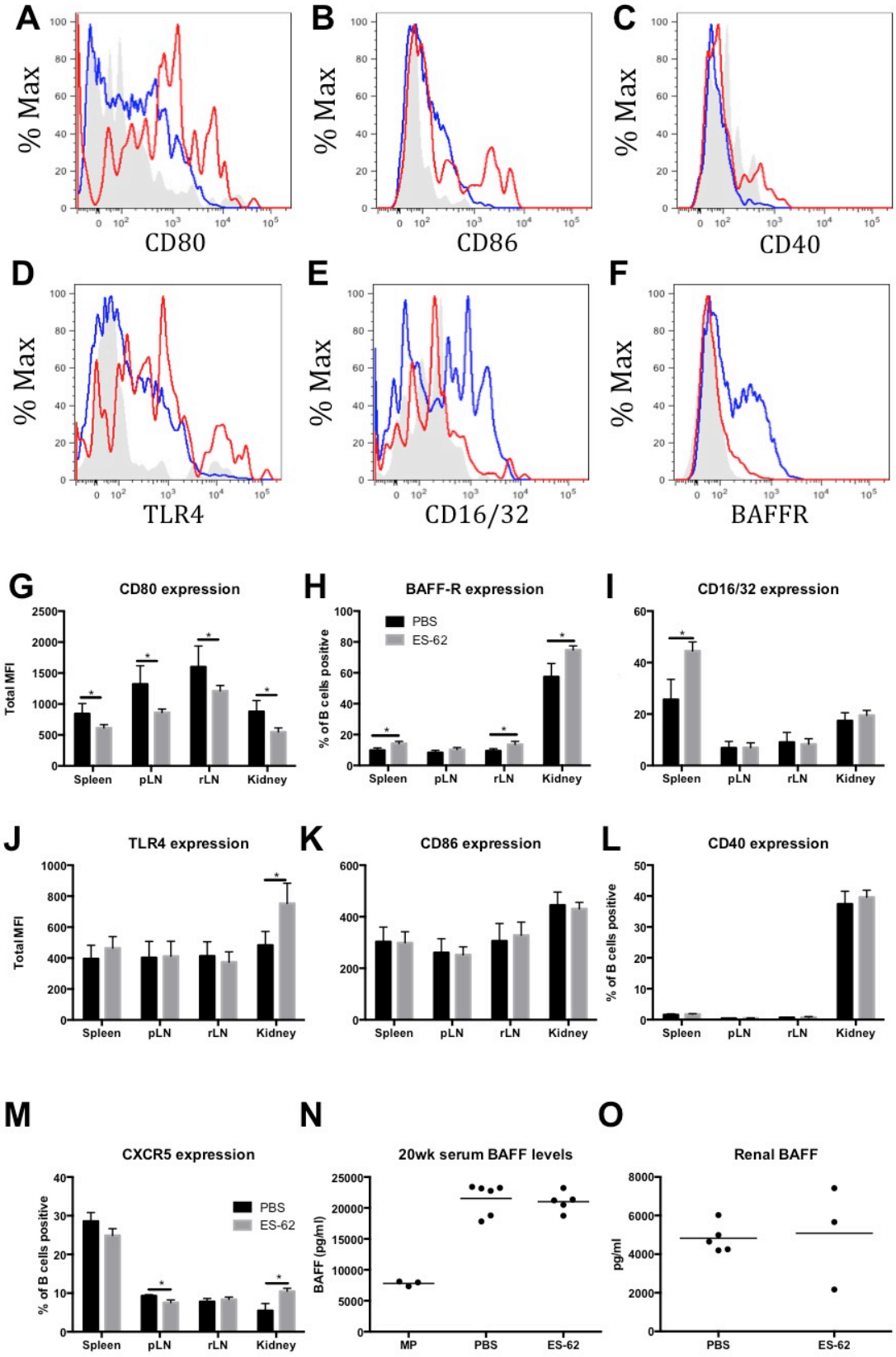

A
% Max
CD80
B
% Max
CD86
C
% Max
CD40
D
% Max
TLR4
E
% Max
CD16/32
F
% Max
BAFFR
G
CD80 expression
Total MFI
Spleen
pLN
rLN
Kidney
H
BAFF-R expression
PBS
ES-62
% of B cells positive
I
CD16/32 expression
J
TLR4 expression
K
CD86 expression
L
CD40 expression
M
CXCR5 expression
N
20wk serum BAFF levels
BAFF (pg/ml)
MP
O
Renal BAFF
pg/ml

Figure 4.8. ES-62 promotes a hyporesponsive B cell phenotype. The expression level of CD80 (A), CD86 (B), CD40 (C), TLR4 (D), CD32 (E) and BAFF-R (F) was determined on B cells circulating in the blood in MRL/*lpr* mice treated with (blue line; 4 mice pooled) or without (red line, 4 mice pooled) ES-62 relative to appropriate isotype controls (grey histogram). The expression levels of CD80 (G), BAFF-R (H), CD32 (I), TLR4 (J), CD86 (K), CD40 (L) and CXCR5 (M) on B cells in the spleen, pLN, rLN and kidney of MRL/*lpr* mice treated with (grey bar, n=18) or without (black bar, n=11) ES-62 was determined by flow cytometry. The concentration of BAFF was measured in the serum of MRL/MP mice, or MRL/*lpr* mice treated with or without ES-62 (N), or renal interstitial fluid of mice treated with or without ES-62 (O) by ELISA (R&D). The data are representative of three independent experiments (except M-O) and are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

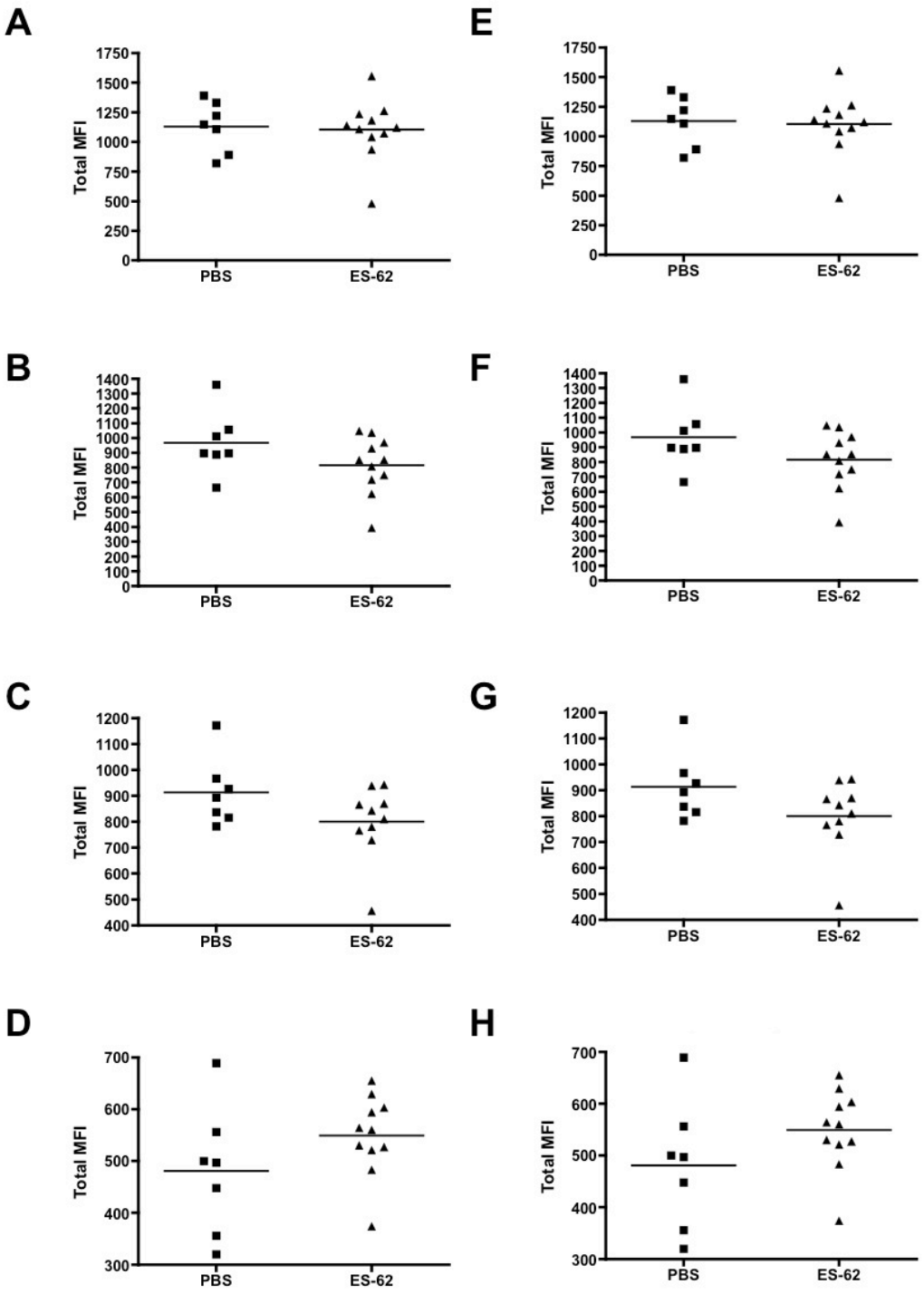
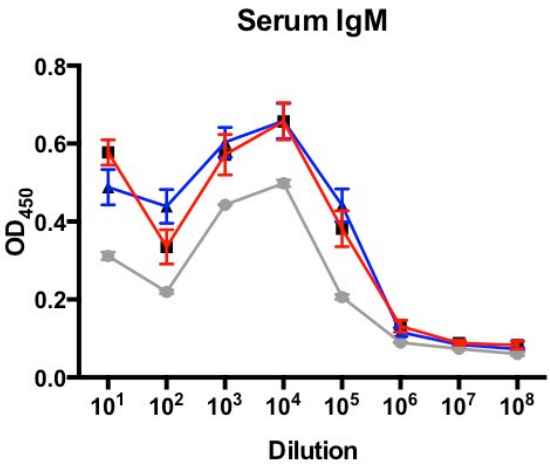
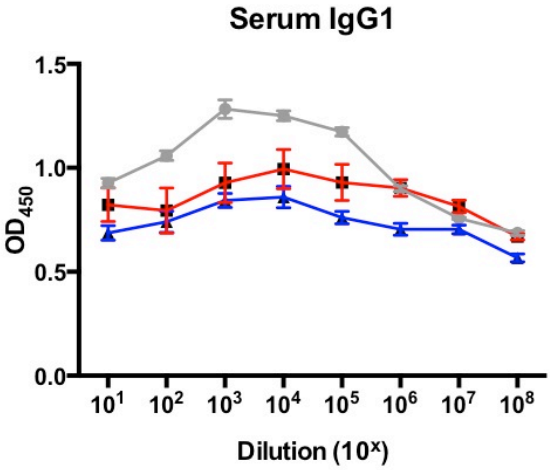

A
Total MFI
1750
1500
1250
1000
750
500
250
0
PBS
ES-62
B
Total MFI
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
PBS
ES-62
C
Total MFI
1200
1100
1000
900
800
700
600
500
400
PBS
ES-62
D
Total MFI
700
600
500
400
300
PBS
ES-62
E
Total MFI
1750
1500
1250
1000
750
500
250
0
PBS
ES-62
F
Total MFI
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
PBS
ES-62
G
Total MFI
1200
1100
1000
900
800
700
600
500
400
PBS
ES-62
H
Total MFI
700
600
500
400
300
PBS
ES-62

Figure 4.9. ES-62 does not modulate the levels of IgM or IgD expression. The mean fluorescence intensity (MFI) of IgM (A-D) and IgD (E-H) expression by B cells was measured, by flow cytometry, in the spleen (A & E), pLN (B & F), rLN (C & G) and kidneys (D & H) of 21-week old MRL/*lpr* mice. The data are representative of three independent experiments and are presented as the mean values of individual mice ± SEM.

A


Serum IgM
0.8
0.6
0.4
0.2
0.0
OD450
10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8
Dilution

B


Serum IgG1
1.5
1.0
0.5
0.0
OD450
10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8
Dilution (10^x)

C

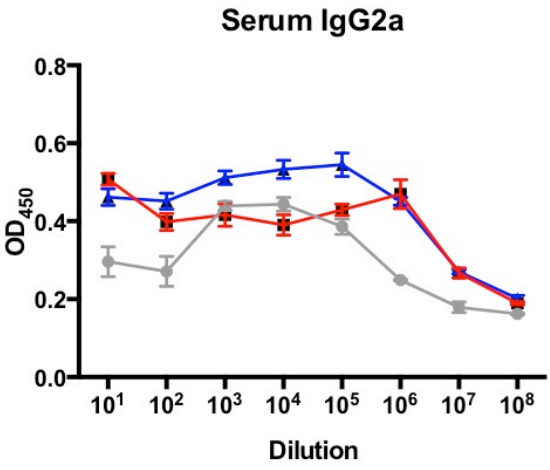
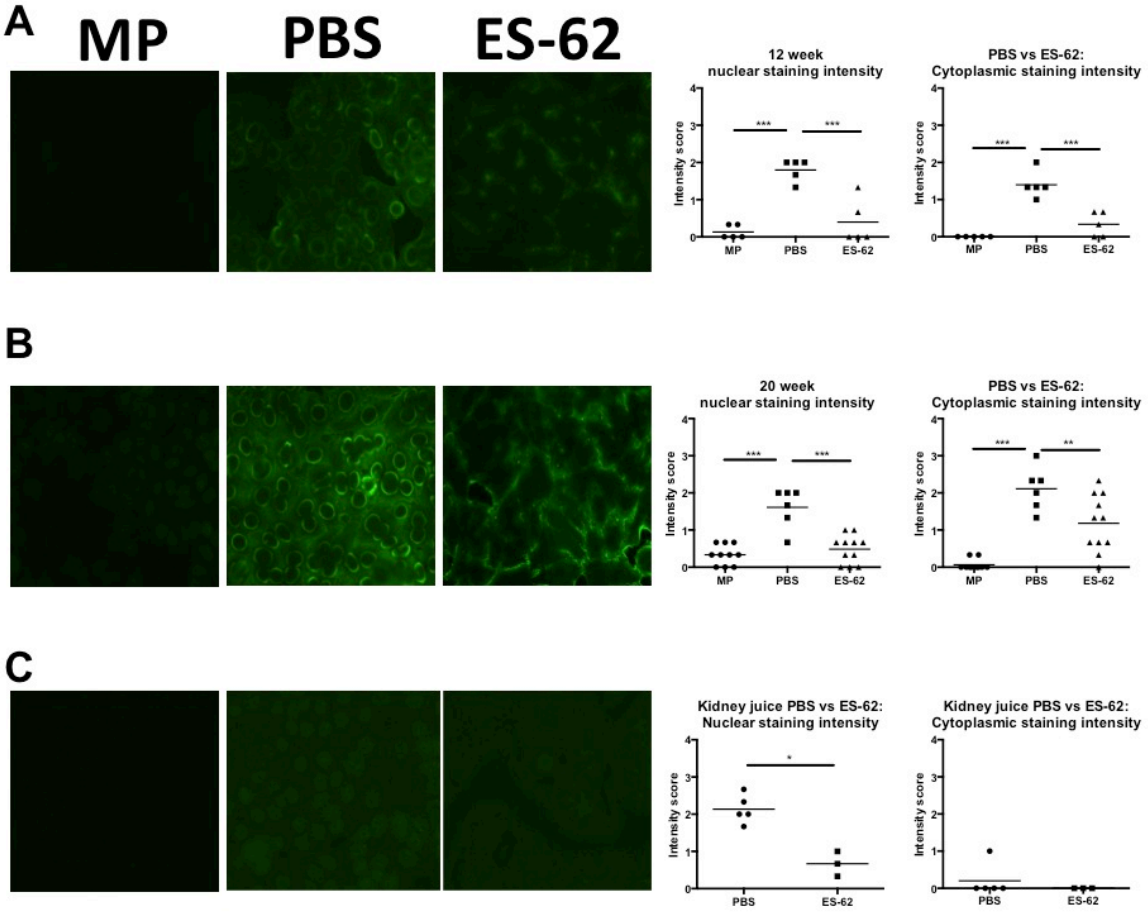

Serum IgG2a
0.8
0.6
0.4
0.2
0.0
OD450
10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8
Dilution

Figure 4.10. ES-62 does not modulate total antibody production. The levels of IgM (A), IgG1 (B) and IgG2a (C) antibodies in serum from 21-week old MRL/MP mice (grey line) or 21-week old MRL/*lpr* mice treated with ES-62 (blue line) or PBS (red line) was measured by ELISA.


A
MP
PBS
ES-62
12 week
nuclear staining intensity
PBS vs ES-62:
Cytoplasmic staining intensity
Intensity score
B
20 week
nuclear staining intensity
PBS vs ES-62:
Cytoplasmic staining intensity
C
Kidney juice PBS vs ES-62:
Nuclear staining intensity
Kidney juice PBS vs ES-62:
Cytoplasmic staining intensity

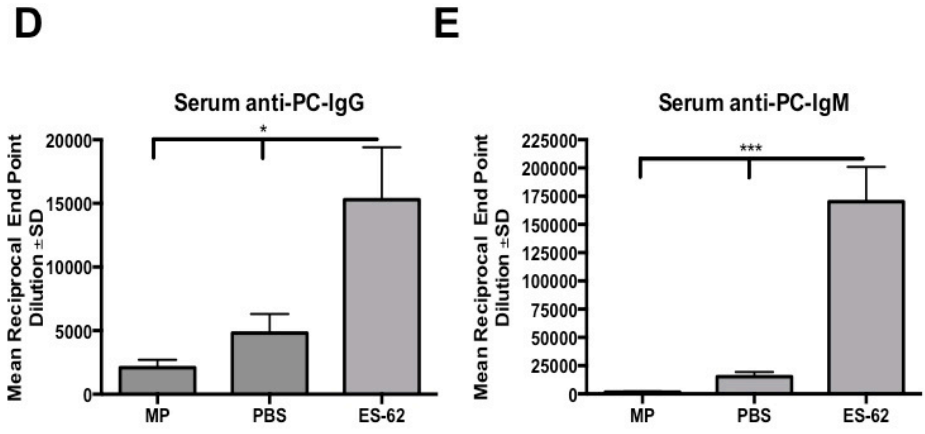

D
Serum anti-PC-IgG
Mean Reciprocal End Point Dilution ±SD
MP
PBS
ES-62
E
Serum anti-PC-IgM

Figure 4.11. ES-62 suppresses pathogenic ANA responses. Hep-2 slides (Antibodies incorporated) were incubated with serum from 12-week (A) and 20-week (B) MRL/MP or MRL/*lpr* mice treated with PBS or ES-62.The ANA staining intensity of 3 biological replicates of the serum from each mouse was scored in the nucleus and cytoplasm as: none (0), ± (1), positive (2) and bright (3); for MRL/MP (n=10), PBS treated MRL/*lpr* (n=6) and ES-62 treated MRL/*lpr* (n=11) mice. Hep-2 slides were also stained with renal interstitial fluid from 21-week old MRL/MP or MRL/*lpr* mice treated with PBS (n=5) and ES-62 (n=3) treated MRL/*lpr* mice and the ANA staining intensity was scored in the nucleus and cytoplasm (C). The level of anti-PC IgG (D) IgM (E) antibodies were measured by ELISA by Dr. Lamyaa Al-Riyami as described in Chapter 2. Statistical significance was determined using the students t-test where *=$p<0.05$ and ***=$p<0.001$.

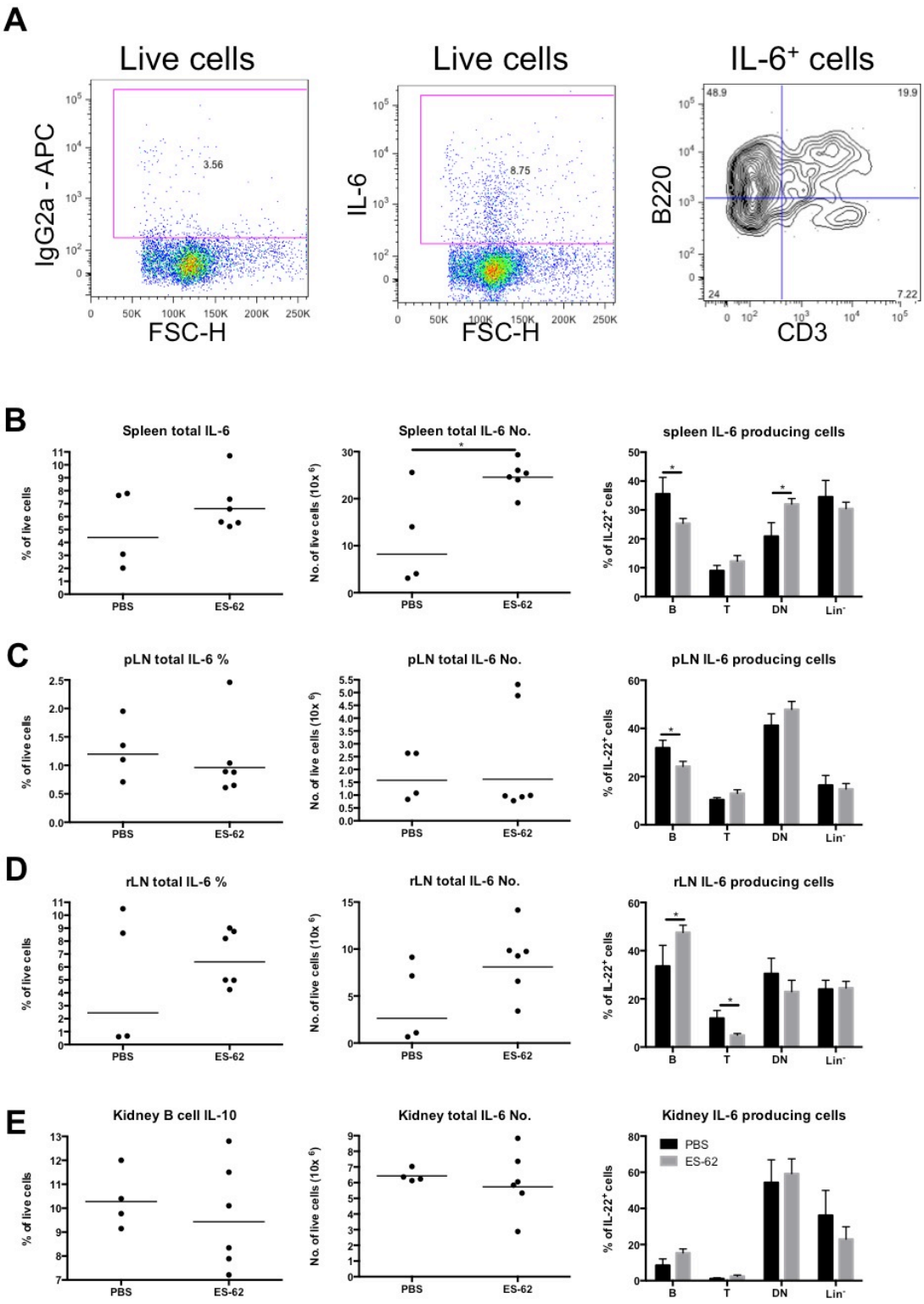

A
Live cells
Live cells
IL-6+ cells
IgG2a - APC
FSC-H
IL-6
B220
CD3
3.56
8.75
48.9
19.9
24
7.22
B
Spleen total IL-6
Spleen total IL-6 No.
spleen IL-6 producing cells
% of live cells
No. of live cells (10x 6)
% of IL-22+ cells
PBS
ES-62
B
T
DN
Lin-
C
pLN total IL-6 %
pLN total IL-6 No.
pLN IL-6 producing cells
D
rLN total IL-6 %
rLN total IL-6 No.
rLN IL-6 producing cells
E
Kidney B cell IL-10
Kidney total IL-6 No.
Kidney IL-6 producing cells
PBS
ES-62

Figure 4.12. The effect of ES-62 on IL-6 production by B cells. Cells were stimulated with PMA and ionomycin for 6 hours, BrefeldinA was added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye, washed and then stained for surface markers, fixed, permeablized and incubated with anti-mouse-IL-6 antibodies. Cells were analysed by flow cytometry (A) and the proportion and number of IL-6^+ cells were determined in the spleen (B), pLN (C), rLN (D) and kidney (E). In each organ, the proportion of $B220^+CD3^-$ B cells, $B220^-CD3^+$ T cells, $B220^+CD3^+$ double negative T cells and $B220^-CD3^-$ Lin^- cells in mice treated with (grey bar, n=8) or without (black bar, n=4) ES-62 was determined by flow cytometry. The data are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

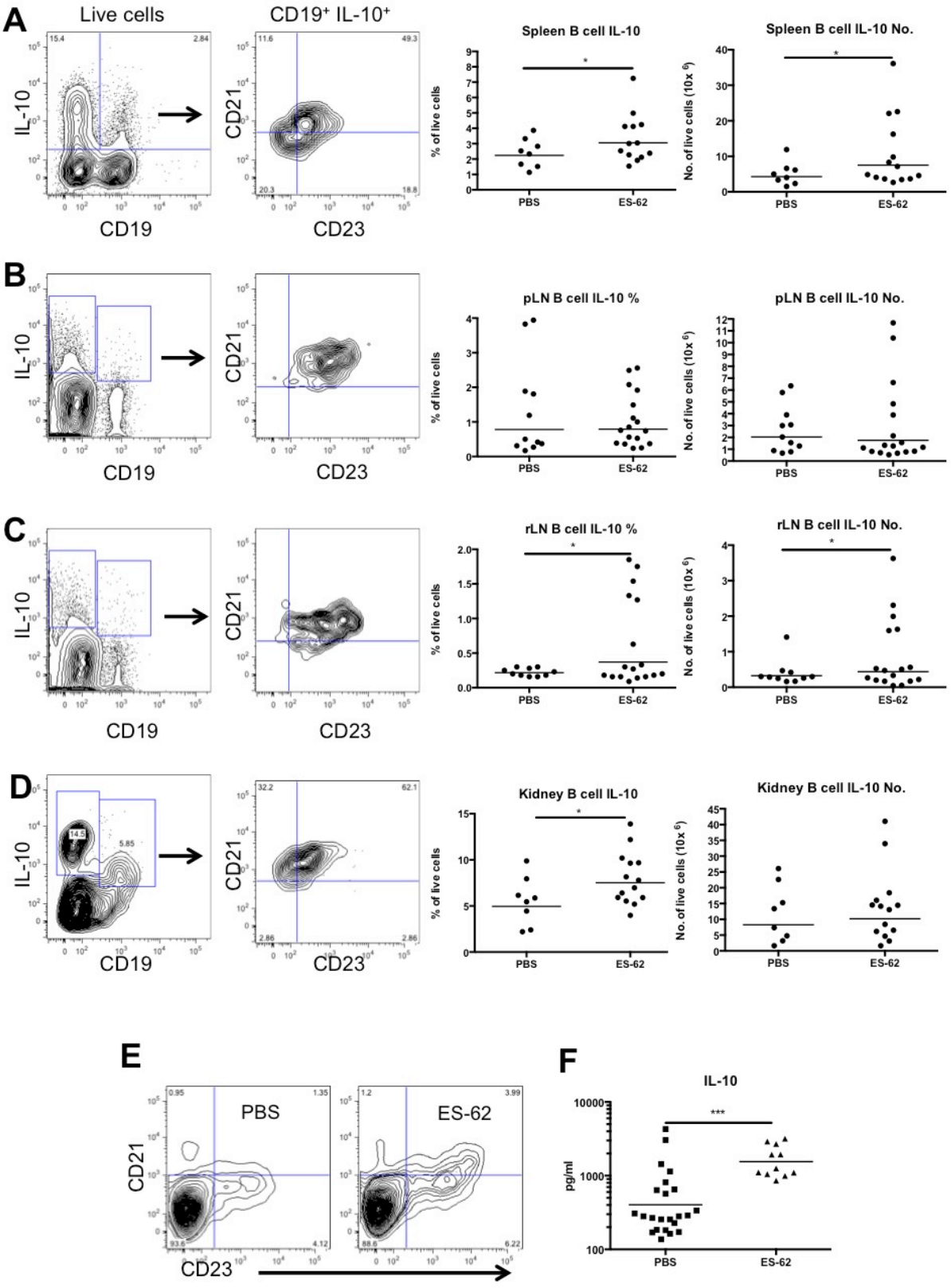

A
Live cells
CD19⁺ IL-10⁺
IL-10
CD19
CD21
CD23
Spleen B cell IL-10
Spleen B cell IL-10 No.
% of live cells
No. of live cells (10x 6)
PBS
ES-62
B
pLN B cell IL-10 %
pLN B cell IL-10 No.
C
rLN B cell IL-10 %
rLN B cell IL-10 No.
D
Kidney B cell IL-10
Kidney B cell IL-10 No.
E
PBS
ES-62
F
IL-10
pg/ml

Figure 4.13. The effect of ES-62 on IL-10-producing B cells. Cells were stimulated with LPS, PMA and ionomycin for 6 hours, BrefeldinA was added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye and stained for surface markers. Cells were then fixed and permeabilized and incubated with anti-mouse-IL-10 antibodies. Cells were analysed by flow cytometry and the proportion and number of $IL\text{-}10^{+}CD19^{+}$ cells were determined in the spleen (A), pLN (B), rLN (C) and kidney (D) of PBS (n=10) or ES-62 (n=13) treated MRL/*lpr* mice. In each organ, the phenotype of the $IL\text{-}10^{+}$ B cells was further analyzed by measuring CD21 and CD23 expression. The data are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$. Blood from mice treated with or without ES-62 were pooled (4 per group) and the proportion of $CD19^{+}CD21^{+/-}CD23^{+/-}$ B cells circulating in the blood was determined (E); data are representative of two independent experiments (E). The concentration of IL-10 in the renal interstitial fluid of individual was measured by ELISA (F). Statistical significance was determined using the students t-test where *=$p<0.05$ and ***=$p<0.001$.

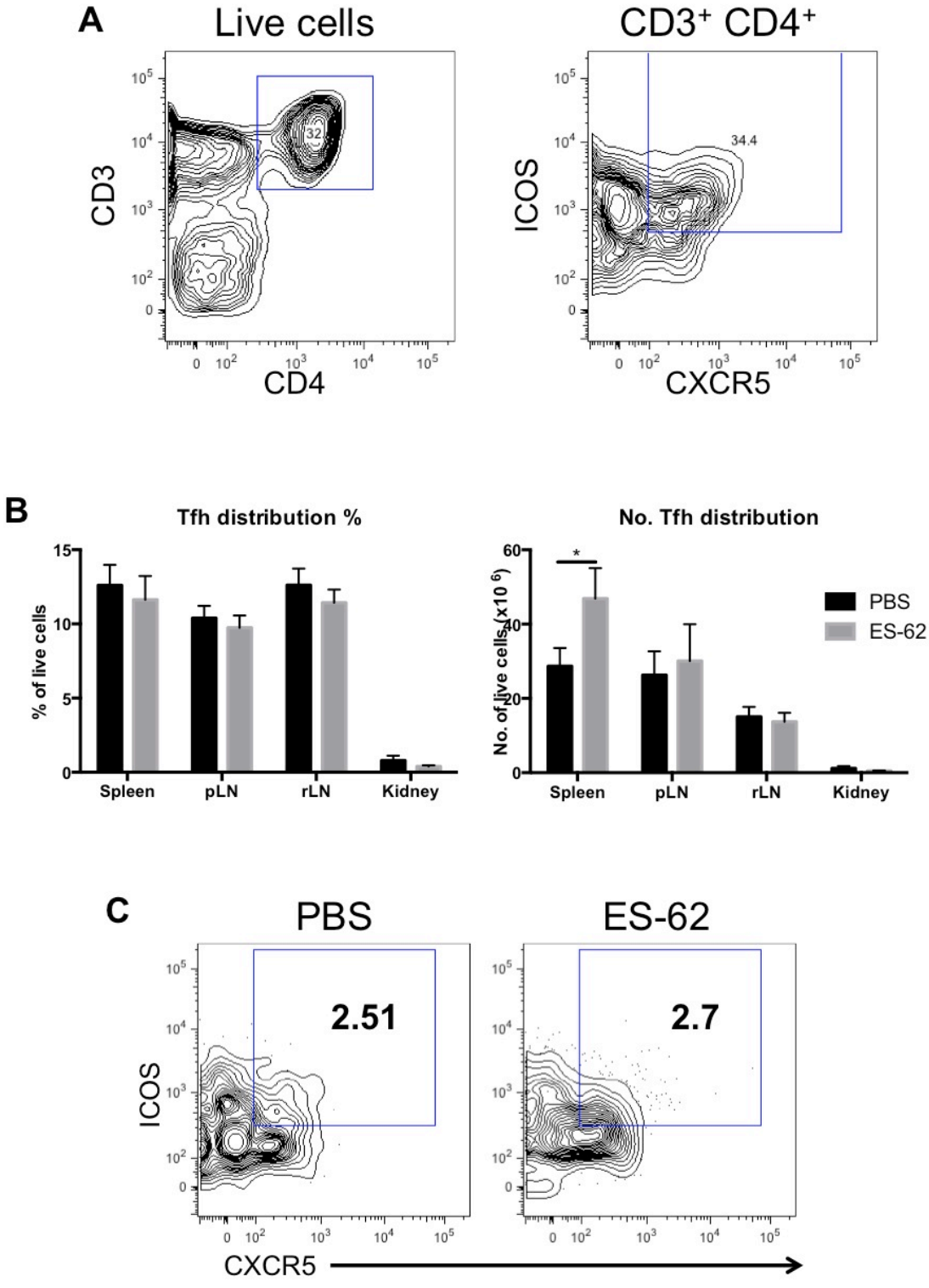

A
Live cells
CD3+ CD4+
CD3
CD4
ICOS
CXCR5
32
34.4
B
Tfh distribution %
No. Tfh distribution
% of live cells
No. of live cells (x10 6)
PBS
ES-62
Spleen
pLN
rLN
Kidney
*
C
PBS
ES-62
2.51
2.7
ICOS
CXCR5

Figure 4.14. The effect of ES-62 on T follicular helper cells. The proportion and number of $CD3^+CD4^+ICOS^+CXCR5^+$ T follicular helper cells (Tfh) was determined by flow cytometry (A) in the spleen, pLN, rLN, kidney (B) and blood (C; cells were pooled from 4 mice in each group) of 21-week old MRL/*lpr* mice treated with (grey bar, n=10) or without (black bar, n=7) ES-62. Data are represent 2 independent experiments, apart from the analysis of the blood, and are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

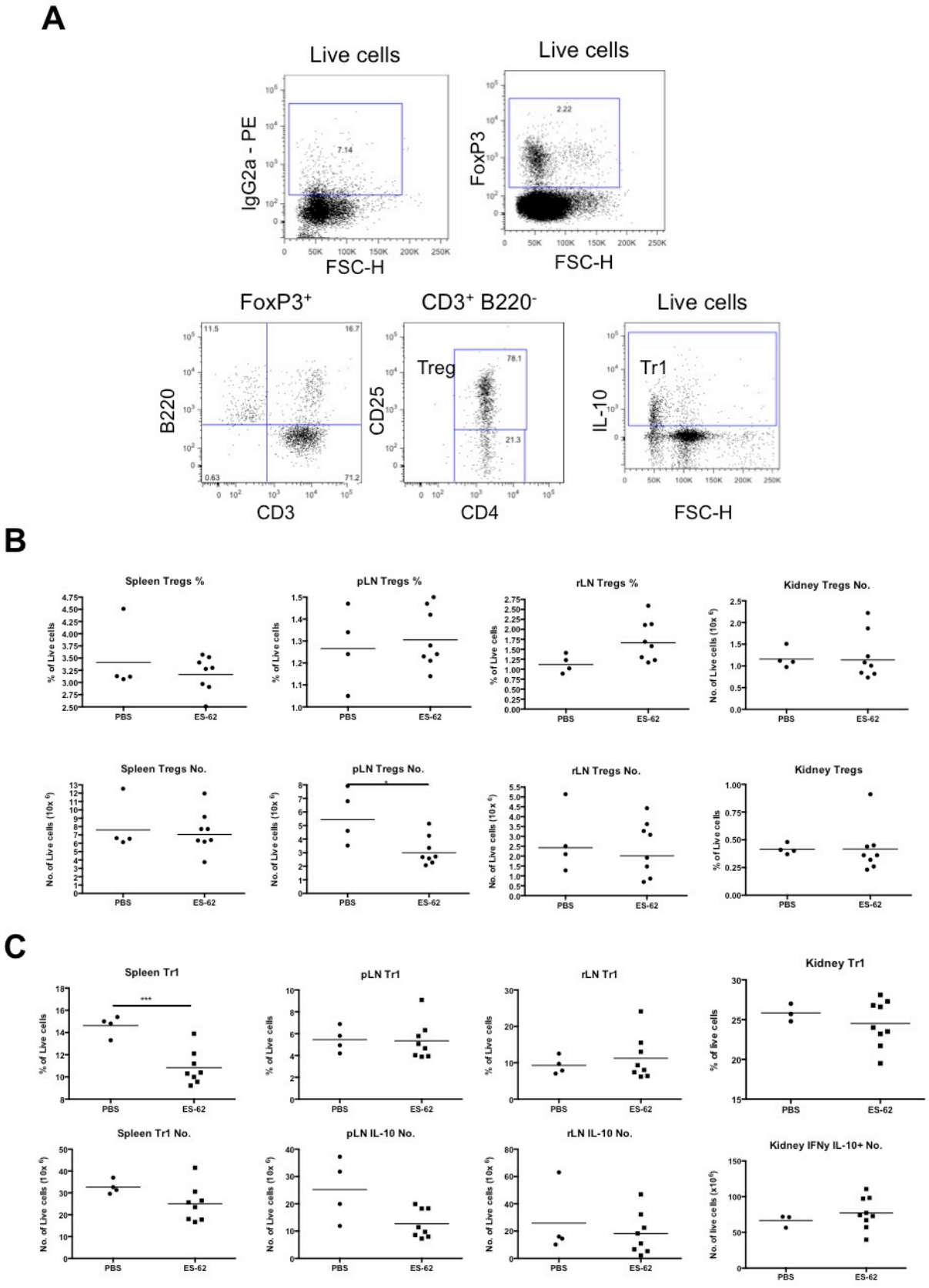

A
Live cells
IgG2a - PE
FSC-H
7.14
Live cells
FoxP3
FSC-H
2.22
FoxP3+
B220
CD3
11.5
16.7
0.63
71.2
CD3+ B220-
CD25
CD4
Treg
78.1
21.3
Live cells
IL-10
FSC-H
Tr1
B
Spleen Tregs %
pLN Tregs %
rLN Tregs %
Kidney Tregs No.
Spleen Tregs No.
pLN Tregs No.
rLN Tregs No.
Kidney Tregs
% of Live cells
No. of Live cells (10x 6)
PBS
ES-62
C
Spleen Tr1
pLN Tr1
rLN Tr1
Kidney Tr1
Spleen Tr1 No.
pLN IL-10 No.
rLN IL-10 No.
Kidney IFNy IL-10+ No.
% of live cells
No. of live cells (x10⁶)

Figure 4.15. The effect of ES-62 on regulatory T cells. Unstimulated cells were assessed for FoxP3 expression whilst for the analysis of IL-10 producing T cells, cells were stimulated with PMA and ionomycin for 6 hours with Brefeldin A being added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye and stained for surface markers before being fixed and permeabilized, incubated with anti-mouse-IL-10 antibodies and analyzed by flow cytometry (A). The proportion and number of $FoxP3^{+}B220^{-}CD3^{+}CD4^{+}CD25^{+}$ regulatory T cells (B; Tregs) or $IL\text{-}10^{+}B220^{-}CD3^{+}$ Tr1 cells (C) was determined in the spleen, pLN, rLN and kidney of mice treated with PBS (n=4) or ES-62 (n=8). The data are presented as the mean values of individual mice ± SEM and statistical significance was determined using the student's t-test.

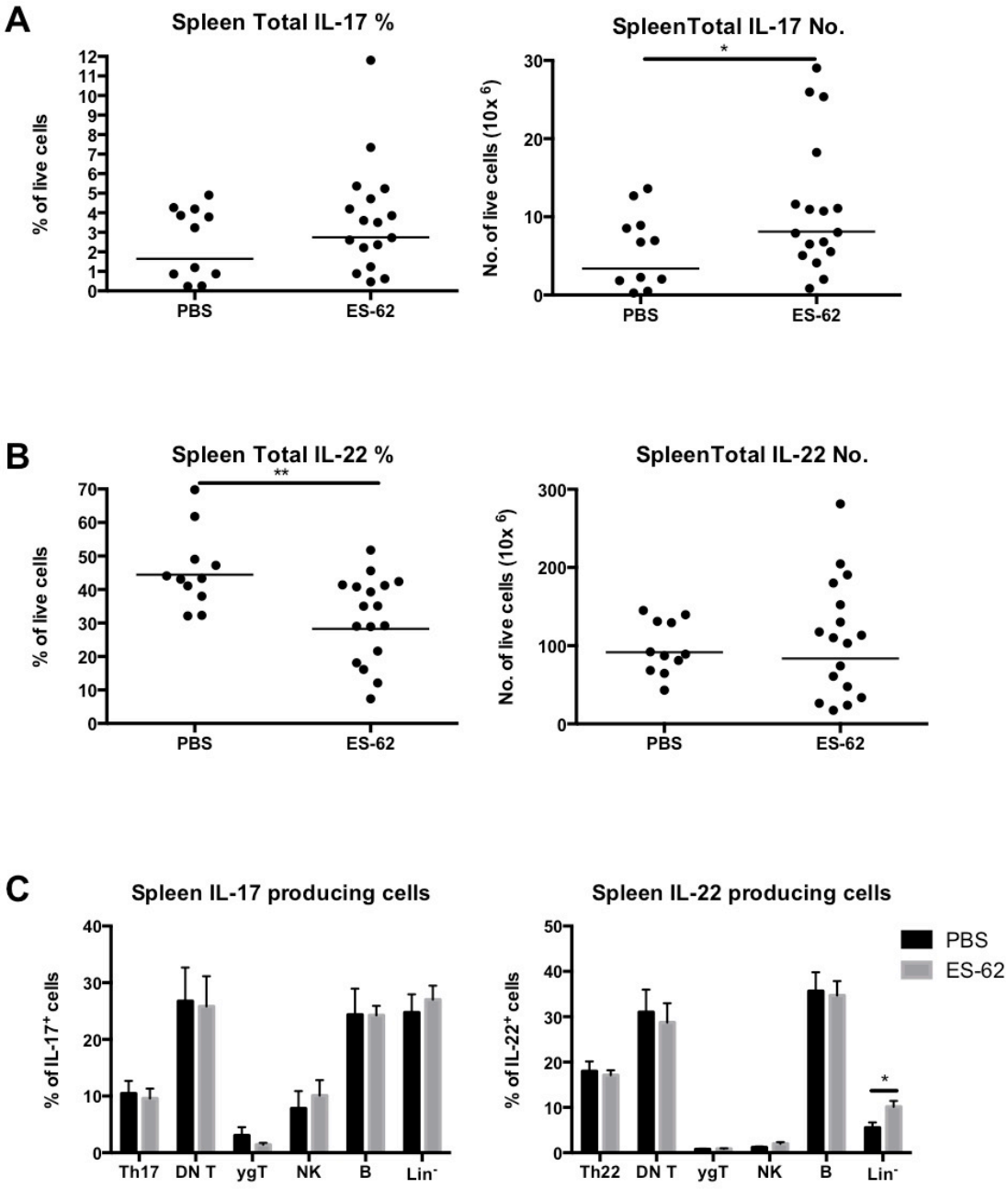

A
Spleen Total IL-17 %
% of live cells
PBS
ES-62
SpleenTotal IL-17 No.
No. of live cells (10x 6)
*
B
Spleen Total IL-22 %
**
SpleenTotal IL-22 No.
C
Spleen IL-17 producing cells
% of IL-17+ cells
Th17
DN T
ygT
NK
B
Lin-
Spleen IL-22 producing cells
% of IL-22+ cells
Th22
PBS
ES-62

Figure 4.16. The effect of ES-62 on IL-17- and IL-22-producing cells in the spleen. Splenocytes were stimulated with PMA and ionomycin for 6 hours with BrefeldinA being added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye and stained for surface markers before being fixed, permeablized and incubated with anti-mouse-IL-17A or anti-mouse-IL-22 antibodies. The proportion and number of IL-17^+ or IL-22^+ cells in the spleens of individual mice treated with PBS (n=11) or ES-62 (n=18) was determined by flow cytometry. The data are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$ and **=$p<0.01$.

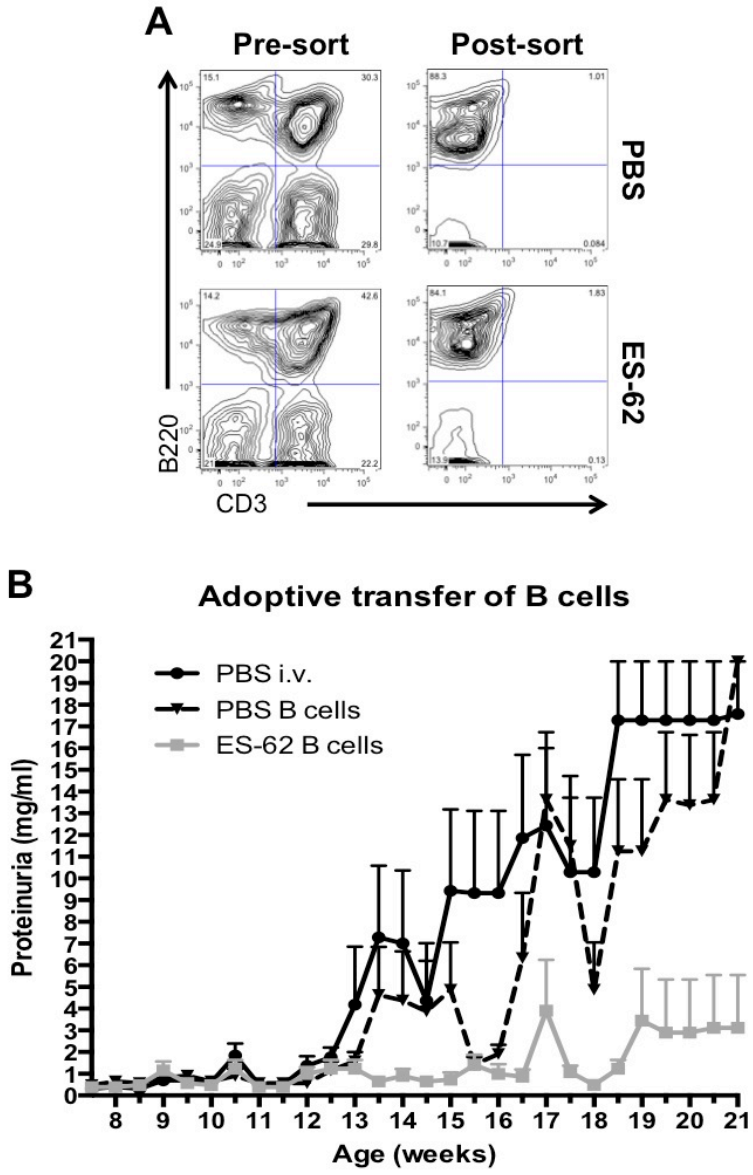

A
Pre-sort
Post-sort
PBS
ES-62
B220
CD3
B
Adoptive transfer of B cells
PBS i.v.
PBS B cells
ES-62 B cells
Proteinuria (mg/ml)
Age (weeks)

Figure 4.17. Adoptively transferred B cells from ES-62 treated mice prevent the development of proteinuria in recipient mice. Splenocytes were pooled from 21-week old MRL/*lpr* mice treated with ES-62 (n=8) or PBS (n=8) and $CD43^-$ B cells were purified (Table 1; approximately 85% ± 4.17 pure) and injected (5 x 10^6 cells in 100μl of sterile PBS) into the tail veins of 7-week old MRL/*lpr* mice. Proteinuria of the recipient mice (i.v. PBS, n= 6; PBS B cells, n= 8; ES-62 B cells, n=8) was measured twice weekly until 21-weeks of age at which point the mice were sacrificed.

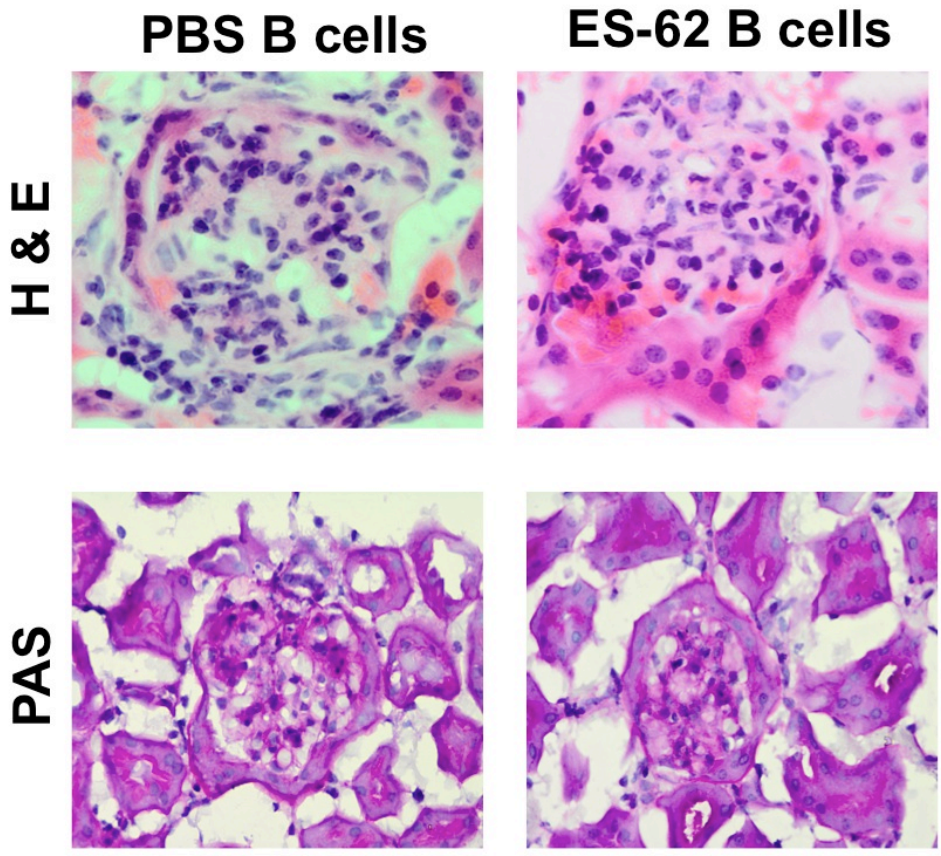

PBS B cells
ES-62 B cells
H & E
PAS

Figure 4.18. Adoptively transferred B cells do not suppress the development of glomerulonephritis. The kidneys from 21-week old MRL/*lpr* mice that received B cells from PBS or ES-62 treated donors, were fixed and snap frozen before being sectioned (7μm) and stained by either H&E or PAS.

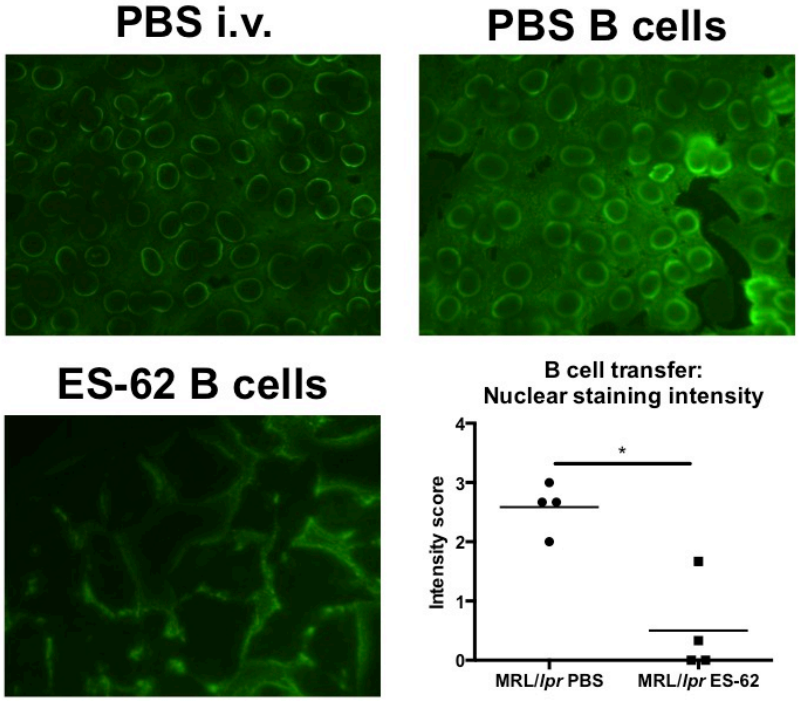

PBS i.v.
PBS B cells
ES-62 B cells
B cell transfer:
Nuclear staining intensity
Intensity score
4
3
2
1
0
*
MRL/lpr PBS
MRL/lpr ES-62

Figure 4.19. Adoptive transfer of B cells from ES-62 treated mice suppresses pathogenic ANA responses. Hep-2 slides (Antibodies incorporated) were incubated with serum from 21-week MRL/*lpr* mice that received i.v. B cells from PBS- or ES-62-treated donors or i.v. PBS as a control (A). The ANA staining intensity of 3 biological replicates of the serum from each mouse was scored in the nucleus and cytoplasm as: none (0), ± (1), positive (2) and bright (3); for MRL/*lpr* mice that received B cells from PBS (n=4) or ES-62 (n=4) treated donors. Statistical significance was shown using the student's t-test where *=$p<0.05$.

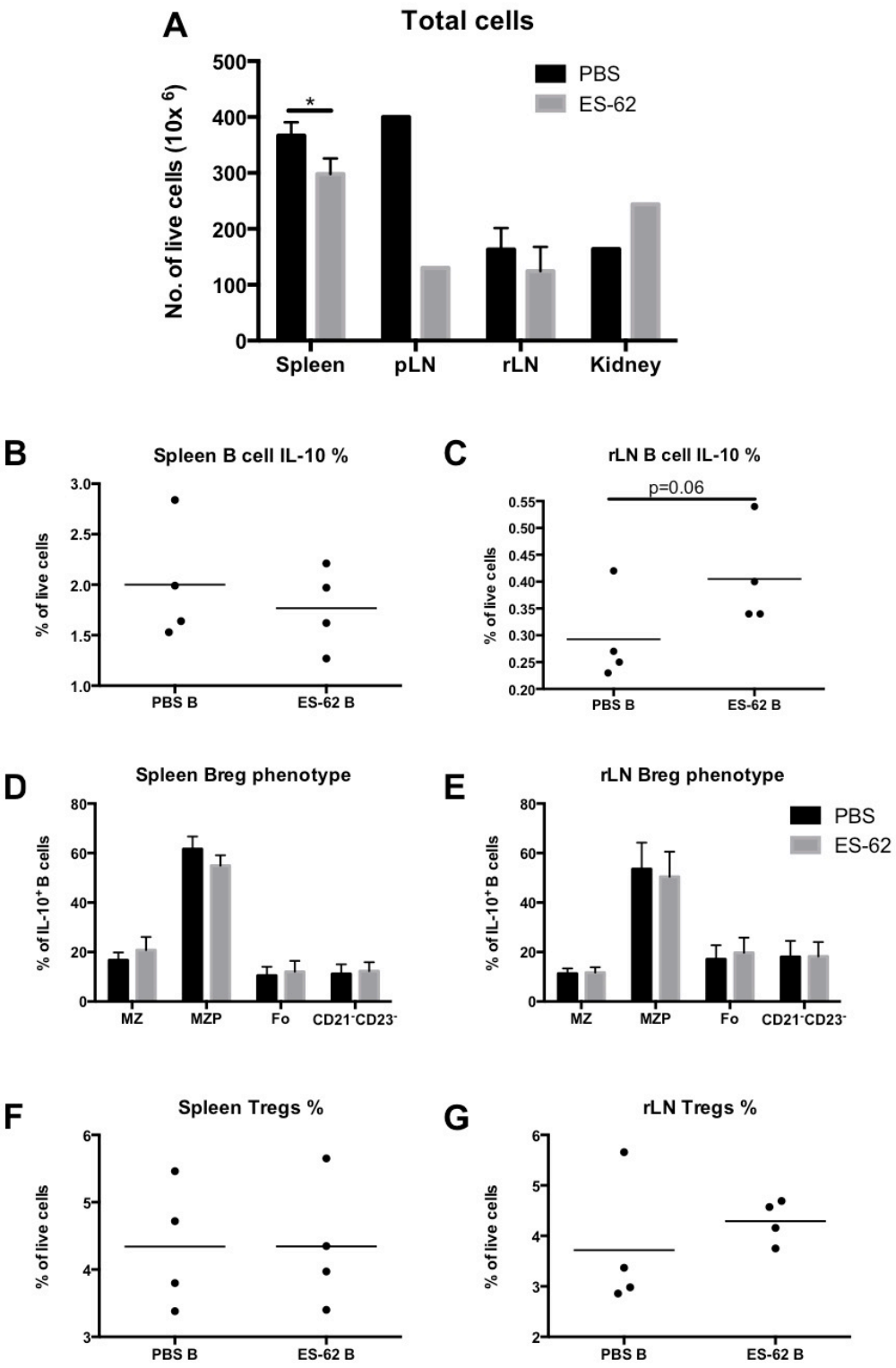

A
Total cells
No. of live cells (10x 6)
500
400
300
200
100
0
*
PBS
ES-62
Spleen
pLN
rLN
Kidney
B
Spleen B cell IL-10 %
% of live cells
3.0
2.5
2.0
1.5
1.0
PBS B
ES-62 B
C
rLN B cell IL-10 %
p=0.06
% of live cells
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
PBS B
ES-62 B
D
Spleen Breg phenotype
% of IL-10+ B cells
80
60
40
20
0
MZ
MZP
Fo
CD21-CD23-
E
rLN Breg phenotype
% of IL-10+ B cells
PBS
ES-62
MZ
MZP
Fo
CD21-CD23-
F
Spleen Tregs %
% of live cells
6
5
4
3
PBS B
ES-62 B
G
rLN Tregs %
% of live cells
6
5
4
3
2
PBS B
ES-62 B

Figure 4.20. The effect of the adoptive transfer of B cells from ES-62 treated donor mice on regulatory B and T cell populations. The number of cells in the spleen, pLN (n=4 pooled), rLN and kidney (n=4 pooled) of 21-week MRL/*lpr* mice, that received B cells from PBS (black bar, n-10) or ES-62 (grey bar, n=10) treated donors, were counted (A). Cells were the stimulated with PMA and ionomycin for 6 hours, BrefeldinA was added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye and stained for surface markers. Cells were then fixed, permeablized and incubated with anti-mouse-IL-10 antibodies. For the analysis of regulatory T cells, unstimulated cells were stained with the Live/Dead cell discrimination fixable dye. The surface markers of cells were stained before the cells were washed, fixed and permeablized and stained for FoxP3 using a FoxP3 staining kit (eBioscience, CA). Cells were analysed by flow cytometry and the proportion of IL-10^+ B cells were determined in the spleen (B) and rLN (C). The proportion of IL-10^+ B cells with a MZ ($CD21^+CD23^-$), MZP ($CD21^+CD23^+$), Fo ($CD21^{low/-}CD23^+$) or $CD21^-CD23^-$ phenotype was determined in the spleen (D) and rLN (E) where data are presented as the mean values of individual mice ± SEM. The proportion of $CD3^+CD4^+CD25^+FoxP3^+$ regulatory T cells was determined in the spleen (F) and rLN (G) by flow cytometry. Data are representative of 2 independent experiments and statistical significance was determined using the student's t-test.

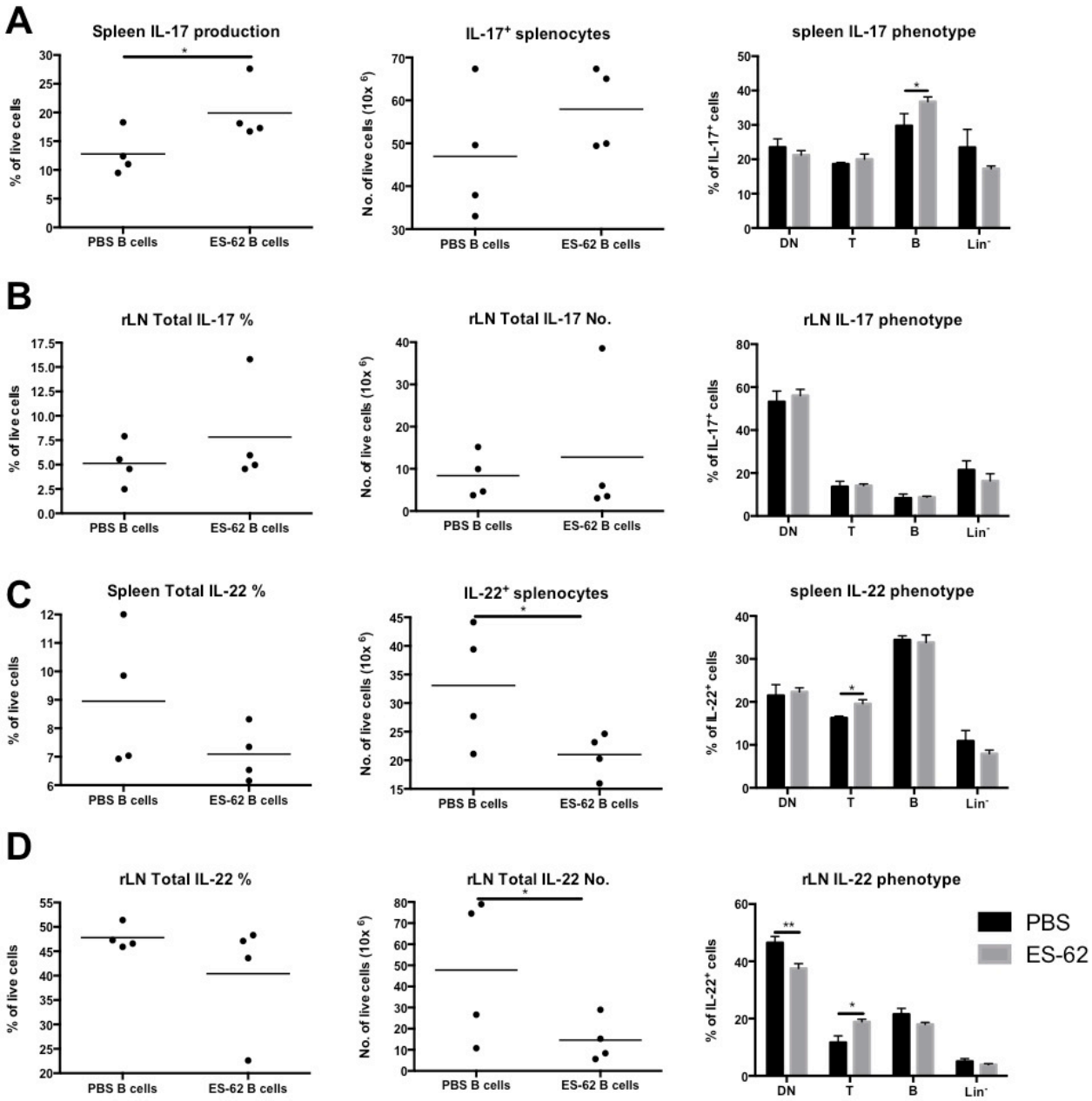

A
Spleen IL-17 production
% of live cells
PBS B cells
ES-62 B cells
IL-17+ splenocytes
No. of live cells (10x 6)
spleen IL-17 phenotype
% of IL-17+ cells
DN
T
B
Lin-
B
rLN Total IL-17 %
rLN Total IL-17 No.
rLN IL-17 phenotype
C
Spleen Total IL-22 %
IL-22+ splenocytes
spleen IL-22 phenotype
% of IL-22+ cells
D
rLN Total IL-22 %
rLN Total IL-22 No.
rLN IL-22 phenotype
PBS
ES-62

Figure 4.21. The effect of the adoptive transfer of B cells from ES-62 treated donor mice on IL-17A and IL-22 production. Cells from the spleen or rLN of 21-week MRL/*lpr* mice that received B cells from PBS (n=4) or ES-62 (n=4) treated donors were stimulated with PMA and ionomycin for 6 hours, BrefeldinA was added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye and stained for surface markers. Cells were then fixed, permeablized and incubated with anti-mouse-IL-17A or anti-mouse-IL-22 antibodies before being washed and analysed by flow cytometry. The proportion, number and phenotype of IL-17^+ (A & B) or IL-22^+ (C & D) cells was determined in the spleen (A & C) and rLN (B &D). The data are representative of two independent experiments and are presented as the mean values of individual mice ± SEM and statistical significance was determined using the students t-test where *=$p<0.05$.

5. ES-62 induces hypo-responsiveness of renal cells to pro-inflammatory stimuli

5.1. Introduction

SLE is a systemic autoimmune disease mediated by the deposition of immune complexes in target organs: the kidney is a major site of pathology and glomerulonephritis a serious manifestation that presents in patients (1,2). In terms of immune complexes, those involving anti-dsDNA autoantibodies are particularly associated with disease severity (3). These antibodies usually bind to DNA released from necrotic cells, however in the kidney, due to interactions with collagen type IV or heparin sulphate at the glomerular basement membrane, circulating DNA becomes embedded in the glomerular matrix and provides a focused source of antigen for the circulating anti-dsDNA antibodies, allowing formation of large immune complexes (4,5). Consequent activation of antibody receptors (6), in concert with complement (7,8) and toll-like (9,424) receptors, drives inflammation in the kidney. The resulting proinflammatory cytokine response leads to the infiltration of innate and adaptive cells that drive pathology by the production of antibodies, cytokines and the failed clearance of apoptotic or antigenic debris; the collateral damage of which perpetuates further inflammatory responses (2,10).

The data presented in the preceding chapters suggest that ES-62-mediated suppression of proteinuria in the MRL/*lpr* mouse model of SLE appears to be associated with resetting of the IL-17A/IL-22 inflammatory axis and the effector: regulatory B cell balance. However, surprisingly, histological analysis of the kidneys from such ES-62-treated mice did not reveal any striking improvement in glomerular proliferation, cellular infiltration, basement membrane thickening or complement and antibody deposition. It was therefore the aim of this chapter to determine whether the protection against proteinuria afforded by ES-62 reflected desensitisation of renal cells to pathogenic pro-inflammatory stimuli, in particular those targeting TLRs, FcRs and complement receptors.

5.2. Results

5.2.1. The upregulation of pro-inflammatory receptor expression by kidney cells during disease progression is suppressed in MRL/*lpr* mice treated with ES-62

Prophylactic treatment of MRL/*lpr* mice with ES-62 prevents the development of proteinuria: however, this is not associated with any global improvement in renal histology as evidenced by glomeruloproliferation, glomerular basement membrane thickening, cellular infiltration, immune complex or complement deposition (Fig. 3.3). It was therefore hypothesized that ES-62 might act to desensitize effector cells, both resident and infiltrating, to inflammatory signals in the kidneys and thus a more comprehensive analysis of the responses of such kidney cells was carried out. Consistent with reports that signaling via TLR, FcR and complement receptors perpetuate chronic inflammation in the kidney (6-9,424), analysis of kidney cells from MRL/MP (21-week), MRL/*lpr* (7-week) and MRL/*lpr* (21-week) mice showed that the levels of expression of these receptors, as well as that of the co-stimulatory molecule CD86, increased with disease progression (Fig. 5.1A-E). Expression of MyD88, the key signal transducer for IL-1R/TLR family that is involved in the response to endogenous DAMPs in lupus (425) and a known target of ES-62 mediated immune modulation (322,336,337), was similarly increased (Fig. 5.1F). This is consistent with previous findings that MyD88 is essential for disease development in lupus-prone mice (426,427) and required for renal graft rejection (428). Interestingly, therefore, expression of MyD88 was significantly reduced in cells from the kidneys of ES-62 relative to PBS treated mice (Fig. 5.2A & B). Moreover, *ex vivo* culture of the kidney cells from 21-week old MRL/*lpr* mice overnight with ES-62 resulted in a clear inhibition of LPS (as a surrogate of endogeneous DAMPs) stimulated up regulation of MyD88, TLR4, C5aR and C3aR, but not CD16/32 expression (Fig. 5.2C-G). To address whether these observations reflected ES-62 mediated hyporesponsiveness of resident kidney cells, explant cultures of renal fibroblasts were expanded from the kidneys of PBS or ES-62 treated MRL/*lpr* mice for two weeks and then, again as a surrogate for DAMP signaling, cultured with and without LPS for a further 24 hours before being examined for pro-inflammatory receptor expression. This analysis revealed that renal fibroblasts from ES-62 treated mice exhibited reduced expression of MyD88,

C5aR and CD64 (FcgRI), albeit not significantly, but not CD16, CD86 or TLR4 (Fig. 5.3A-C and data not shown). The finding that fibroblasts from ES-62-treated mice produced less IL-6 in response to LPS than those from PBS-treated mice highlighted the potential functional relevance of the modulation of MyD88 (Fig. 5.3D). Moreover, renal fibroblasts from both ES-62 and rIL-17A treated mice exhibited reduced MyD88 expression compared to those from PBS and rIL-22 treated mice and this reflected a reduction in LPS-stimulated MCP-1 (CCL2) production (Fig. 5.3E & F). To further explore the functional relevance of such receptor modulation, renal fibroblasts were also cultured with a variety of potentially disease-relevant pro-inflammatory stimuli including rIL-6, rIL-17, rIL-22, rC3a, rC5a and in addition, FcγIIIR (CD16) was activated by antibody-mediated cross-linking; however, all of these stimuli failed to elicit MCP-1 or IL-6 production.

5.2.2. Renal fibroblast proliferation and wound healing

One of the pathological features of glomerulonephritis is hyper-cellularity but no significant difference in this parameter could be detected between ES-62- or PBS-treated mice by histological analysis (H & E staining). Interestingly therefore, it was observed that *ex vivo* expansion of fibroblasts from ES-62-treated mice routinely resulted in lower yields of cells which proliferated less (as indicated by their DNA synthesis) than those from PBS-treated MRL/*lpr* mice (Fig. 5.4A & B). To address whether this potentially reflected differences in the regulation of epithelial barrier permeability, the ability of renal fibroblasts from PBS and ES-62 treated mice to heal artificial wounds was evaluated over 72 hours (Fig. 5.4C). Despite their reduced capacity for proliferation, renal fibroblasts from ES-62 treated mice initiated wound repair significantly earlier than cells from either PBS- or rIL-22-treated mice (Fig. 5.4C). Nevertheless, and perhaps reflecting that IL-22 has been implicated in wound repair of epithelial cells (132,429), significantly higher levels of "healing" was observed with the cells from rIL-22 treated mice between 48-72 h. However, it appeared that these cells were insensitive to contact inhibition as they were observed to visually exceeded the confines of monolayer confluency (Fig. 5.4C). Although these observations do not reconcile the apparent discrepancies between fibroblast proliferation and histological hyper-cellularity, they may suggest that cells from ES-62-treated mice retain wound healing properties that promote the maintenance of barrier permeability without exhibiting the deregulated

fibroblast hyperplasia observed with renal fibroblasts from rIL-22 treated mice that could lead to fibrosis and contribute to kidney pathology

5.2.3. Analysis of infiltrating kidney cells

The observed desensitization of pro-inflammatory cytokine and chemokine responses by resident kidney cells from ES-62-treated mice suggested that this could result in reduced recruitment of inflammatory cells to this site of inflammation, breaking the perpetuating cycle of chronic inflammation. Thus, as ES-62 did not appear to induce any striking decrease in cellular infiltration as evidenced by H & E histological staining of kidney sections (Fig. 3.3), it was decided to determine whether the improvement in proteinuria reflected changes in the composition and/or functional phenotype of the cellular infiltrates.

Indeed, as reported in Chapters 3 & 4, it was found that the levels of effector T cells (Fig. 3.6), ILC (Fig. 3.9) and effector B cells (Fig.4.5) were reduced in the kidneys of ES-62 treated mice. These data are consistent with ES-62 resetting the effector: regulatory balance of B and T cell responses (Chapter 3 and 4). Moreover, analysis of granulocyte populations revealed that whilst the proportion of $CD11b^{hi}GR1^{hi}$ cells were considerably elevated in the blood (Fig. 5.5A), the proportions of $CD11b^{hi}GR1^{hi}$, $CD11b^{hi}GR1^{low}$ and $CD11b^{hi}GR1^{-}$ cells were significantly reduced in the kidneys of ES-62 treated mice at 21-weeks (Fig. 5.5B). As there were no significant differences in their levels in spleen, pLN, rLN or bone marrow at this time point, these data may suggest a block in their survival or migration to the kidney (Fig. 5.5C & F). By contrast, prior to the onset of disease at 12-weeks, when IL-17A production was generally reduced by ES-62, although these granulocytes could not be detected in the kidneys, the levels of $CD11b^{high}GR1^{low}$ cells in the spleen, $CD11b^{high}GR1^{low}$ and $CD11b^{high}GR1^{-}$ cells in pLN and $CD11b^{high}GR1^{low}$ and $CD11b^{high}GR1^{-}$ cells in rLN were reduced in ES-62-treated mice (Fig. 5.5G-I), perhaps suggesting that ES-62 prevents the early recruitment and activation of pathogenic granulocytes. However, although the levels of $CD45^{+}F4/80^{+}CD11b^{+}$ macrophages in the spleen and LNs were largely unaltered in ES-62 treated mice at 21-weeks (Fig. 5.6A-C), the levels of macrophages were raised in blood and kidneys of ES-62 treated mice (Fig. 5.6D &

E), which, as an imbalance in the ratio of M1: M2 macrophages has been reported in lupus prone mice (430), may reflect a modulation of the functional phenotype of these macrophages.

ES-62 has previously been reported to suppresses PAMP-driven pro-inflammatory responses by macrophages (320,331,333) and so to address whether it indeed modulated the functional phenotype of macrophages in lupus, bone marrow-derived macrophages (BMM) from MRL/*lpr* mice were cultured for 24 hours with our without LPS. Such BMM from MRL/*lpr* mice produced large quantities of TNFα, IL-1β, IL-6, IL-27, IL-10, IL-12p70 and IL-23 in response to LPS. Interestingly, pre-treatment with ES-62 for only 2 hours resulted in inhibition of LPS induced IL-1β, IL-6 and IL-12p70 production (Fig. 5.7A-G). Furthermore, in the BMM as is the renal fibroblasts, ES-62 reduced LPS up-regulated the expression of MyD88 (Fig. 5.7H).

5.2.4. Small molecular analogues of phosphorylcholine suppress proteinuria in the MRL/*lpr* mouse

ES-62 is a large immunogenic molecule and therefore not an ideal therapeutic candidate. The complexity of the lifecycle required to produce this immunomodulatory molecule further questions its feasibility as a therapy. Small molecular analogues (SMA) based on the immunomodulatory moiety of ES-62, phosphorylcholine (PC), have therefore been derived and tested in a variety of inflammatory settings (International Patent Application No. PCT/GB2013/051988). Two lead compounds, S3 and S5 (Fig. 5.8A), identified from initial screens of anti-inflammatory properties against BMM (reduction of LPS-, BLP- and CpC-mediated IL-12p40 and IL-6 production and down-regulation of MyD88), were examined for their therapeutic potential in the MRL/*lpr* model alongside SMA15, a structurally related molecule that did not exhibit anti-inflammatory activity or down-regulate MyD88 expression in this screen (Fig. 5.8B and Al-Riyami, Rzepecka, Rodgers, Harnett & Harnett; unpublished data). To address their therapeutic potential in lupus, MRL/*lpr* mice were monitored for 16 weeks until they began to show signs of pathology (proteinuria ~3 mg/ml) before administering the SMAs (each mouse received 1 μg of S3, S5 or SMA15 s.c., twice-weekly until 21-weeks). Both S3 and

S5 significantly suppressed the development of proteinuria (Fig. 5.9A) and rLN lymphadenopathy (Fig. 5.9B); interestingly, therefore, both S3 and S5 also reduced MyD88 levels in the kidney cells (Fig. 5.9C). By contrast, the mice treated with SMA15, if anything, experienced exacerbated disease that was associated with mild to severe skin inflammation at the site of injection.

Interestingly, as with ES-62, the suppression of proteinuria in the MRL/*lpr* mouse was not associated with substantial improvements in renal histology in terms of glomerulonephritis, cellular infiltration and glomerular basement membrane thickening (Fig. 5.10A). Nor was it associated with a consistent reduction in ANA responses (Fig. 5.10B), although it is interesting to note that some of the mice (3/5) receiving S5 showed a similar Hep2 staining pattern to ES-62 suggesting that they may have switched to developing antibodies that recognized/cross-reacted with membrane, as opposed to nuclear or cytoplasmic antigens: however this propensity did not appear to be linked to proteinuria level. By contrast, serum from mice that received SMA15 showed strong nuclear staining indicating high titers of anti-DNA responses and all of these mice exhibited high levels of proteinuria.

To further address their mechanism of action, the effect of the SMAs on the other key immunoregulatory sites modulated by ES-62 were investigated but these preliminary studies did not identify any clear target. Nevertheless, they suggested that S3 and S5 did not act by inducing IL-10 producing B cell or regulatory T cell populations (Fig. 5.11A & B). Moreover, although there was some evidence that S3 and S5 reduced the levels of IL-17A-producing cells in the rLN and kidney and S3 had similar effects on IL-22-producing cells (Fig. 5.11C & D), there was no significant effect of either S3 or S5 on the levels of IL-10, IL-17A or IL-22 in the renal interstitial fluid of these mice (Fig. 5.10E-G). Surprisingly, however, those treated with SMA15 contained significantly higher concentrations of IL-22 and significantly lower concentrations of IL-10 and IL-17A, findings perhaps consistent with their high levels of proteinuria and skin inflammation. Reflecting the late administration after the onset of disease pathology, collectively these data suggest that, rather than targeting pathogenic events resulting in the breaking of tolerance, the therapeutic effects of S3 and S5 may be mediated predominantly through their effects on the renal cells, perhaps by modulation of MyD88 expression.

5.3. Discussion

Lupus nephritis is the major cause of mortality in SLE patients and occurs in approximately 50% of patients; thus novel therapies to improve efficiency with reduced toxicity in treating this pathology are urgently required (431,432). In the MRL/*lpr* mouse model of SLE, ES-62 suppresses the development of proteinuria and therefore improves the functions of the kidneys, despite any apparent substantial effect on renal histology. Rather, this appears to be due to a reduction in the production of the pro-inflammatory cytokine IL-22, and the resultant recruitment of inflammatory leukocytes that infiltrate the kidneys, as well as reduced generation of ANA. However, as the parasite product does not appear to suppress the level of immune complex and complement deposition that is generally associated with ANA production, other factors may be involved such as modulation of signaling of their proinflammatory receptors (C3aR, C5aR, FcgRI and FcgRIII) in the inflamed kidneys by the parasite product. Perhaps, as ES-62 appears to modulate the repertoire of antibodies in the MLR/*lpr* mouse, favoring development of anti-PC over ANA responses (Fig.4.11), the resultant immune complexes deposited in the kidneys of MRL/*lpr* treated with ES-62 may be smaller and/or less pathogenic than those formed by interactions with the DNA embedded in the glomerular basement membrane. Nevertheless, such immune complexes would still be expected to stimulate renal cells via their cross-linking of FcR: thus, by suppressing the expression of FcRs, such as CD16 and CD64, ES-62 may render these renal cells hyporesponsive to such inflammatory stimuli. Moreover, the accompanying suppression of MyD88 expression by ES-62 would further reduce their responsiveness, as immune complex-mediated renal damage has been reported to require synergistic signaling via FcRs and TLRs (433).

Both the classical and alternative complement pathways are active in the nephritic kidneys of SLE patients. The classical pathway appears to be involved in regulating the clearance of apoptotic debris and hence, is generally viewed as a positive factor in lupus nephritis (32). The alternative pathway, on the other hand, is generally thought to contribute towards lupus nephritis by the activation and recruitment of inflammatory cells (35) and consistent with this, inhibition of the alternative pathway can prevent glomerular nephritis in the MRL/*lpr* and NZB/W F1

mouse models of SLE (434,435). Thus, although ES-62 does not appear to suppress the deposition of complement in the kidneys of MRL/*lpr* mice, by reducing the expression of C3aR and C5aR, the parasite product may render renal cells hyporesponsive to these pro-inflammatory stimuli. Indeed, suppression of C3aR signaling has been shown to prevent the induction of pathogenic IL-23 responses (436,437) and this may be relevant to the reduction of pathogenic IL-22-producing cells (126) observed in the kidneys of ES-62 treated mice (Chapter 3). Likewise, C5aR signaling acts in synergy with TLR4 and MyD88 signaling to drive IL-17F production by macrophages (438), and in addition, drives IL-17A production by activating DC production of IL-6 and TGFβ (439): thus, suppression of C5a responses may play a role in desensitizing the kidney to the pathogenic effects of IL-17A in the initiation phase of disease.

TLR1-9 and TLR11 are expressed and upregulated in the kidneys of lupus prone mice (440). Reflecting this, TLR signaling aggravates glomerulonephritis (441-443) presumably as a result of its role in neutrophil recruitment to the kidney (443,444). Furthermore, TLR signaling via MyD88 promotes IL-1α, IL-1β, IL-6, TNFα and MCP-1 production in the kidney (433,445) and is required for the development of pristane-induced nephropathy (446). Collectively, therefore, the reduction of MyD88 observed in BMM, renal fibroblasts and kidney cells from ES-62-treated MRL/*lpr* mice would provide a mechanism, by preventing neutrophil recruitment and proinflammatory cytokine production, to explain, at least in part, improved renal function in such mice.

Indeed, TLR4 expression has been shown to correlate with levels of MCP-1 and IL-6 in the kidneys of patients suffering from chronic kidney disease (447). Similarly, serum and urinary levels of MCP-1 correlate with disease activity, and suppression of synthesis of this chemokine prevents mesangial cell proliferation (448,449). As IL-17A promotes the recruitment of inflammatory cells, such as monocytes and neutrophils, to the kidney through the induction of CCL2 (MCP1) (450), it was perhaps surprising to find that whilst ES-62 increased the concentration of IL-17A in the renal interstitial fluid of 21-week MRL/*lpr* mice, this did not result in an increase in infiltrating neutrophils or monocytes. However, this may be explained by the observation that LPS induced production of MCP1 was

lower in renal fibroblasts from either ES-62- or rIL-17-treated mice, presumably reflecting the decreased expression of MyD88 by such cells. This latter finding perhaps suggests that ES-62 exploits a homeostatic (IL-17-mediated) mechanism for first initiating and then limiting and resolving inflammation in the kidney.

The biological function of the kidney glomerulus is to filter the blood of nitrogen-containing urea, water and salt. In order to prevent the escape of macromolecules and cells into the urine, the glomerular basement membrane must form a tight barrier (451). The reduced development of proteinuria in MRL/*lpr* mice treated with ES-62 is an indication that the parasite product improves renal function; ES-62 must therefore promote epithelial barrier function, potentially by affecting tight junctions and this could be achieved by several potential mechanisms. Firstly, whilst MyD88 signaling appears to be important, via MyD88-ARNO-ARF6 signaling, for the disruption of vascular stability (452), it has also been found to promote epithelial cell homeostasis (453), with recent findings demonstrating that MyD88, MAL and PKC signaling is required for the maintenance of epithelial tight junction barriers (454). Whilst ES-62 has been shown to modulate MyD88 and PKC isoform in a number of cell types (311), it appears that the parasite product does not influence MAL expression (Tay, Eason & Harnett; unpublished data). It may be possible therefore that whilst suppressing pro-inflammatory TLR signaling, unbalanced MyD88 and Mal signaling in the kidneys of ES-62 treated mice may enhance tight barrier formation. Alternatively, inhibition of C5aR signaling has been shown to promote the maintenance and viability of the blood brain barrier in the MRL/*lpr* mouse (455,456) and hence, suppression of C5a signaling, by reducing C5aR expression, may allow ES-62 to exert similar effects in the kidney. Finally, resetting of the balance of IL-17A and IL-22 production by ES-62 may also have affected tight barrier formation, as IL-23 has been reported to be required for the development of glomerulonephritis in the anti-glomerular basement membrane (αGMB) model (457). Although, IL-22 has been shown to promote epithelial barrier formation and resistance to injury in the lung (132), it has been widely reported to play both protective and pathogenic roles depending on the inflammatory context and hence during chronic IL-22 signaling the fibroblast proliferation required for wound healing may lead to fibrosis and act to disrupt barrier formation.

ES-62 is a large immunogenic compound that is unsuitable as a therapeutic and therefore small molecular analogues (SMAs) have been designed, based on the PC-moiety that appears to be responsible for most of the immunomodulatory actions of the parasite product. Two of these compounds, S3 and S5, suppressed the development of proteinuria in MRL/*lpr* mice even although they were administered following the onset of pathology: although their mechanism of action has not yet been defined, as with ES-62, protection was associated with reduction of MyD88 expression by renal cells. Interestingly, the SMAs did not improve renal histology or appear to reduce ANA production, although serum from some of the mice receiving S5 exhibited a similar pattern of membrane staining as that from ES-62 treated mice. As glomerulonephritis remains a major problem for SLE patients, new drugs to prevent kidney failure are urgently required. Exploitation of the natural relationship between parasite immunomodulatory proteins and the immune system may therefore provide the basis for the development of novel immunomodulatory drugs. As ES-62 can improve renal function despite clear kidney pathology, drugs based on its mechanism of action may therefore prolong the working life of such kidneys and subsequently have a clear impact on the quality of life of SLE patients.

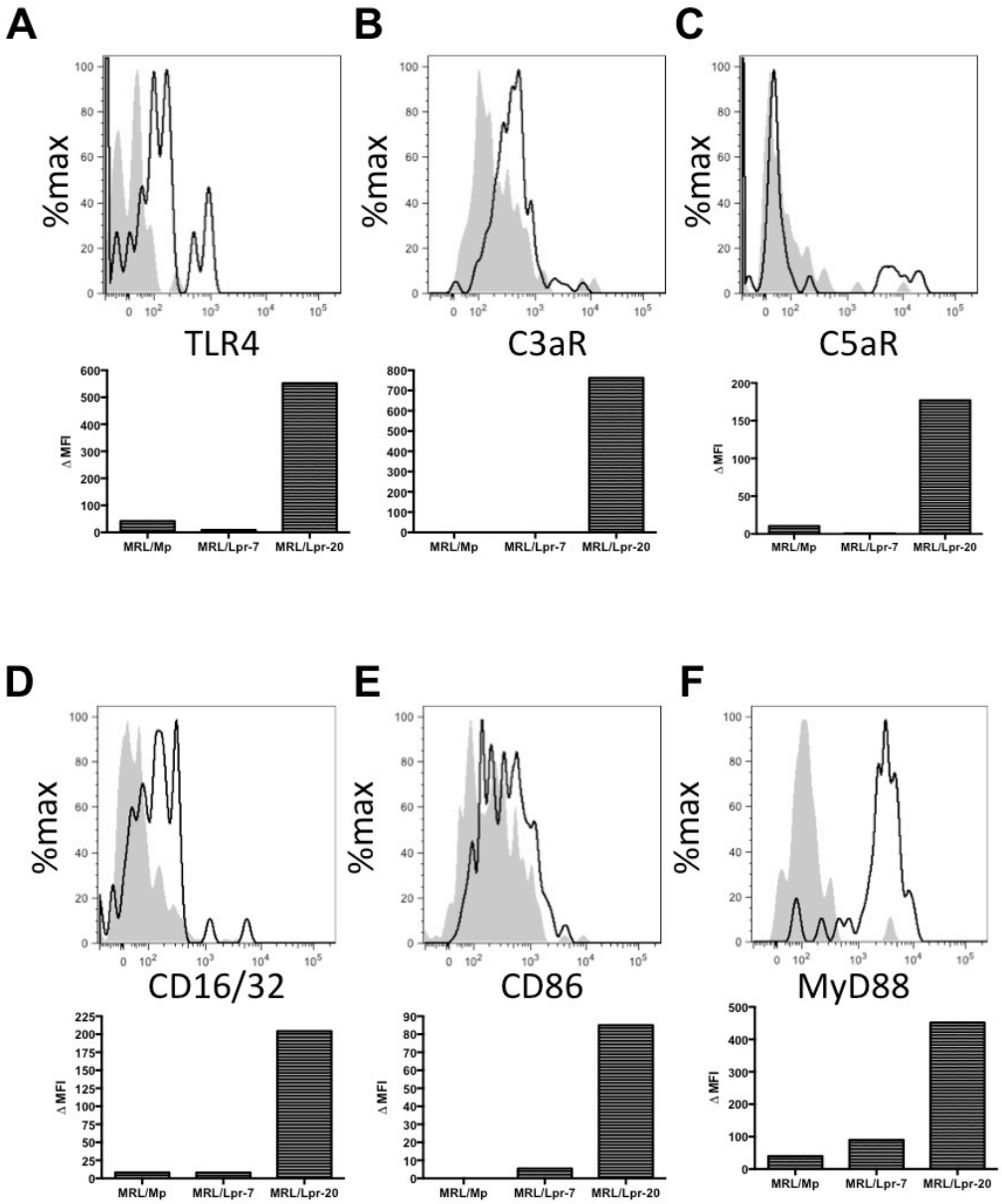

A
B
C
%max
TLR4
C3aR
C5aR
Δ MFI
MRL/Mp
MRL/Lpr-7
MRL/Lpr-20
D
E
F
CD16/32
CD86
MyD88

Figure 5.1: Pro-inflammatory receptor expression by renal cells. Kidney cells from 21-week old MRL/MP, 7- or 21-week old MRL/*lpr* mice were pooled (4 mice per group) and the expression of TLR4, CD16/32, C3aR, C5aR, MyD88 and CD86 evaluated by flow cytometry. Exemplar plots are shown for 21-week MRL/*lpr* mice, positive stains are represented as open histograms and the isotype as a filled grey histogram. The data are presented as MFI of expression of each marker relative to its isotype control.

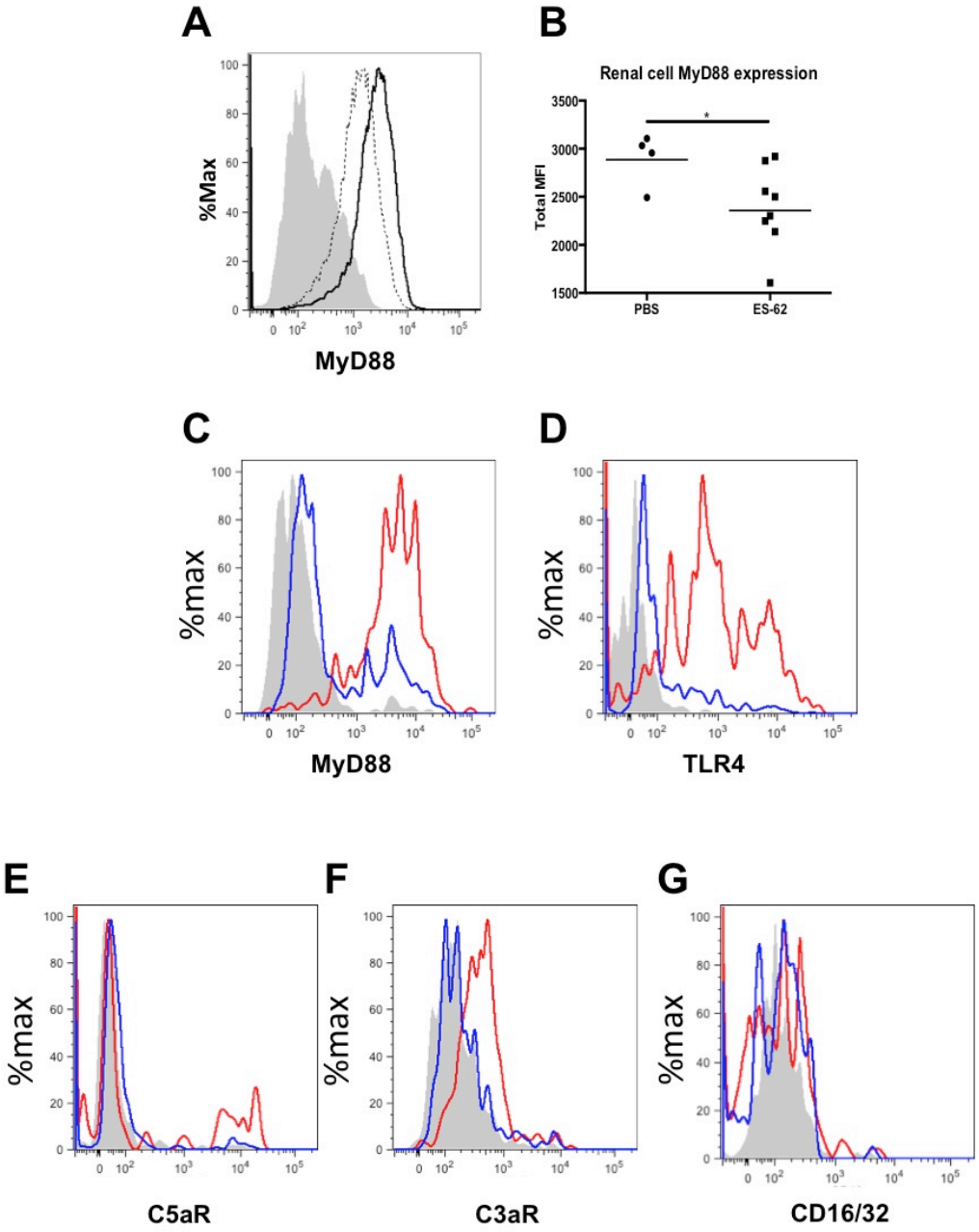

A
%Max
MyD88
B
Renal cell MyD88 expression
Total MFI
PBS
ES-62
C
%max
MyD88
D
%max
TLR4
E
%max
C5aR
F
%max
C3aR
G
%max
CD16/32

Figure 5.2: The expression of pro-inflammatory receptors by renal cells is reduced in mice treated with ES-62. MyD88 expression was evaluated in renal cells from MRL/*lpr* mice treated with or without ES-62 by flow cytometry (A & B). Kidney cells from PBS treated MRL/*lpr* mice (n=4 pooled) were pre-treated with (blue line) or without (red line) ES-62 and then cultured for 18 hours in the presence of LPS (1μg/ml), the expression of MyD88 (C), TLR4 (D), C5aR (E), C3aR (F) and CD16/32 (G) was the determined by flow cytometry and compared to relevant isotype controls (grey histogram). Data are representative of two independent experiments and the student's t-test was used to show statistical significance (B).

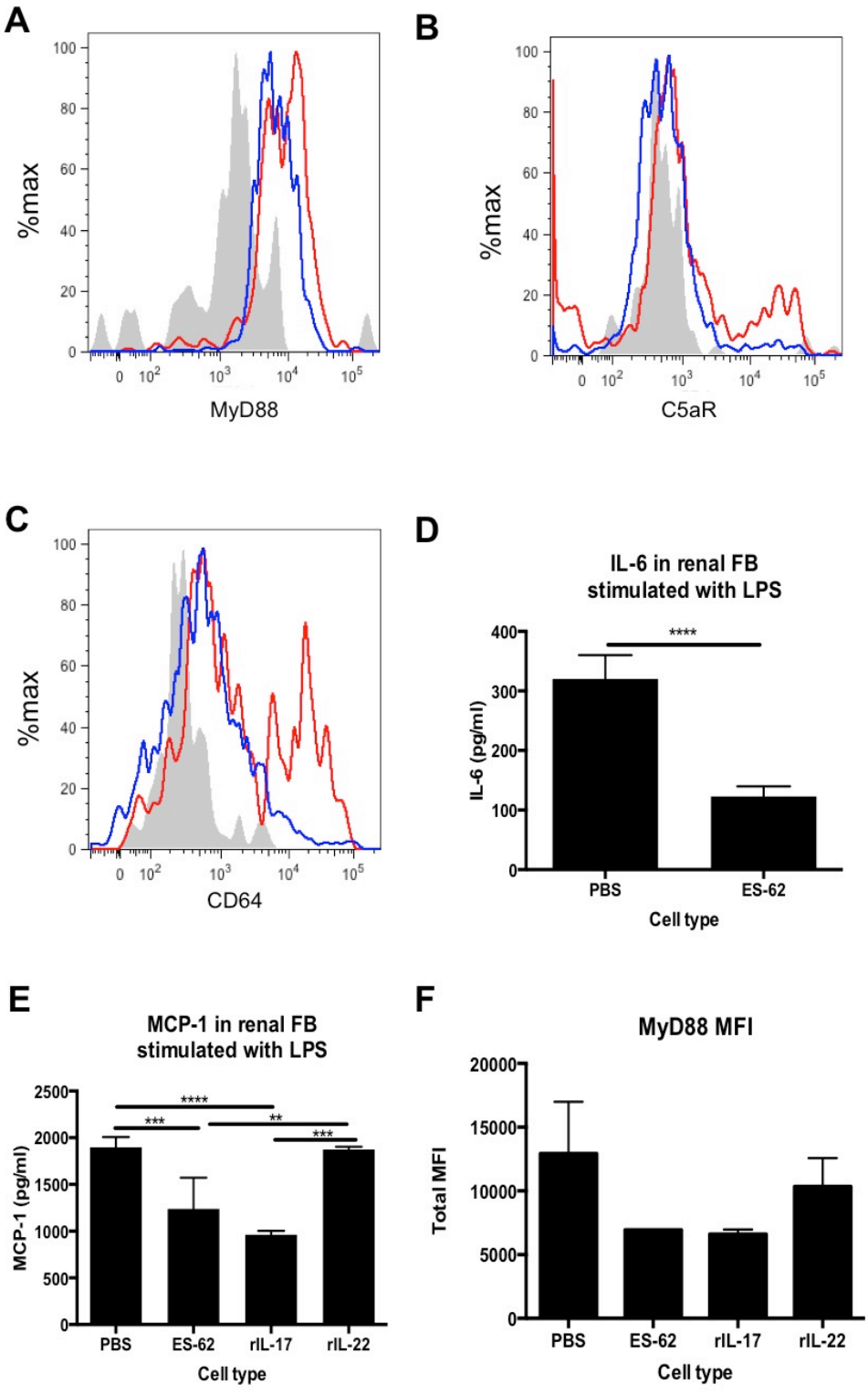

A
%max
MyD88
B
%max
C5aR
C
%max
CD64
D
IL-6 in renal FB
stimulated with LPS
IL-6 (pg/ml)
****
PBS
ES-62
Cell type
E
MCP-1 in renal FB
stimulated with LPS
MCP-1 (pg/ml)
****
***
**
***
PBS
ES-62
rIL-17
rIL-22
Cell type
F
MyD88 MFI
Total MFI
PBS
ES-62
rIL-17
rIL-22
Cell type

Figure 5.3: ES-62 renders renal fibroblasts hypo-responsive. Renal fibroblasts were expanded from the kidneys of ES-62 (blue line) or PBS (red line) treated MRL/*lpr* mice, the expression of MyD88 (A), C5aR (B) and CD64 (C) were evaluated by flow cytometry and compared to an appropriate isotype control. Representative plots are shown alongside graphs of three biological replicates from one of two independent experiments (A-C). The concentration of IL-6 (D) and MCP-1 (E) were measured in the supernatants from renal fibroblasts stimulated with LPS for 18 hours. The total mean fluorescence intensity (total MFI) of MyD88 in these cells was measured by flow cytometry (F). The student's t-test (D) and one-way ANOVA (E) tests were used to show statistical significance where **=$p<0.001$ and ***=$p<0.001$.

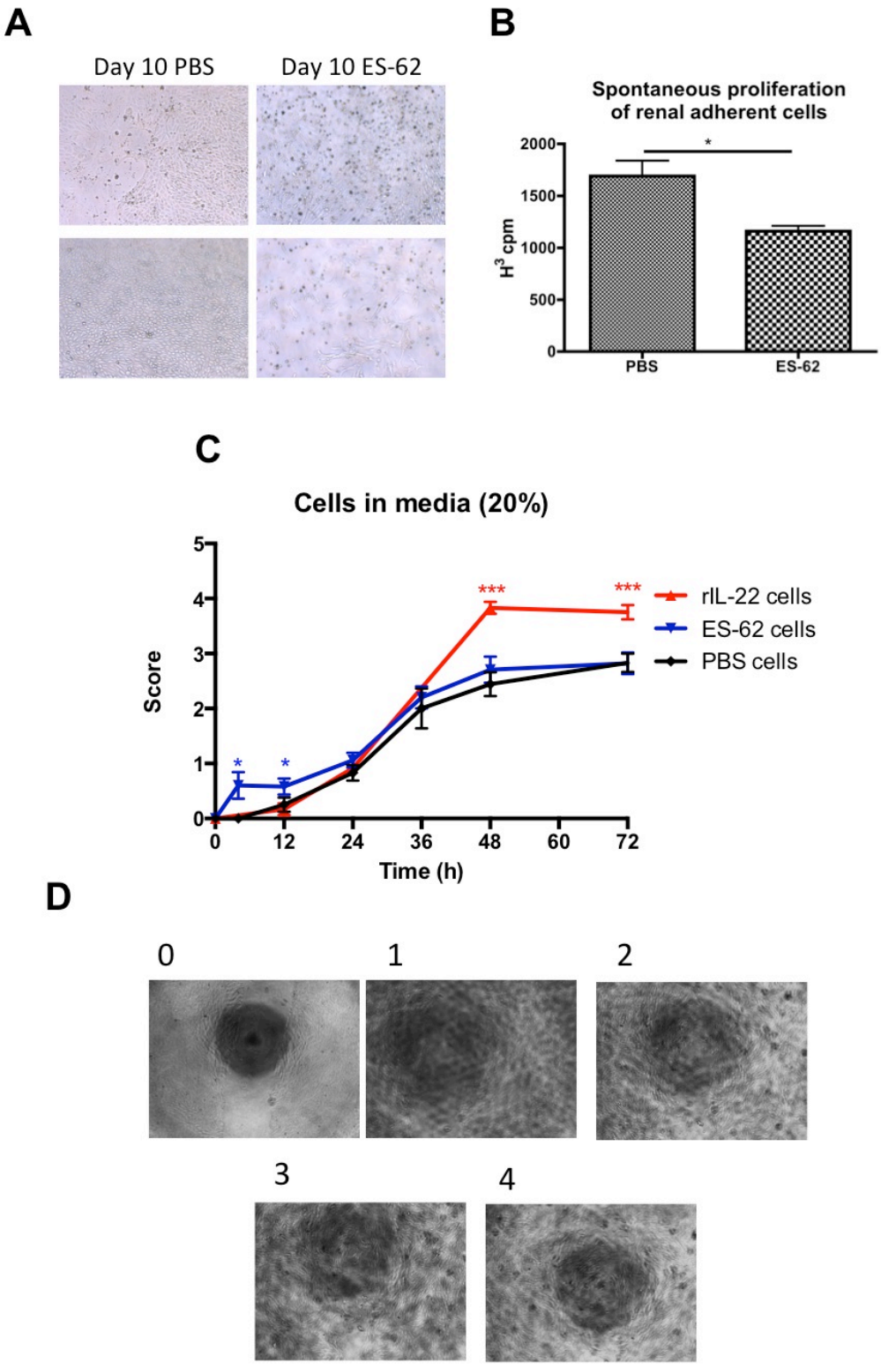

A
Day 10 PBS
Day 10 ES-62
B
Spontaneous proliferation of renal adherent cells
H^3 cpm
2000
1500
1000
500
0
*
PBS
ES-62
C
Cells in media (20%)
Score
Time (h)
0
12
24
36
48
60
72
***
***
*
*
rIL-22 cells
ES-62 cells
PBS cells
D
0
1
2
3
4

Figure 5.4: Renal fibroblast proliferation is altered in ES-62 treated mice. Renal fibroblasts expanded from the kidneys of 21-week old PBS or ES-62 treated MRL/*lpr* mice were photographed after 10 days, using the Olympus IX51 light microscope at x10 optical zoom (A). Renal fibroblasts were cultured with [^{3}H] thymidine for 24 hours before the cells were harvested and and incorporation into DNA measured by a microbeta plate reader. Renal fibroblasts from mice treated with PBS, ES-62 or rIL-22 were cultured in a 96-well plate until they reached 80-90% confluency, at which point a 10 μm wound was created using a pipette tip; the rate of recovery (C) was then scored over 72 hours on the basis of clean cut (0), cellular extensions across cut (1), cellular extensions and wound narrowing (2), narrow wound, almost closed (3) and healed (4) (D). Statistical significant was shown using the 2-way ANOVA where *=$p<0.05$ and ***=$p<0.0001$.

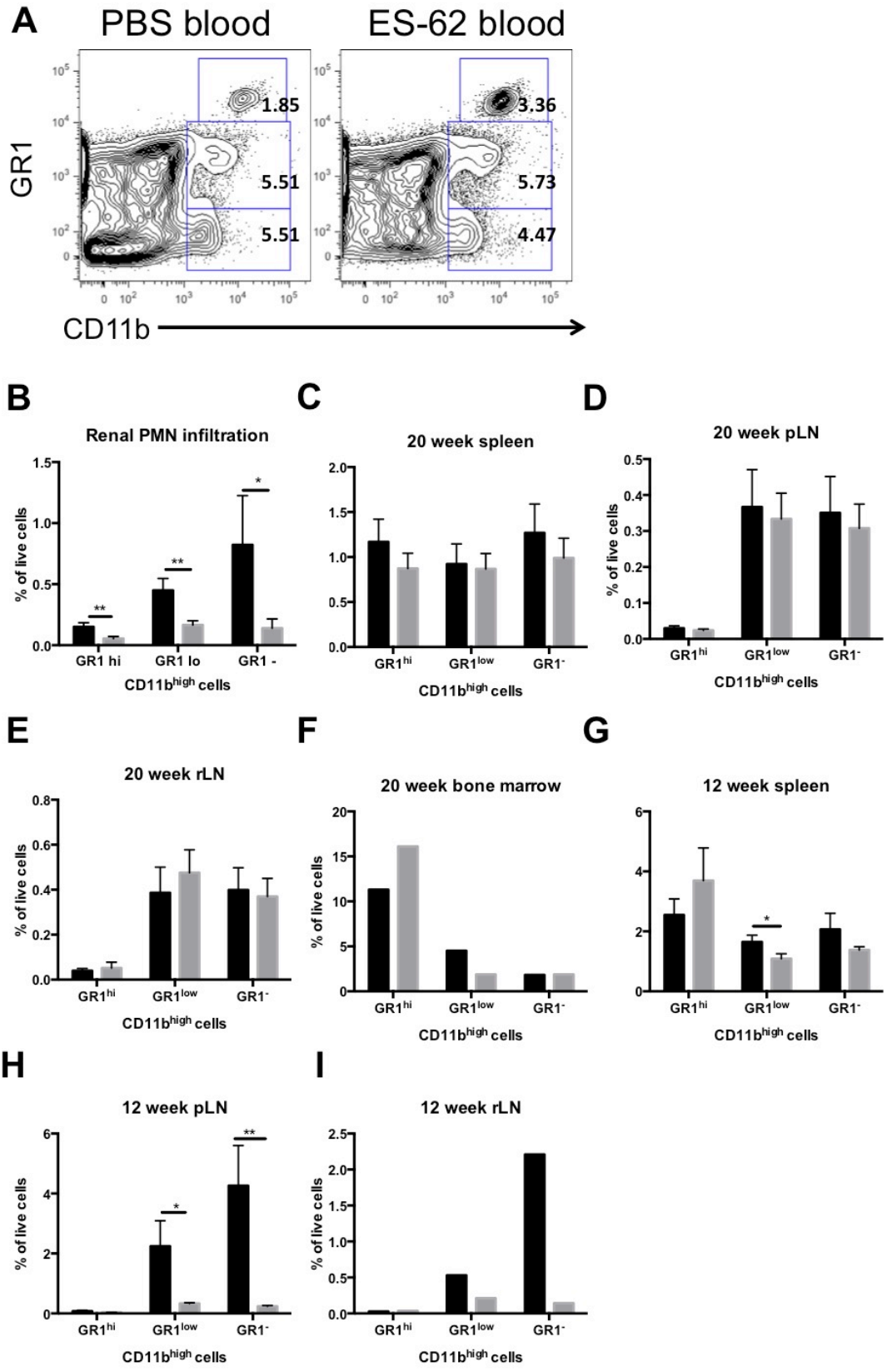

A
PBS blood
ES-62 blood
GR1
CD11b
1.85
5.51
5.51
3.36
5.73
4.47
B
Renal PMN infiltration
% of live cells
GR1 hi
GR1 lo
GR1 -
CD11b^high cells
C
20 week spleen
GR1^hi
GR1^low
GR1^-
CD11b^high cells
D
20 week pLN
GR1^hi
GR1^low
GR1^-
CD11b^high cells
E
20 week rLN
GR1^hi
GR1^low
GR1^-
CD11b^high cells
F
20 week bone marrow
GR1^hi
GR1^low
GR1^-
CD11b^high cells
G
12 week spleen
GR1^hi
GR1^low
GR1^-
CD11b^high cells
H
12 week pLN
GR1^hi
GR1^low
GR1^-
CD11b^high cells
I
12 week rLN
GR1^hi
GR1^low
GR1^-
CD11b^high cells

Figure 5.5: ES-62 modulated the population dynamics of myeloid cells. Cells from 21-week old PBS (black bar, n=11) or ES-62 (grey bar, n=18) treated MRL/*lpr* mice were stained with anti-GR1 and anti-CD11b antibodies to resolve the $CD11b^{high}GR1^{high}$, $CD11b^{high}GR1^{int}$, $CD11b^{high}GR1^{low}$ and $CD11b^{high}GR1^{-}$ granulocyte populations in the blood (A, sample pooled from 4 mice for each group and is representative of 2 independent experiments), kidneys (B), spleens (C), pLN (D), rLN (E) and bone marrow (F, 4 mice pooled from each group); and the spleen (G), pLN (H) and rLN (I, 4 mice pooled from each group) of 12-week MRL/*lpr* mice.

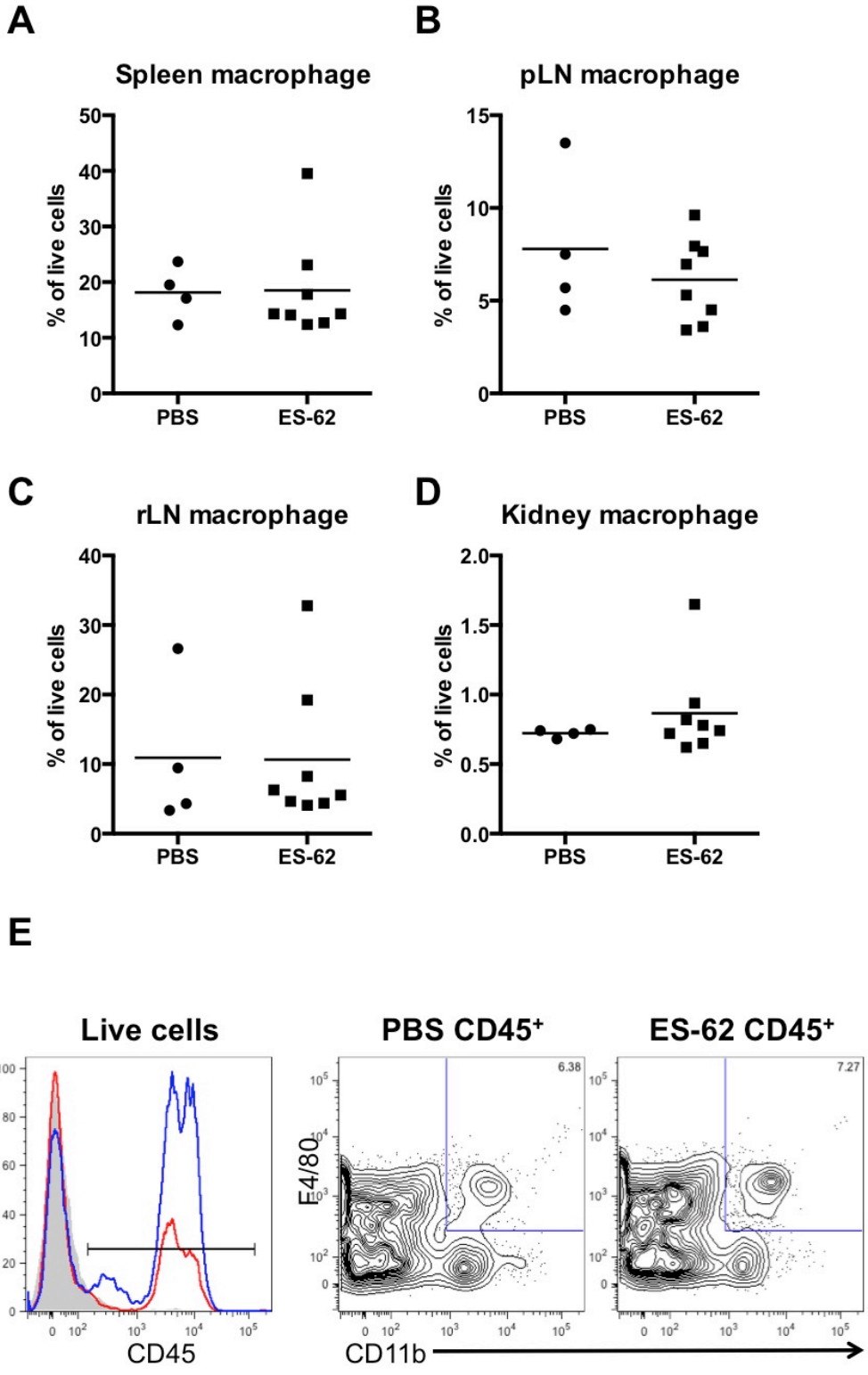

A
Spleen macrophage
% of live cells
50
40
30
20
10
0
PBS
ES-62
B
pLN macrophage
% of live cells
15
10
5
0
PBS
ES-62
C
rLN macrophage
% of live cells
40
30
20
10
0
PBS
ES-62
D
Kidney macrophage
% of live cells
2.0
1.5
1.0
0.5
0.0
PBS
ES-62
E
Live cells
PBS CD45+
ES-62 CD45+
%max
100
80
60
40
20
0
CD45
E4/80
CD11b
6.38
7.27

Figure 5.6: Macrophages populations in PBS and ES-62 treated mice. The proportion of $CD45^+F4/80^+CD11b^+CD64^+$ macrophages present in the spleen (A), pLN (B), rLN (C), kidney (D) and in the blood (E) of 21-week MRL/*lpr* mice treated with or without ES-62 was determined by flow cytometry; where live cells were first identified as being $CD45^+$ (red line = PBS, blue line = ES-62, grey histogram = isotype) and then selected on the basis of being $F4/80^+CD11b^+$.

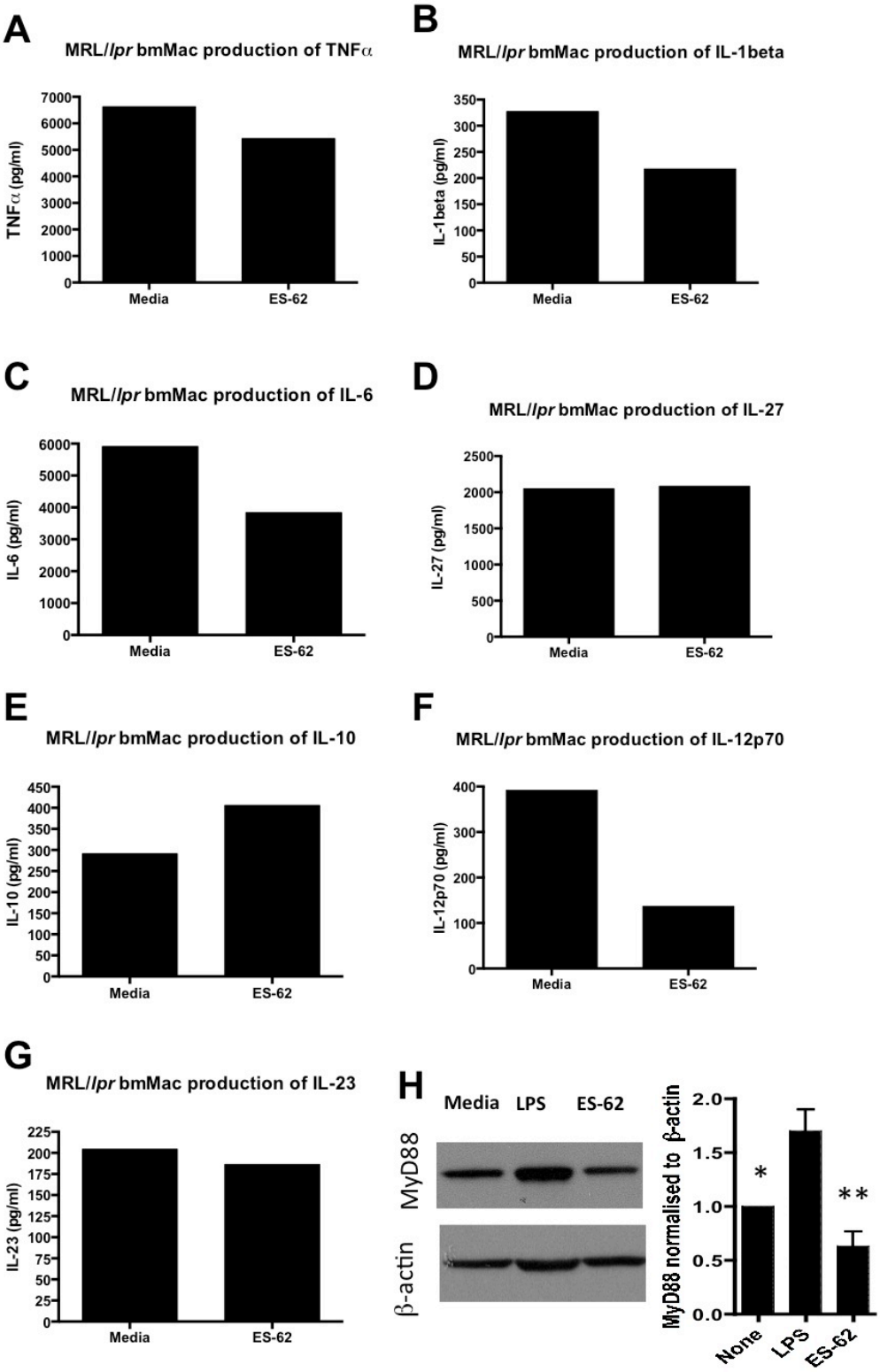
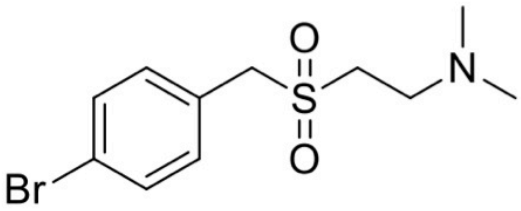

A
MRL/lpr bmMac production of TNFα
TNFα (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
Media
ES-62
B
MRL/lpr bmMac production of IL-1beta
IL-1beta (pg/ml)
350
300
250
200
150
100
50
0
Media
ES-62
C
MRL/lpr bmMac production of IL-6
IL-6 (pg/ml)
6000
5000
4000
3000
2000
1000
0
Media
ES-62
D
MRL/lpr bmMac production of IL-27
IL-27 (pg/ml)
2500
2000
1500
1000
500
0
Media
ES-62
E
MRL/lpr bmMac production of IL-10
IL-10 (pg/ml)
450
400
350
300
250
200
150
100
50
0
Media
ES-62
F
MRL/lpr bmMac production of IL-12p70
IL-12p70 (pg/ml)
400
300
200
100
0
Media
ES-62
G
MRL/lpr bmMac production of IL-23
IL-23 (pg/ml)
225
200
175
150
125
100
75
50
25
0
Media
ES-62
H
Media
LPS
ES-62
MyD88
β-actin
MyD88 normalised to β-actin
2.0
1.5
1.0
0.5
0.0
*
**
None
LPS
ES-62

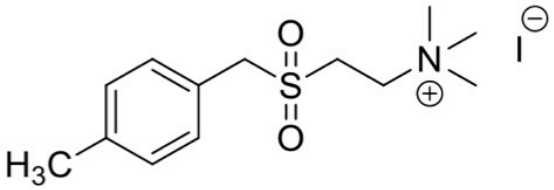
Figure 5.7: ES-62 suppresses pro-inflammatory cytokine production by bone marrow derived macrophages. Macrophages derived from bone marrow precursors from the femur and tibia of 21-week old MRL/*lpr* mice, were pre-treated with media or ES-62 for 2 hours before being cultured with LPS for 18 hours. The concentration of TNFα (A), IL-1β (B), IL-6 (C), IL-27 (D), IL-10 (E), IL-12p70 (F) and IL-23 (G) was then measured in the culture supernatants by ELISA; data are from duplicate biological replicates and are representative of 3 independent experiments. The level of MyD88 expression was determined by Western blot, using the ab2068 antibody (Abcam), and the protein level, relative to β-actin, was measured using the ImageJ software package from 7 independent experiments; statistical significance was shown using the 1-way ANOVA with the Tukey post-test where *=$p<0.05$ and **=$p<0.001$ (H).

A

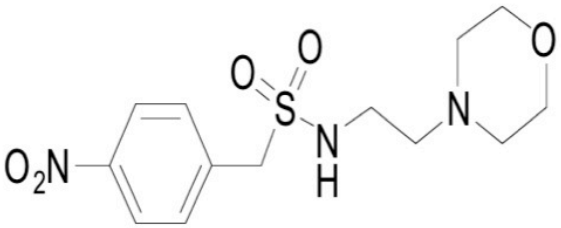
S3



S5



SMA15



B

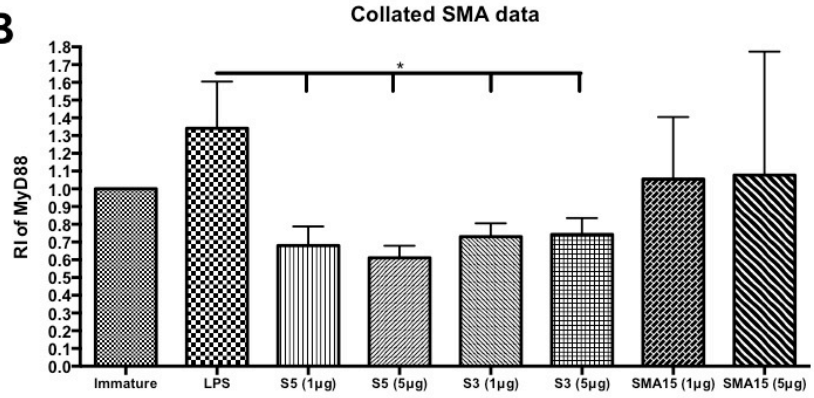


Figure 5.8: The effect of S3, S5 and SMA15 on MyD88 protein levels in BMM. Diagrams of the chemical structures of S3, S5 and SMA15 (A). BMM from MRL/*lpr* mice were cultured with media, LPS, S3 (at 1μg/ml or 5μg/ml), S5 (at 1μg/ml or 5μg/ml) or SMA15 (at 1μg/ml or 5μg/ml) for 18 hours; cells were washed, lysed and the relative intensity of MyD88 (ab2068), relative to β-actin, was analyzed in protein lysates by Western blotting. Image J analysis from 5 experiments showed that S3 and S5 significantly downregulate MyD88 expression (A).

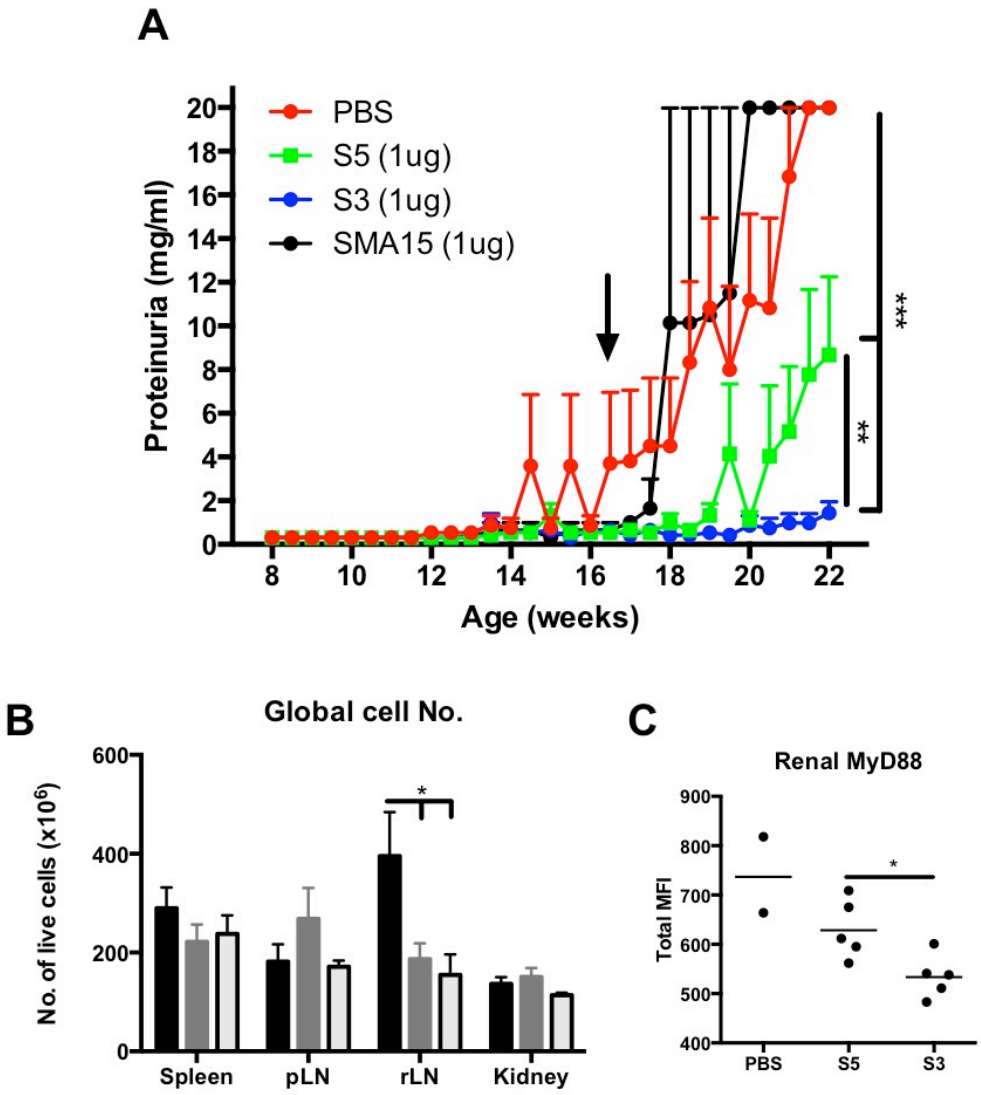

A
PBS
S5 (1ug)
S3 (1ug)
SMA15 (1ug)
Proteinuria (mg/ml)
Age (weeks)
***
**
B
Global cell No.
No. of live cells (x10^6)
Spleen
pLN
rLN
Kidney
C
Renal MyD88
Total MFI
PBS
S5
S3

Figure 5.9: S3 and S5 prevent the development of proteinuria in the MRL/*lpr* mice. Female MRL/*lpr* mice were monitored for onset (1-3 mg/ml) of proteinuria which occurred at 16-weeks of age (arrow), at which point the mice began to developed proteinuria levels of approximately 1-3 mg/ml. Mice received 1μg of S3 (n=6), S5 (n=6), SMA15 (n=3, one of these mice died at week 18) (all at 1 μg in 100μl) or PBS (n=4; 100 μl) injected sub-cutaneously twice weekly. Proteinuria was measured twice weekly throughout the model and mice were culled at week 22 (A). The number of cells in the spleen, pLN, rLN and kidney was counted using a hemacytometer (B) and the level of MyD88 expression by kidney cells was determined by flow cytometry (B). The two-way ANOVA and student's t-test statistical analysis were used to determine statistical significance where *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$.

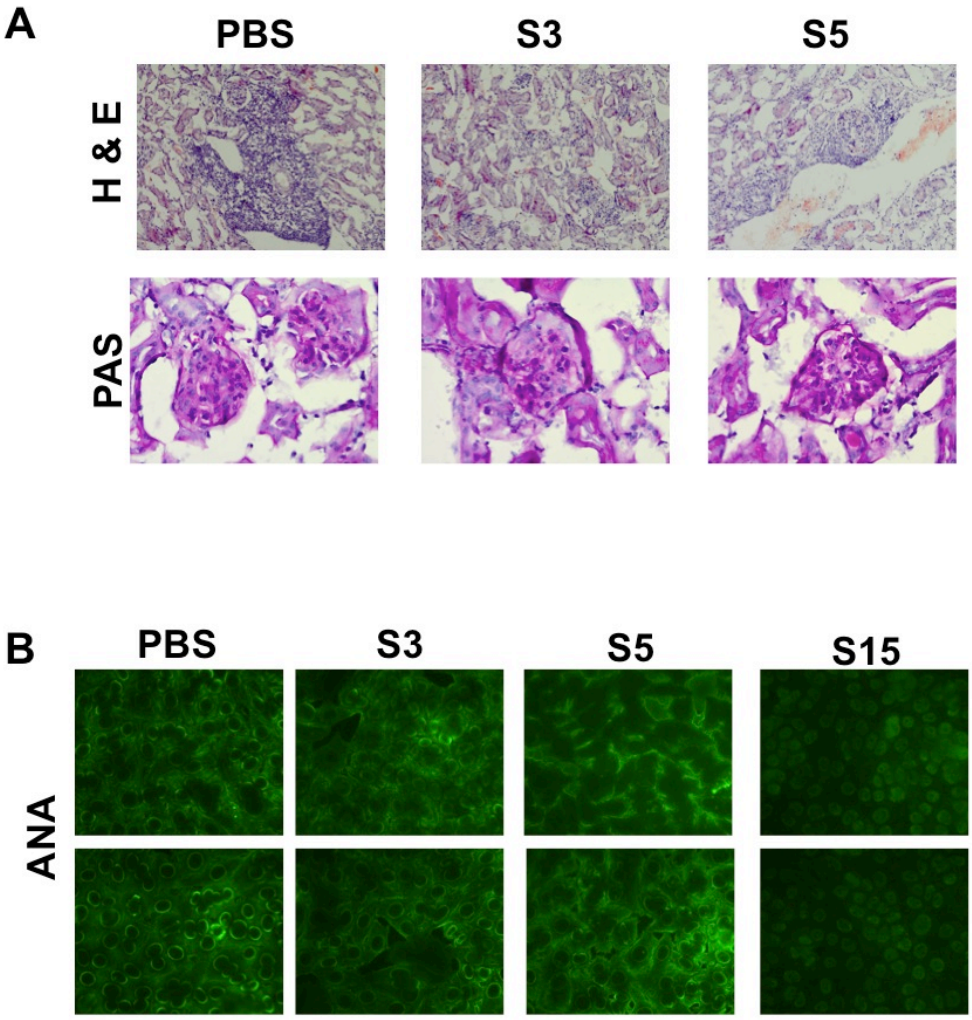

A
PBS
S3
S5
H & E
PAS
B
PBS
S3
S5
S15
ANA

Figure 5.10: Treatment with S3 or S5 does not improve renal histology. Kidney sections from mice treated with PBS, S3 or S5 were stained by H & E or PAS and visualized using the Olympus IX51 light microscope at x10 optical zoom (A). Serum from the mice treated with PBS, S3, S5 or SMA15 was used to stain Hep-2 slides for analysis of ANA:staining intensity was then evaluated using an axiovert fluorescence microscope at 10x optical zoom (B).

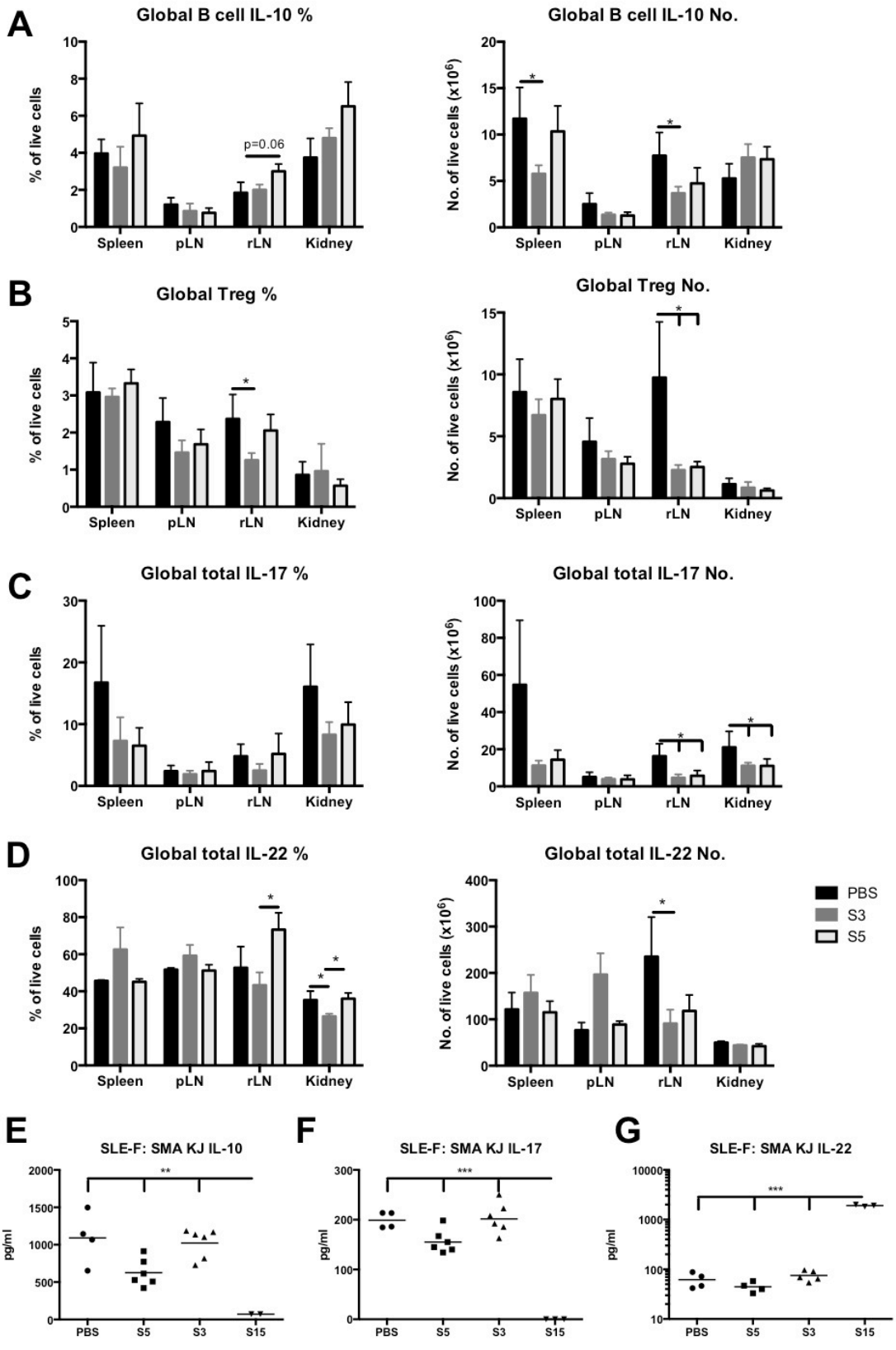

A
Global B cell IL-10 %
% of live cells
p=0.06
Spleen
pLN
rLN
Kidney
Global B cell IL-10 No.
No. of live cells (x10^6)
B
Global Treg %
Global Treg No.
C
Global total IL-17 %
Global total IL-17 No.
D
Global total IL-22 %
Global total IL-22 No.
PBS
S3
S5
E
SLE-F: SMA KJ IL-10
pg/ml
S15
F
SLE-F: SMA KJ IL-17
G
SLE-F: SMA KJ IL-22

Figure 5.11: Treatment with S3 or S5 does not modulate the key immunological parameters targeted by ES-62. Cells from the spleen, pLN, rLN or kidneys of 21-week female MRL/*lpr* mice treated with PBS, S3 or S5 were stimulated with PMA plus ionomycin (and LPS for IL-10 (A)) for 6 hours, with BrefeldinA added after 30 minutes to block intracellular trafficking. Cells were stained with the Live/Dead cell discrimination fixable dye and stained for surface markers. Cells were then fixed and permeabilized and incubated with the relevant anti-mouse-cytokine or FoxP3 antibodies. Cells were analyzed by flow cytometry and the proportion and number of IL-10$^+$CD19$^+$ (A), CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ Tregs (B), IL-17$^+$ (C) and IL-22$^+$ (D) cells were determined. The concentration of IL-10 (E), IL-17A (F) and IL-22 (G) in the interstitial fluid of PBS, S3, S5 and S15 treated mice was measured by ELISA. The student's t-test was used to determine statistical significance where *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$.

6. ES-62 modulation of collagen-induced arthritis in mice is associated with an increase in IL-10-producing B cells and reduced plasma cell infiltration of the joints

6.1. Introduction

The data presented in the previous chapters demonstrates that ES-62 protects against the development of autoimmune lupus-like pathology in the MRL/lpr mouse, by resetting the balance of effector: regulatory B cells and consequently IL-17A/IL-22 responses. Moreover, transfer of B cells from ES-62-, but not PBS-treated MRL/lpr mice was sufficient to recapitulate the protection afforded by ES-62. Protection was determined in terms of proteinuria, but such mice may sometimes also exhibit arthritis and this was also prevented by exposure to ES-62 (McGrath, Harnett & Harnett, unpublished data). Thus, as the mice in the cohorts involved in the B cell transfer studies did not develop arthritis, and also to determine whether this action of ES-62 was specific to the B cell-driven pathology in Lupus, it was decided to determine whether ES-62 generally acted to reset the effector: regulatory B cell balance in other autoimmune pathologies such as the mouse collagen-induced arthritis (CIA) model of Rheumatoid Arthritis (RA) in which it has been previously shown to confer protection by acting to suppress pathogenic Th1/Th17 responses (336). This model was chosen as the success of rituximab as a therapy for autoimmune disease had refocused interest on the pathogenic and protective roles of B cells in RA (458) with recent studies highlighting the importance of IL-10-producing regulatory B cells (Bregs) in the prevention and amelioration of CIA and also antigen-induced arthritis (AIA), via the suppression of Th1/Th17 responses and the promotion of Tr1 and Treg differentiation (245,345,458,459). In this context, it was interesting, therefore, that previous studies had found that whilst exposure to ES-62 resulted in enhanced spontaneous ex vivo production of IL-10 by splenocytes from mice with CIA, it induced hypo-responsiveness of normal and CIA-derived splenic B cells and reduced the levels of pathogenic collagen-specific IgG2a antibodies (319). It was therefore the aim of this chapter to determine whether the protective effects of ES-62 in CIA are associated with an increase in the levels of IL-10-producing B cells and suppression of effector B cells, to address whether ES-62 may act, at least in part, to modulate the balance between effector and regulatory B cell responses in this mouse model of RA.

6.2. Results

6.2.1. ES-62 reduces the levels of Germinal Centre B cells in the spleens of mice with CIA

The associated between ES-62-mediated protection against CIA (Fig. 6.3A & B) and the modulation of B cell populations was investigated (Fig. 6.1 & 2). There was no significant modulation by the helminth product of either the proportion or number of $CD19^+$ B cells in the spleen (Fig. 6.4A; numbers ($x10^6$) ± SEM: Naive, 25.13 ± 2.26; PBS, 26.86 ± 1.76; ES-62, 28.27 ± 2.68) or draining LN (dLN) (results not shown) and consistent with this, no significant changes were observed in the transitional (T1-T3), marginal zone precursor (MZP) or marginal zone (MZ) populations in the spleen (Fig. 6.4B & C and results not shown). However, ES-62 significantly increased the levels of $CD19^+CD21^{low/-}CD23^{high}$ follicular B cells (Fo; Fig. 6.4B & C) and further analysis showed that whilst exposure to ES-62 had no effect on follicular type-2 (Fo2) B cells (results not shown), it increased the levels of follicular type-1 B cells (Fo1; Fig. 6.4 D; numbers ($x10^6$) ± SEM: PBS, 6.08 ± 0.64; ES-62, 7.97 ± 0.91). The reduction in Fo1 B cells was associated with corresponding reductions in germinal centre B cells (GC; Fig. 6.4E; numbers ($x10^6$) ± SEM are: PBS, 6.28 ± 1.36; ES-62, 2.95 ± 0.39) and $CD3^+CD4^+ICOS^+CXCR5^+$ follicular helper T cells (Tfh; from 2.42 to 1.49% of live splenocytes; Fig. 6.4F) in the spleen. By contrast, ES-62 had no effect on the levels of $CD19^+B220^+IgM^-IgD^-IgG^+$ cells, which may represent a subset of memory B cells and were generally reduced in mice with CIA (Fig. 6.3G). Collectively, these data suggest that ES-62 may act to reduce the generation of pathogenic antibodies by blocking the activation of follicular B cells and their consequent differentiation into GC B cells (460).

6.2.2. ES-62 modulates the recruitment of B cells to the joints of mice with CIA

As pathogenic B cells migrate to the joints and even form ectopic germinal centres in response to B cell recruitment and survival factors such as BAFF, CXCL12 and CXCL13 secreted by synovial fibroblasts (458), whether the above effects of in

vivo exposure to ES-62 on the profile of B cells were reflected in the arthritic joint was investigated. This revealed that both the proportion (Fig. 6.5A & B) and absolute numbers (Fig. 6.5C) of $CD19^+$ B cells found in the joints were significantly reduced by ES-62 treatment. This reduction was reflected in a $CD19^+CD23^+$ B cell population (Fig. 6.5D & E), which further analysis revealed to be follicular type 1 B cells (Table 6.1). There was also a clear decrease in $CD19^-CD138^+$ (from 9.27 to 2.47% live cells) and $CD19^+CD138^+$ (from 15.6 to 6.51% live cells) cells infiltrating the joints of mice treated with ES-62 (Fig. 6.5F & G) suggestive of a reduction in plasma cells. Consistent with this, further analysis, excluding the myeloid and T cell lineages expressing CD138 (Fig. 6.5H), revealed that exposure to ES-62 indeed suppressed the proportions (Fig. 6.5I-J) and numbers (Table 6.1) of $CD19^-B220^-CD138^+$ (from 8.31 to 3.69% live cells) and $CD19^+B220^{low/-}CD138^+$ (from 1.37 to 0.72% live cells) plasma cell respectively phenotypically similar to the long-lived plasma cell and short-lived plasma cell/plasmablast functional populations, reported previously (352,355,411). This presumably reflected reduced development and/or migration of such cells, as suggested by the significant increases in the levels of Fo1 (Fig. 6.4D) and $CD19^-B220^-CD138^+$ plasma cells (numbers ($x10^6$) ± SEM: Naïve, 0.75 ± 0.22; PBS, 1.28± 0.31; ES-62, 1.58± 0.26) found in the spleen, since ES-62 did not modulate the levels of early $CD19^+B220^+CD138^+$ "pre-plasma cells" which have been reported are subject to a tolerance checkpoint that is defective in the autoimmune-prone MRL/*lpr* mouse (354) (Fig. 6.4K).

In addition to investigating its effects on the levels of B cells found in the joint, modulation of the functional phenotype of such infiltrating cells was also assessed. Thus, whilst ES-62 did not significantly modulate the expression of BAFF-R, CD80, CD86 or TLR4 (either in terms of percentage positive cells or levels of expression) on splenic $CD19^+$ B cells, expression of CD80, TLR4 and, to a lesser extent, CD86, but not BAFF-R, by $CD19^+$ B cells in the joint was down-regulated (Fig. 6.6 and data not shown), mirroring a tendency towards a decrease in expression of both CD80 and TLR4 by $CD19^+$ DLN cells (Data not shown). Further analysis revealed that this reflected selective down-regulation of TLR4, CD80 and CD86 expression on $CD19^+CD23^{high}CD21^{low/-}$ follicular B cells but not $CD19^+CD23^-CD21^{high}$ or $CD19^+CD23^{high}CD21^{high}$ B cells (Fig. 6.6A). Moreover, although B1,

GC and plasma cells also expressed TLR4, such expression was not modulated by exposure to ES-62 (Fig. 6.6A)

Collectively, therefore, these data suggest that ES-62 may act to prevent development and migration of pathogenic B cells to the site of inflammation, with the residual B cells that infiltrate the joint being rendered functionally hypo-responsive.

6.2.3. IL-10-producing B cells are elevated in the spleens of mice with CIA receiving ES-62

IL-10 producing regulatory B cells (Bregs) have been reported to curb pathogenic Th1/Th17 responses in CIA and it has been proposed that these cells protect against disease by promoting the development of Tr-1 regulatory T cells (345). Although the ES-62-mediated suppression of B cell development and migration (Fig. 6.4 & 6.5) and consequent inhibition of generation of pathogenic IgG2a responses observed in CIA (335) might reflect the ability of the parasitic worm product to induce hypo-responsiveness of B2 cells by uncoupling the BCR from ErkMAPkinase signalling, both in vitro and in vivo, it is also interesting to note that it has previously been shown to induce the production of IL-10 by peritoneal B1 B cells (324). As B1 cells are not thought to play a central role in systemic autoimmunity and autoantibody production in CIA (461), although there is no unambiguous phenotype for B1 cells in the spleen (199), it is therefore possible that the reduced levels of $CD19^{+}IgM^{+}CD43^{high}CD5^{+}$ and $CD19^{+}IgM^{+}CD43^{high}CD5^{+}$ cells, that have been reported to be B1a and B1b cells respectively (200,353,462,463), found in the spleens (Fig. 6.7A-C) and DLNs (Fig. 6.7D) of ES-62-treated mice with CIA could reflect their egress and migration to the joints to mediate IL-10-dependent anti-inflammatory effects. Perhaps consistent with this, although the levels of B cells infiltrating the joint were reduced following treatment with ES-62, the proportion of such B1-like cells was slightly increased (PBS: 1.42%, 1.3%; ES-62: 1.58%, 1.51%, where data are expressed as the proportion (%) of live cells, that are $CD43^{+}CD19^{+}IgM^{+}$ B1-like cells, harvested from the joints of 6-7 mice/group in 2 independent experiments).

Analysis of IL-10-producing B cells (Fig. 6.8A) revealed that whilst induction of CIA resulted in a significant reduction in the levels of these cells, relative to naïve mice, this was not the case for those treated with ES-62. Indeed, exposure to ES-62 led to a significant enhancement in the level of IL-10-producing B cells in mice with CIA, restoring them to levels comparable with those found in healthy naive mice (Fig. 6.7C; numbers ($x10^6$) ± SEM: Naïve, 1.81 ± 0.22; PBS, 1.48 ± 0.12; ES-62, 1.99 ± 0.26). $CD19^+IL\text{-}10^+$ B cells in the spleens of naïve and CIA mice (Fig. 6.8A) reflected a mixed population comprising phenotypes consistent with marginal zone precursor, marginal zone, follicular B cells and $CD19^+CD21^{low/-}CD23^+$ B cells (Fig. 6.8B). Almost all of these cells expressed CD1d with the highest expression on the MZ ($CD21^+CD23^-$) and MZP ($CD21^+CD23^+$) populations (Fig. 6.8E) and some of the $IL\text{-}10^+$ cells expressed CD5 with the highest expression on the follicular and $IL\text{-}10^+CD19^+CD21^-CD23^-$ populations; both of these markers have previously associated with certain IL-10-producing B cells (464,465). Rather surprisingly, the ability of ES-62 to return the levels of IL-10-producing B cells towards that existing in naïve, non-arthritic mice, did not appear to involve a preferential increase of any of these phenotypes, perhaps suggesting it acts rather to regulate B cell responses in a "homeostatic" manner. Although the protective effects of IL-10-producing B cells in CIA have previously been reported to be associated with the induction of Tr-1 regulatory cells (345), our analysis showed the IL-10 production by splenocytes to be predominantly B cell-derived (Fig. 6.8A) and that the $CD19^-$ $IL\text{-}10^+$ population was not increased by treatment with ES-62 (Fig. 6.8D), suggesting that Tr-1 cells were not being induced in this case. Likewise, and consistent with the previously reported lack of Breg-mediated induction of Tregs in the CIA model (345), further investigation indicated that in vivo treatment with ES-62 did not result in enhanced levels of FoxP3-expressing $CD4^+$ regulatory T cells in the DLN (McGrath, Harnett & Harnett, unpublished data), as shown following induction of Bregs in AIA (419).

6.3. Discussion

The successful clinical implementation of B cell-depletion therapies in recent years has reignited interest in the pathogenic and protective roles of B cells in RA. In particular, much interest has focused on the ability of IL-10-producing regulatory B cells to prevent development of pathogenic Th1/Th17 responses and induce regulatory T cells that result in the suppression of disease in the antigen (methylated BSA)-induced arthritis (AIA) and CIA mouse models of RA (245,345,419). Interestingly, therefore it has recently emerged that one of the strategies exploited by parasitic helminths to dampen host immune responses and hence promote their survival is the induction of IL-10-producing Bregs: moreover, the protection afforded against allergic inflammatory disease by such worms has also been associated, at least in part, by their induction of Bregs(353,466-469). Thus, the current findings that exposure to ES-62 results in elevated levels of IL-10-producing B cells may provide a rationale for previous findings that the parasite product exerts its protective effects in CIA via suppression of Th1, Th17 and IL-17-producing gd T cell responses, as well as being consistent with an earlier observation that it promotes spontaneous IL-10 production by splenocytes from mice with CIA (319,335,336). Interestingly, although regulatory B cells have been proposed to mediate at least some of their protective effects in experimental arthritis via the generation of natural Tregs (AIA) and/or induced Tr1 (AIA and CIA) cells (245,345,419), there was no evidence that ES-62 induces any IL-10-producing regulatory T cells in DBA/1 mice with CIA. Although perhaps surprising, this failure to induce Tregs is supported by preliminary data from the C57BL/6 model of chronic CIA (470), in which in vivo exposure to ES-62 does not increase the levels of either $FoxP3^+$ or IL-10-producing $CD4^+$ T cells in the DLN of such mice (McGrath & Harnett, unpublished data) and is consistent with their lack of induction in previous studies investigating ES-62-mediated hyporesponsiveness to the model antigen Ovalbumin (OVA) in both the DO.11.10 transfer model (37) and the OVA-induced airway inflammation model (338) of asthma. Nevertheless, whilst ES-62 does not increase the levels of Tregs in mice with CIA, it is not possible to rule out at this stage that it may act to reverse/overcome the impaired (cell contact-mediated) suppressive ability of Tregs reported in RA (471).

Perhaps surprisingly, the increase in IL-10-producing B cells resulting from exposure to ES-62 did not reflect induction of a particular phenotype of B cells associated with regulatory function: although modest, these statistically significant increases in IL-10 producing B cells are consistent with the numbers seen in other studies in the absence of enrichment by anti-CD40 or LPS/IL-21 stimulation (244,405,472) and suggest that ES-62 may be acting in a homeostatic manner to reset the balance of effector and regulatory B cells back towards that observed in healthy DBA/1 mice. However, and consistent with this proposal, whilst the ES-62-mediated suppression of the level of GC B cells was associated with a 30-40% reduction of Tfh cells, preliminary data suggest that the residual Tfh cells produced higher levels of the cytokine, IL-21 that appears to be critical to for the generation of functional Bregs that combat autoimmunity (405). Thus, as regulatory function of these IL-10 producing B cells has not been formally demonstrated in this study, it is not possible at this stage to rule out that ES-62 is also reversing the defective regulatory function of one or more Breg phenotypes observed in CIA and RA patients (418,473). Nevertheless, it was shown previously that whilst ES-62 induces high levels of IgG1, but not IgG2a, antibodies in naive wild type BALB/c mice, in IL-$10^{-/-}$ mice the helminth product induces both IgG1 and IgG2a antibodies. This suggests that IL-10 plays an important role in the suppression of IgG2a antibodies directed against ES-62 (345). Interestingly, therefore, neither the ES-62-mediated suppression of DC-priming of OVA-specific Th1 responses(330) nor the suppression of TLR-mediated IL-12 responses of macrophages exposed to ES-62 either in vitro or in vivo (331) appears to be due to autocrine production of IL-10 by APC. By contrast, whilst exposure to ES-62 in vivo induces hypo-responsiveness of splenic B2 cells, peritoneal B cells from such mice produced enhanced spontaneous and BCR-stimulated IL-10 responses (324,326) and although these peritoneal cells will predominantly comprise B1 cells, there is increasing evidence that they likely also included some B2 cells (463).

Collectively, therefore, these data suggest that exposure to ES-62 leads to hypo-responsiveness of effector B2 cell responses and an increase in IL-10-producing B cells that in CIA, given the complex interplay between B cell-derived IL-10 and pathogenic IL-17Aresponses in the regulation of inflammation and autoantibody responses (103,244,247,345,403,405,419), results in the reduction of plasma cells

that may contribute to the suppression of pathogenic autoantibodies and inflammation associated with the ES-62-mediated protection against CIA. For example, the ability of B cell-derived IL-10 to impact on APC such as DC in vivo may contribute to the effects of ES-62 on the DC-dependent priming of Th1/Th17 and IL-17-producing gd T cells (330,336,338). Alternatively, such IL-10 may suppress effector B cell activation with consequent induction of T cell hypo-responsiveness, as the reduction of GC B cells and T_{FH} cells observed in the spleens of ES-62-treated mice is reminiscent of that reported for mast cell-derived IL-10-mediated suppression of T_{FH} cell function (474). This could be particularly important at the site of inflammation with the profound down-regulation of CD80 expression on follicular B cells impacting on the functionality of ectopic GCs given that expression of this B7 molecule by B cells has been shown to be important in the regulation of Tfh development, and consequent GC B cell survival and plasma cell production (475), the latter population being significantly reduced in the joints of ES-62-treated mice. Interestingly therefore, blocking of B7-CD28 interactions has been reported to be sufficient to prevent development of CIA (476). Finally, the strong down-regulation of TLR4 is similarly likely to suppress B cell activation and plasma cell generation at this site (477), and in this way, disrupt the destructive chronic inflammation resulting from cells in the joint expressing upregulated levels of TLRs, including TLR4, and responding to DAMPs, such as HSP22 and tenascin-C (478) found in the synovium of RA patients.

Table 6.1: Exposure to ES-62 in vivo suppresses infiltration of the joints by B2 cells and plasma cells. Data (number of cells x 10^6) are presented from 2 independent experiments where $CD19^+B220^{low/-}CD138^+$ and $CD19^-B220^-CD138^+$ plasma cell and $CD19^+CD21^{low/-}CD23^+AA6.1^-IgM^{low}IgD^{high}$ Fo1 B cell populations infiltrating the joints were analysed by flow cytometric analysis of joint cells harvested from 6-7 mice from each group.

Group	**$CD19^+B220^{low/-}CD138^+$**		**$CD19^-B220^-CD138^+$**		**Fo1**	
Exp	**1**	**2**	**1**	**2**	**1**	**2**
PBS	0.24	0.67	1.5	0.74	0.029	0.11
ES-62	0.035	0.32	0.18	0.36	0.009	0.033

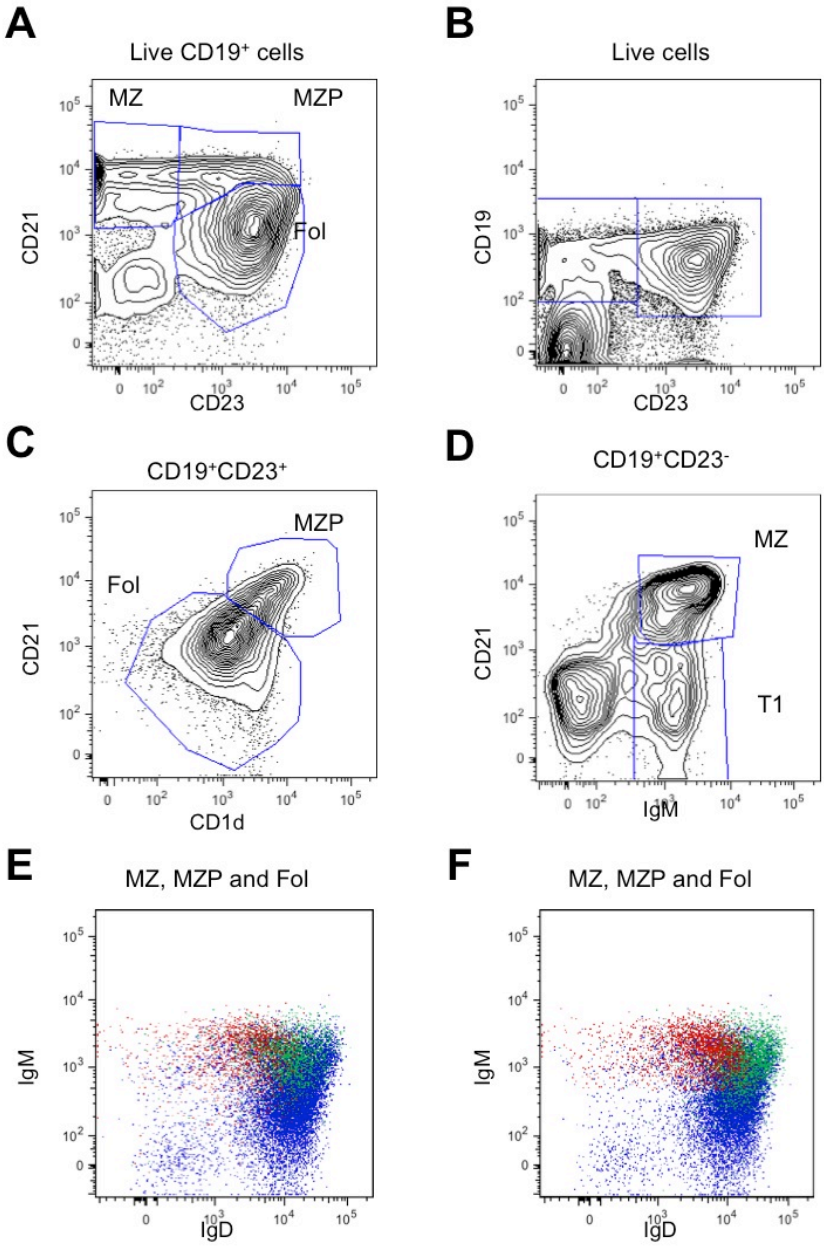

A
Live CD19⁺ cells
MZ
MZP
Fol
CD21
CD23
B
Live cells
CD19
CD23
C
CD19⁺CD23⁺
MZP
Fol
CD21
CD1d
D
CD19⁺CD23⁻
MZ
T1
CD21
IgM
E
MZ, MZP and Fol
IgM
IgD
F
MZ, MZP and Fol
IgM
IgD

Figure 6.1: Gating strategy for analysis of B cell subsets and phenotyping of populations. Cell populations were initially selected on the basis of "Lymphocyte" size and granularity and "doublets" and dead cells were excluded. The conventional method for the analysis of marginal zone precursor (MZP), marginal zone (MZ) and follicular B cells is to resolve $CD19^{+}$ cells by their CD21 and CD23 expression (A). To improve resolution of these subsets we initially gated on $CD19^{+}CD23^{-}$ and $CD19^{+}CD23^{+}$ cells (B) to resolve MZP ($CD21^{hi}CD1d^{hi}$) from Fo ($CD21^{low/-}CD1d^{int/-}$) B cells (C) and MZ ($CD21^{+}IgM^{+}$) and T1 ($CD21^{-}IgM^{+}$) cells (D), respectively, allowing more clearly defined populations of MZ ($IgM^{high}IgD^{low/-}$), MZP ($IgM^{high}IgD^{high}$) and Fo($IgM^{low/-}IgD^{high}$) B cells. This modified method improved the resolution of these populations and the IgM IgD expression profiles between the conventional and contemporary methods: panel E compares the populations from panel A and F compares the populations from panels C and D, Fo1 cells are represented by grey dots, MZP with black dots and MZ with black contour lines.

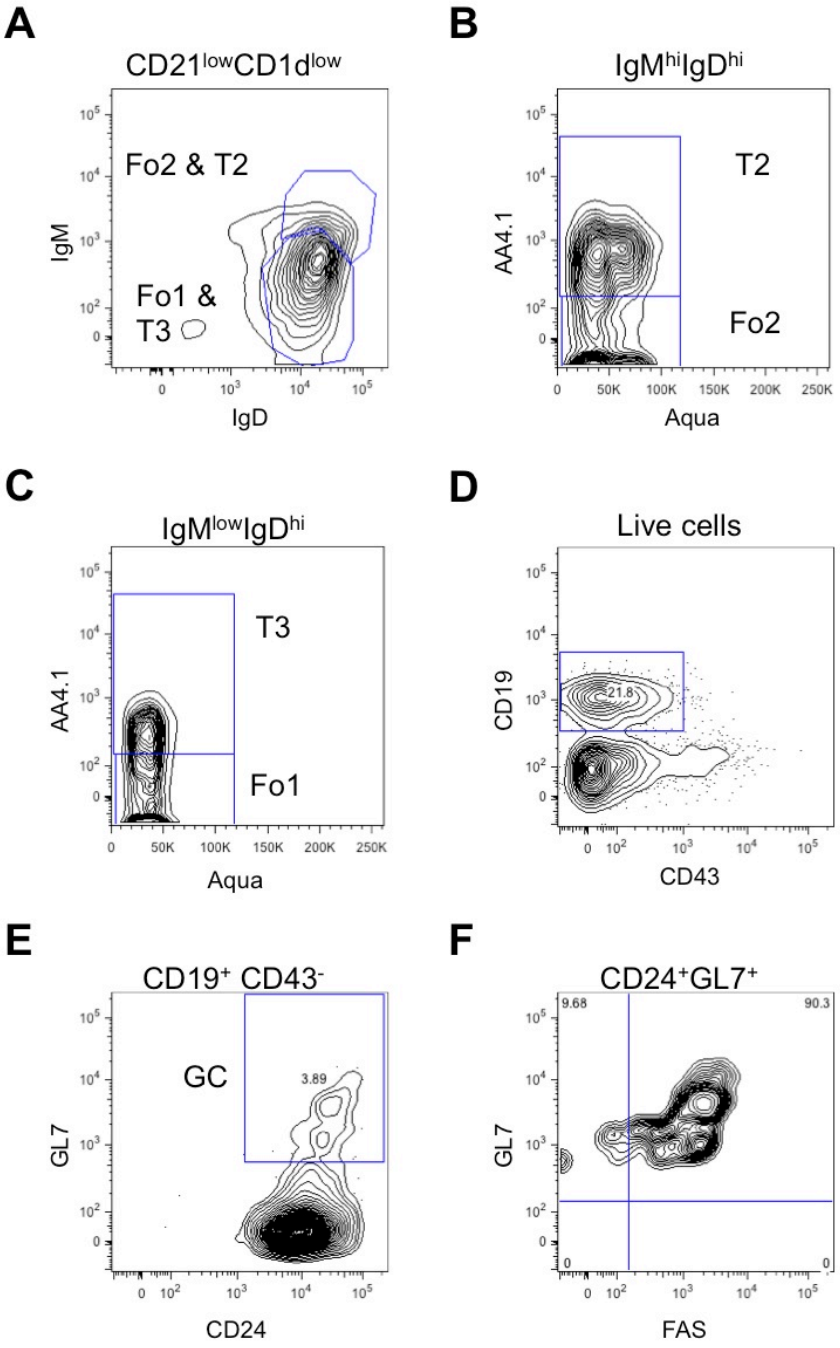

A
CD21lowCD1dlow
Fo2 & T2
Fo1 &
T3
IgM
IgD
B
IgMhiIgDhi
T2
Fo2
AA4.1
Aqua
C
IgMlowIgDhi
T3
Fo1
AA4.1
Aqua
D
Live cells
21.8
CD19
CD43
E
CD19+ CD43-
GC
3.89
GL7
CD24
F
CD24+GL7+
9.68
90.3
0
0
GL7
FAS

Figure 6.2: Gating strategy for analysis of B cell subsets and phenotyping of populations. The follicular (Fo) population identified (Fig6.1C; $CD21^{low/-}CD1d^{low}$) is a heterogenous population that contains the functionally distinct follicular type 1 (Fo 1: $IgD^{high}IgM^{low/-}AA6.1^{-}$) and follicular type 2 (Fo 2: $IgD^{high}IgM^{high}AA6.1^{-}$) as well as the transitional 2 (T2: $IgD^{high}IgM^{high}AA6.1^{+}$) and transitional 3 (T3: $IgD^{high}IgM^{low/-}AA6.1^{+}$) populations. These populations are first separated on the basis of their expression of IgM and IgD (A) and then, AA6.1 (B & C). For the identification of germinal centre (GC) B cells we first identify $CD19^{+}CD43^{-}$ cells (D) and then exclude contaminating non-B cells by gating on the GC cell specific marker GL7 along with the pan B cell marker CD24 (E) before confirming expression of FAS by essentially all (>90%) $CD19^{+}CD43^{-}CD24^{+}GL7^{+}$ GC B cells; we have therefore not included this redundant marker in our analysis (F).

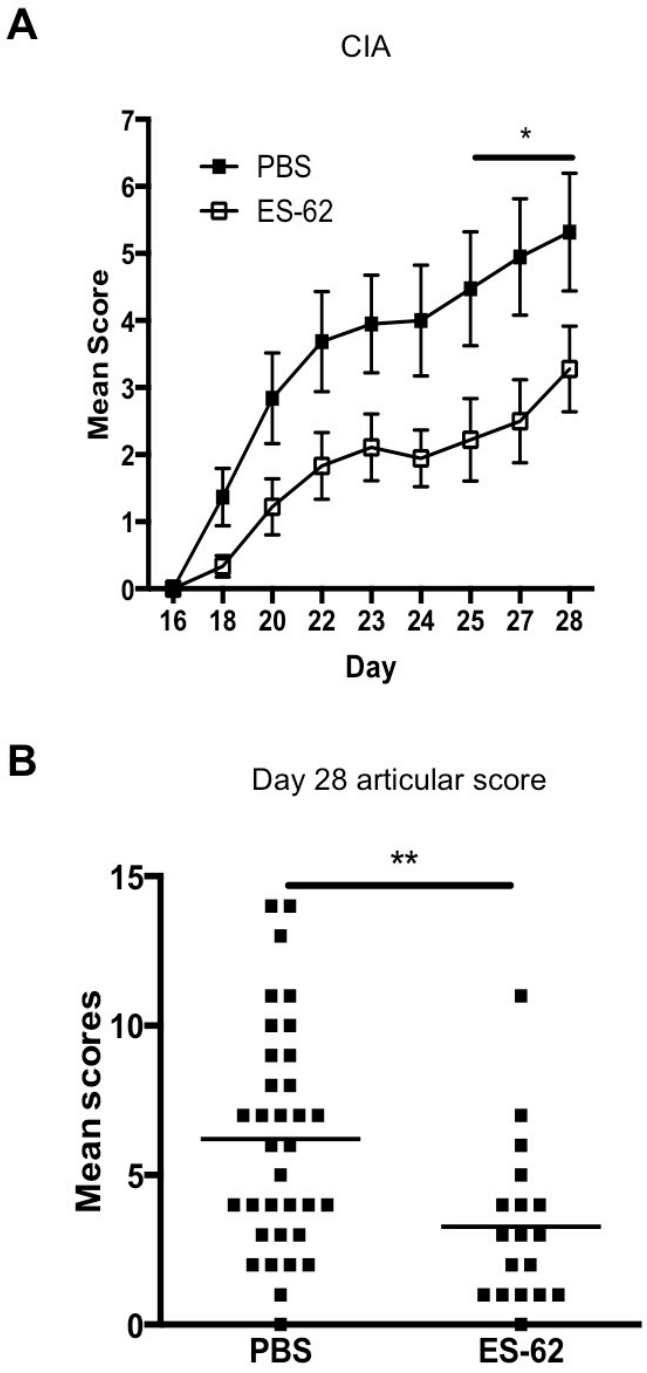

A
CIA
PBS
ES-62
*
Mean Score
Day
16 18 20 22 23 24 25 27 28
B
Day 28 articular score
**
Mean scores
PBS
ES-62

Figure 6.3: ES-62 prevents the development of disease in the CIA mouse model of RA. CIA was induced, as described in the materials and methods section, by Dr. Miguel A Pineda. Mean articular scores (± SEM) of CIA mice treated with PBS (n=34) or ES-62 (n=18) between day 16 and 28 (A) and at time of cull at day 28 (B). The student's t-test was used at each time point to show statistical significance where *=$p<0.05$ and **=$p<0.01$.

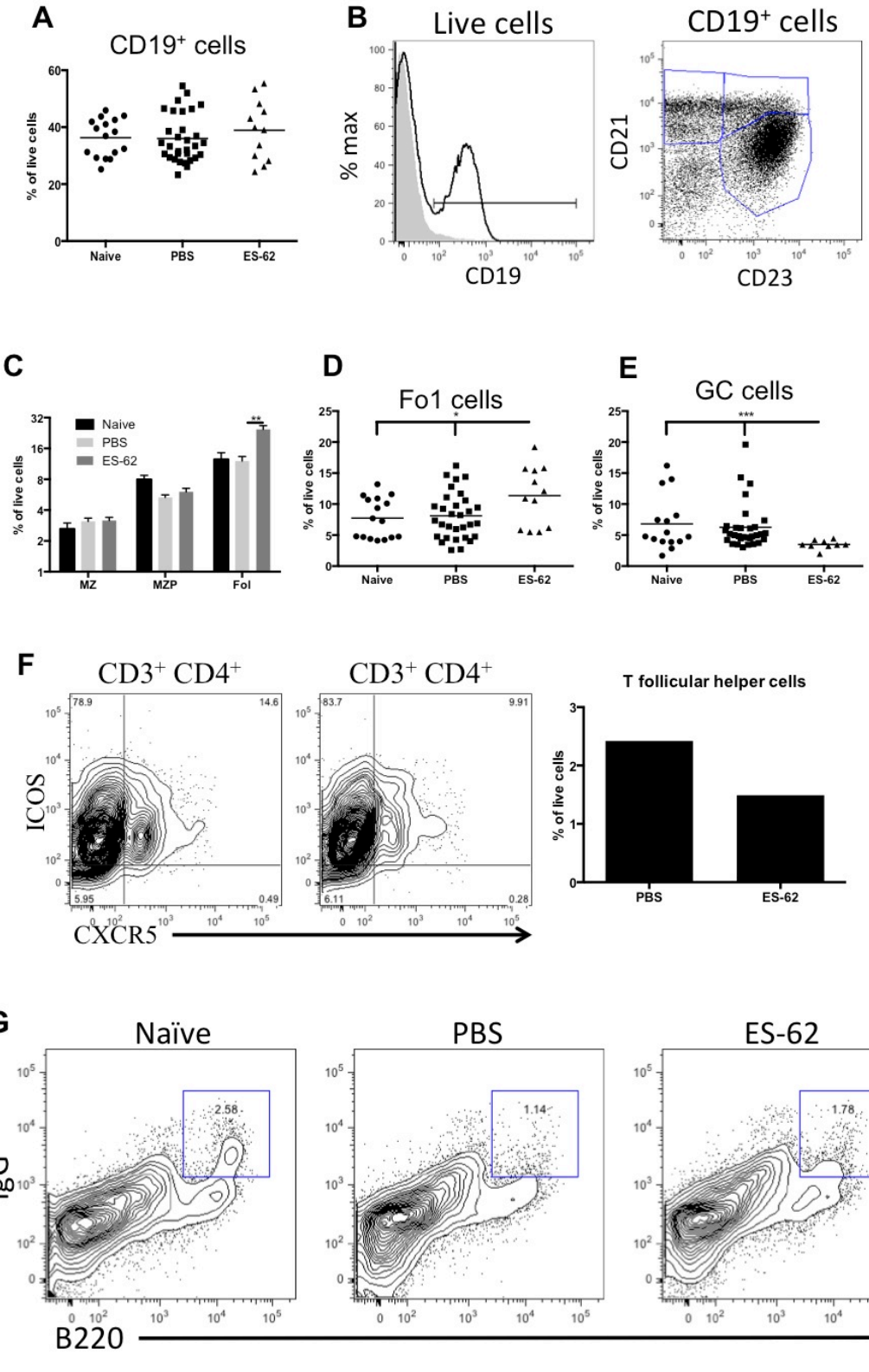

A
CD19+ cells
% of live cells
Naive
PBS
ES-62
B
Live cells
% max
CD19
CD19+ cells
CD21
CD23
C
Naive
PBS
ES-62
% of live cells
MZ
MZP
Fol
D
Fo1 cells
% of live cells
Naive
PBS
ES-62
E
GC cells
% of live cells
Naive
PBS
ES-62
F
CD3+ CD4+
CD3+ CD4+
ICOS
CXCR5
78.9
14.6
5.95
0.49
83.7
9.91
6.11
0.28
T follicular helper cells
% of live cells
PBS
ES-62
G
Naïve
PBS
ES-62
IgG
B220
2.58
1.14
1.78

Figure 6.4: ES-62 reduces the levels of Germinal Centre B cells in the spleens of mice with CIA The percentage of $CD19^+$ B cells (A); representative plots (B) and proportions (C; mean values ± SEM of individual mice where naive, n=16; PBS, n= 31; ES-62, n=12) of MZP, MZ and Fo B cells derived by the conventional method; follicular type 1 B cells (D: $CD19^+CD23^+CD21^{low-/}CD1d^{int}IgM^{low}IgD^{hi}AA6.1^-$) and germinal centre (E: $CD19^+CD43^-CD24^+GL7^+$), as derived by the gating strategy in presented in Fig. 1, in spleens from mice undergoing CIA are shown. The proportion of $CD3^+CD4^+ICOS^+CXCR5^+$ T follicular helper cells were determined by FACS with representative plots shown alongside a graph of the absolute numbers (F) and the proportion of memory B cells in the spleens of naïve mice and CIA mice treated with PBS or ES-62 (G).

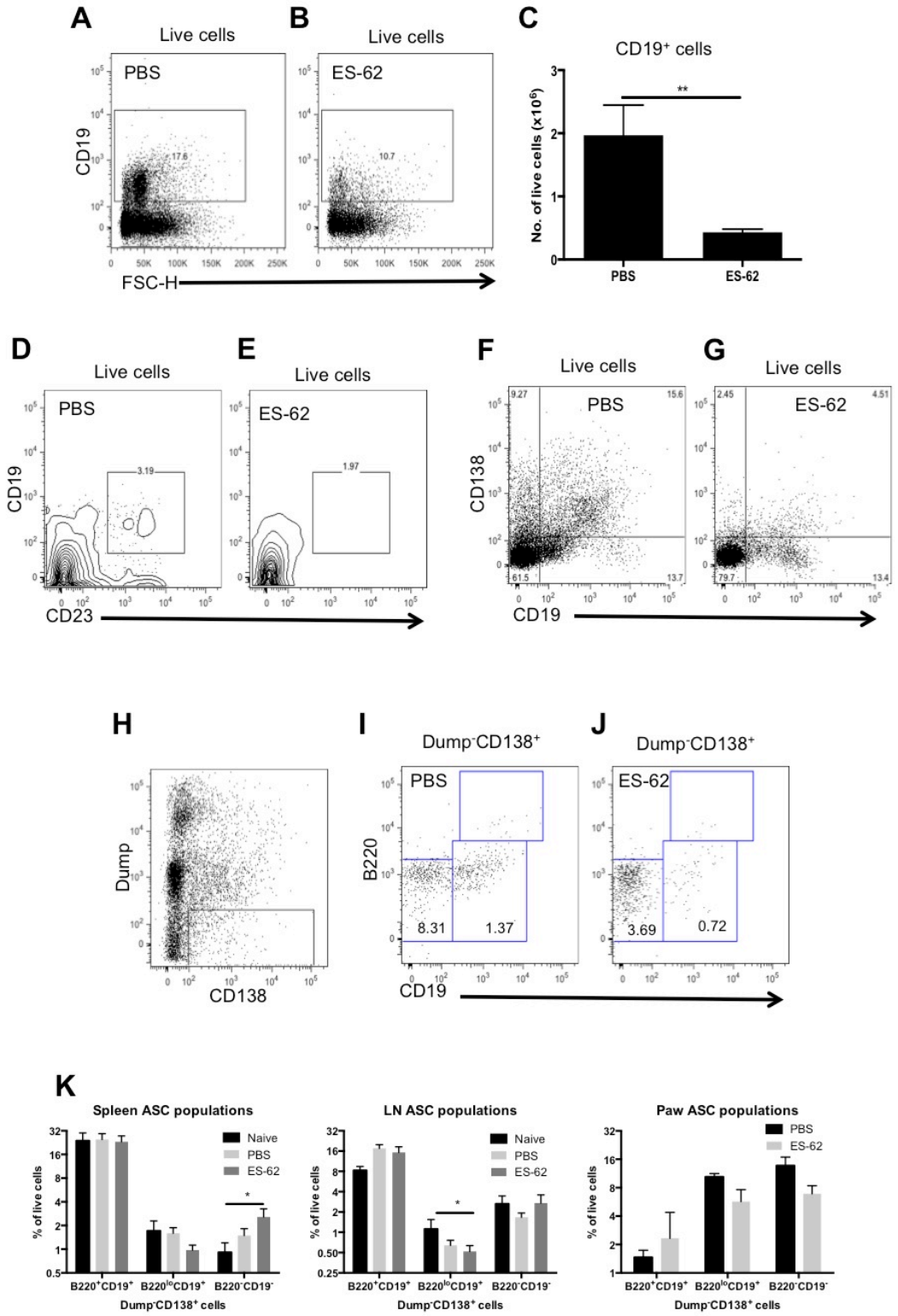

A
Live cells
PBS
CD19
17.6
FSC-H
B
Live cells
ES-62
10.7
C
CD19⁺ cells
No. of live cells (x10⁶)
**
PBS
ES-62
D
Live cells
PBS
CD19
3.19
CD23
E
Live cells
ES-62
1.97
F
Live cells
PBS
CD138
9.27
15.6
61.5
13.7
CD19
G
Live cells
ES-62
2.45
4.51
79.7
13.4
H
Dump
CD138
I
Dump⁻CD138⁺
PBS
B220
8.31
1.37
CD19
J
Dump⁻CD138⁺
ES-62
3.69
0.72
K
Spleen ASC populations
Naive
PBS
ES-62
% of live cells
B220⁺CD19⁺
B220ˡᵒCD19⁺
B220⁻CD19⁻
Dump⁻CD138⁺ cells
LN ASC populations
Naive
PBS
ES-62
% of live cells
B220⁺CD19⁺
B220ˡᵒCD19⁺
B220⁻CD19⁻
Dump⁻CD138⁺ cells
Paw ASC populations
PBS
ES-62
% of live cells
B220⁺CD19⁺
B220ˡᵒCD19⁺
B220⁻CD19⁻
Dump⁻CD138⁺ cells

Figure 6.5: ES-62 modulates the recruitment of B cells to the joints of mice with CIA. Cells extracted from the joints of mice with CIA were analysed for the proportion (A & B) and number (C) of infiltrating $CD19^+$ B cells (A-C; data in C are presented as the means ± SEM of 4 biological replicates pooled from two independent experiments) and consequently for the relative proportions of $CD19^+CD23^+$ (D & E); plasma cells on the basis of CD19 CD138 expression (F & G); exclusion of myeloid and T cell-expressing CD138 cells by use of Dump channel ($CD4^+CD8^+GR1^+F4/80^+CD11b^+$ $CD11c^+$; H); $Dump^-CD19^-B220^-Cd138^+$ plasma cells and $Dump^-CD19^+B220^{low/-}CD138^+$ plasma cells (I & J; values give refer to the % of live cells). The proportion of $Dump^-CD138^+B220^{\pm}CD19^{\pm}$ cells in the spleen, LN and paws are presented (K). The student's t-test was used to show statistical significance where *=$p<0.05$ and **=$p<0.01$.

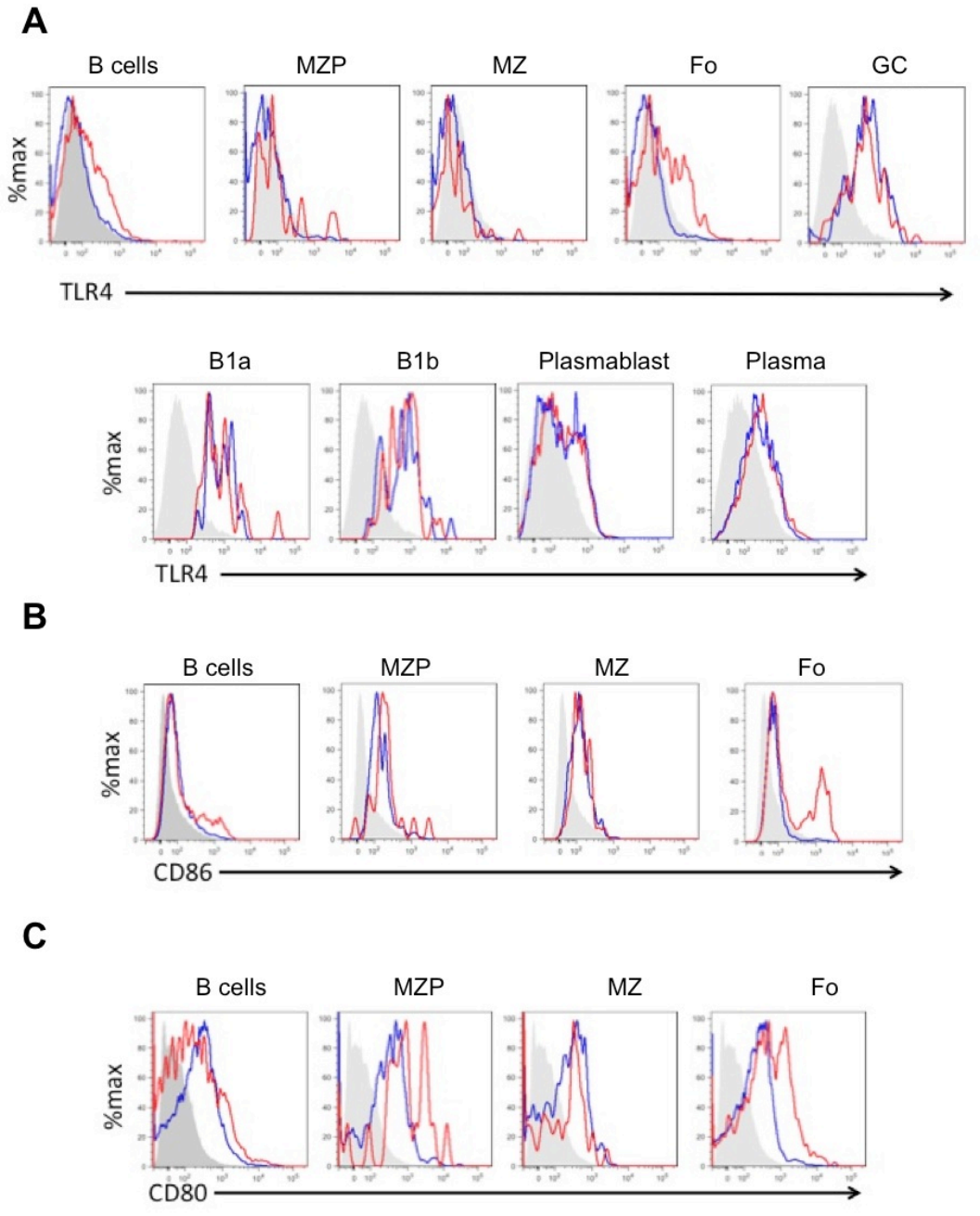

A
B cells
MZP
MZ
Fo
GC
%max
TLR4
B1a
B1b
Plasmablast
Plasma
%max
TLR4
B
B cells
MZP
MZ
Fo
%max
CD86
C
B cells
MZP
MZ
Fo
%max
CD80

Figure 6.6: ES-62 modulates expression of TLR4 and costimulatory molecules on follicular B cells. TLR4 (A), CD86 (B) and CD80 (C) expression by B cells in the joints of PBS and ES-62 treated mice are presented as expression levels relative to isotype control (grey area), for CIA mice treated with ES-62 (blue line) or PBS (red line). Cells from at least 5 mice/group were pooled.

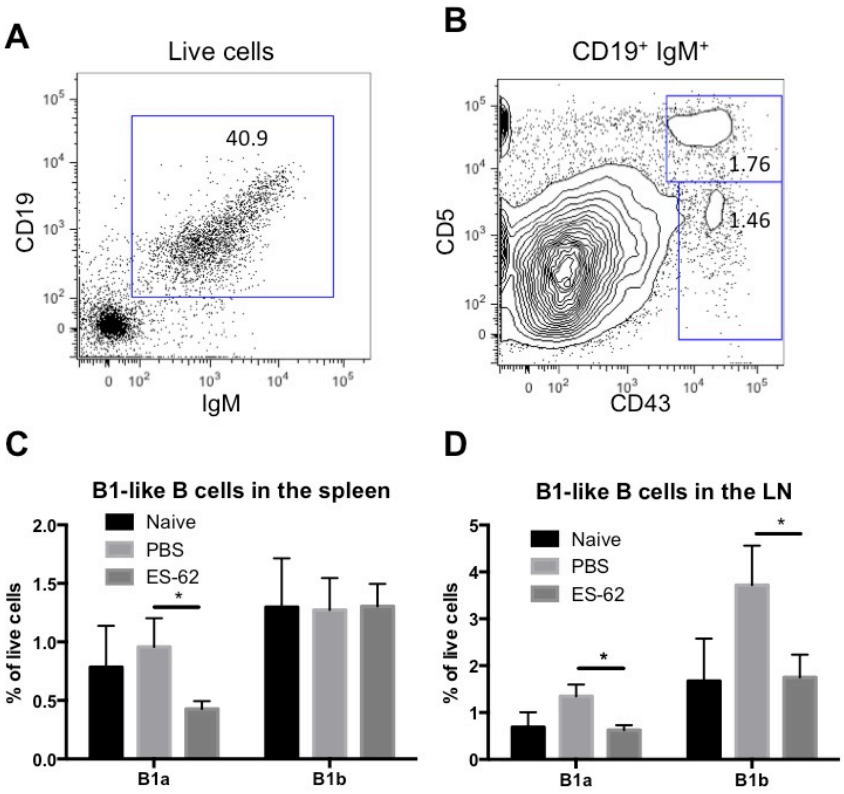

A
Live cells
40.9
CD19
IgM
B
CD19⁺ IgM⁺
1.76
1.46
CD5
CD43
C
B1-like B cells in the spleen
Naive
PBS
ES-62
% of live cells
B1a
B1b
D
B1-like B cells in the LN
Naive
PBS
ES-62
% of live cells
B1a
B1b

Figure 6.7: ES-62 modulates the levels of B1-like cells in the spleen of mice with CIA.

There is no unambiguous phenotype for B1 cells in the spleen but they have been described as $CD19^{high}CD23^{-}CD43^{+}IgM^{high}IgD^{low/-}CD5^{\pm}$ cells where $CD5^{+}$ B1a and $CD5^{-}$ B1b comprise ~2% and 1% of cells in the spleen respectively 6. However, following gating on $CD19^{+}IgM^{+}$(A), analysis of $CD43^{+}CD5^{\pm}$ cells has been widely used to describe B1 cells [25, 26] whilst $CD19^{+}CD5^{+}$ gating has been used to describe B1a cells [28]. Moreover, whilst CD43 can be upregulated on B2 cells, this is usually expressed at a lower level than on B1 cells [27] and we have thus chosen to gate only on $CD43^{high}$ cells (B) in order to exclude any potential $CD43^{+}$ B2 cells. We have therefore phenotyped $CD19^{high}CD43^{+}IgM^{high}IgD^{-}$ B cells as $CD5^{+}$ B1a-like cells and $CD5^{-}$ B1b-like cells and the data show their relative proportions in the spleen (C; naïve, n=10; PBS, n=13 and ES-62, n=11) and DLN (D; naïve, n=4; PBS, n=8 and ES-62, n=8) of the indicated groups of mice.

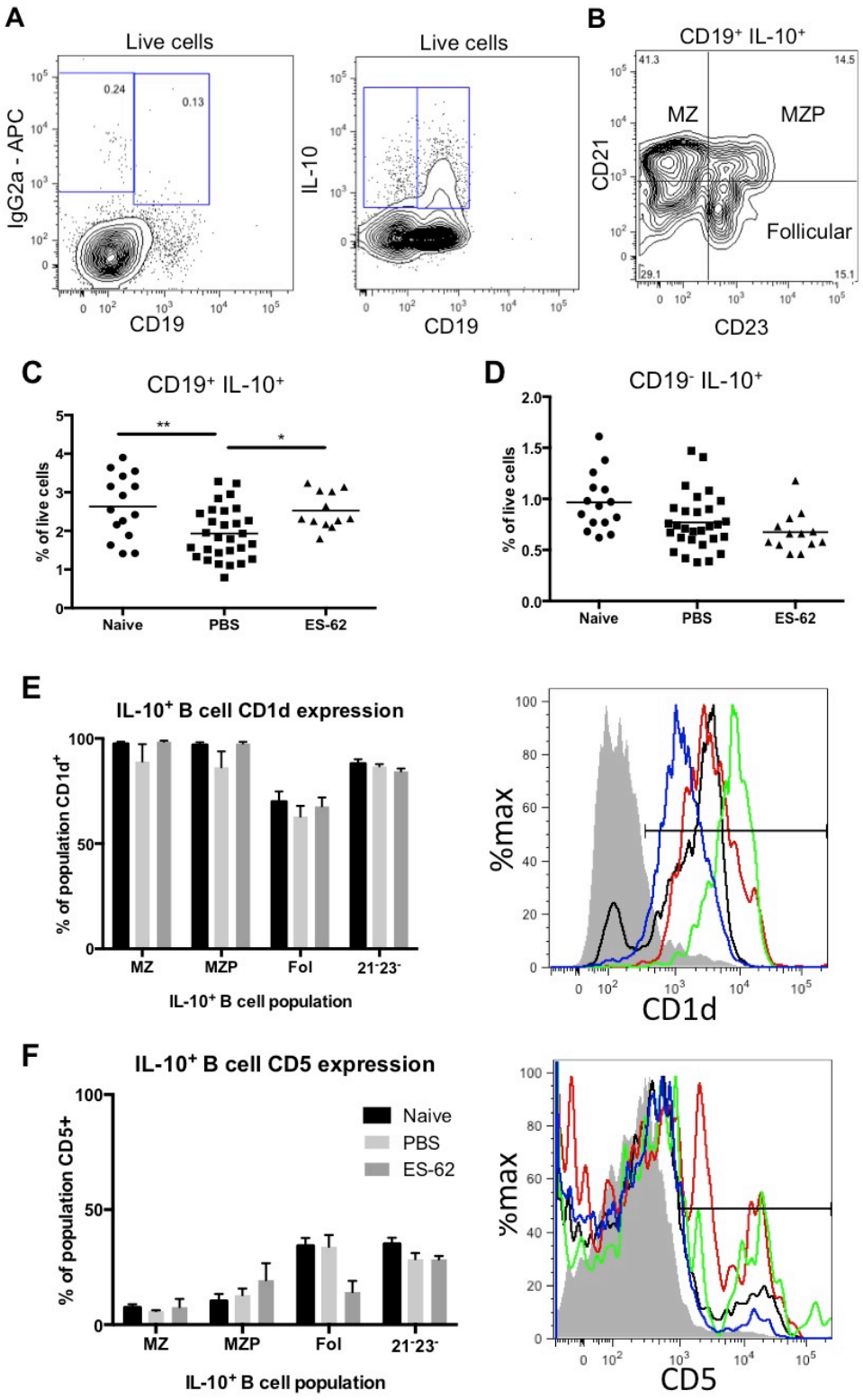

A
Live cells
IgG2a - APC
CD19
0.24
0.13
Live cells
IL-10
CD19
B
CD19+ IL-10+
CD21
CD23
MZ
MZP
Follicular
41.3
14.5
29.1
15.1
C
CD19+ IL-10+
% of live cells
Naive
PBS
ES-62
**
*
D
CD19- IL-10+
% of live cells
Naive
PBS
ES-62
E
IL-10+ B cell CD1d expression
% of population CD1d+
MZ
MZP
Fol
21-23-
IL-10+ B cell population
%max
CD1d
F
IL-10+ B cell CD5 expression
Naive
PBS
ES-62
% of population CD5+
MZ
MZP
Fol
21-23-
IL-10+ B cell population
%max
CD5

Figure 6.8: ES-62 induces IL-10-producing B cells in the spleen of mice with CIA. IL-10-producing $CD19^+$ B cell (A & C) and IL-10-producing $CD19^-$ (A & D) subsets in the spleen were analysed with the proportions of these cells, in spleens of individual naive mice and PBS- and ES-62-treated mice with CIA respectively. Representative plots of the phenotypes of $CD19^+IL\text{-}10^+$ B cells based on their expression of CD21 & CD23 (B) and the level of CD1d (E) and CD5 (F) expression on $IL\text{-}10^+$ MZ (blue line), MZP (red line), Fo (green line) and $CD21^-CD23^-$ (black line) B cells are shown for spleens of mice with CIA. The student's t-test was used to show statistical significance where *=$p<0.05$ and **=$p<0.01$.

7. General discussion

7.1. Developing drugs from parasitic immunomodulators

Filarial nematodes are parasitic helminths that can cause debilitating conditions such as elephantiasis and river blindness (302-304). The low prevalence of autoimmunity in developing countries that are endemic for such parasitic worms may reflect the ability of the parasites to modulate the immune system, a characteristic that has likely evolved to promote survival of the parasite whilst limiting host pathology (1,2,4,5). This relationship has generated interest in the anti-inflammatory products of helminths and consequently, their therapeutic potential. Indeed, understanding the mechanisms by which these helminth-derived molecules modulate the immune response may identify novel pathways for future manipulation (311). Thus, parasite-derived immunomodulatory molecules may provide a platform for the development of novel immune modulating drugs, which would potentially treat disease in a safe and balanced manner, reducing the debilitating side effects associated with conventional therapies.

It is now widely established that infection with parasitic worms can suppress disease in mouse models of autoimmune and allergic inflammatory disease (298,307,308,353). Consistent with this, helminth-infected patients suffering from multiple sclerosis present with less severe disease compared to uninfected multiple sclerosis patients (416). Furthermore, there is a striking negative correlation between the rates of rheumatoid arthritis and incidence of helminth infection in parts of the world where both diseases are common (479). The successful modulation of disease in mouse models has culminated in clinical trials evaluating the benefit of live *T. suis* helminths in the treatment of irritable bowel disease (298,480). These clinical trials have shown promise, however, the potential dangers of prescribing live pathogens to patients and the perception of "eating worms" may prevent the widespread uptake of these treatments. A safer, more controlled and palatable alternative to the therapeutic use of whole worms would therefore be to identify the specific components of the worms that are responsible for the modulation of the immune system (311).

ES-62 is the major excretory-secretory product secreted by the filarial nematode *A. viteae* (481), a PC-containing glycoprotein that exhibits therapeutic potential in

mouse models of RA (collagen-induced arthritis) (335) and asthma (ovalbumin-induced airway hypersensitivity) (228). In both diseases, the suppression of disease was due to modulation of pathogenic IL-17A responses. Consistent with this targeting of IL-17A responses (336,338), in this thesis, ES-62 has been shown to have therapeutic potential in a mouse mode of SLE, which is known to have a mixed Th1/Th2/Th17 phenotype.

7.2. ES-62 suppresses the development of proteinuria in the MRL/*lpr* mouse

SLE is a chronic autoimmune disease with a clear unmet clinical need that affects 5 million people worldwide. SLE is usually treated with immunosuppressive drugs; however, such therapies are not always effective and long-term usage can lead to serious side effects such as infection and cancer (1,2). The exact etiology of SLE is unknown however the disease is largely thought to be due to a defect in the clearance of apoptotic cell debris, resulting in prolonged activation of the innate immune system and activation of auto-reactive lymphocytes. Consistent with this, SLE is characterized by autoantibody responses to double stranded DNA as well as other nuclear and cytoplasmic antigens. The resulting deposition of autoantibody-immune complexes induce localized inflammation in tissues with dense capillary networks, most commonly the skin, joints and kidneys. Consequently, lupus nephritis is one of the major causes of morbidity and mortality in SLE patients (10).

To study the therapeutic potential of ES-62 in SLE, we utilized the MRL/*lpr* mouse, a naturally occurring mutant of the parental MRL/MP mouse, the genome of which contains several mutations in SLE disease susceptibility loci that result in the development of lupus-like disease within 12-18 months that exhibits characteristics of human disease including inflammation of the kidney, joints, skin and cardiovascular system. The *lpr* mutation results in accelerated disease, due to a loss of FAS signaling, with the mice developing severe disease within 5 months, with lupus nephritis the major cause of death in this animal model of SLE (14). The concentration of protein in the urine (proteinuria) directly correlates with glomerulopathy and leukocyte infiltration into the kidney; the twice-weekly prophylactic administration of ES-62 significantly reduces development of

proteinuria in the MRL/*lpr* mouse. Remarkably, however, despite such a drastic improvement in renal function, the level of glomeruloproliferation, glomerular basement membrane thickening, cellular infiltration, complement and immune complex deposition appeared relatively unaffected by the parasite product.

Thus, as ES-62 did not substantially protect against these pathological signs of renal inflammation, it was hypothesized that the parasite product may act to modulate the functional responses of effector cells in the kidney to these pro-inflammatory stimuli. Indeed, the expression of the C3aR, C5aR, FcγRI and FcγRIII by the renal cells and renal fibroblasts was reduced by both the *in vitro* and *in vivo* administration of ES-62. ES-62 has previously been shown to target TLR signaling via the adaptor protein MyD88 (320,322), which has been strongly implicated in the renal pathology of SLE (428,482). It was therefore of interest to also observe a reduction in the level of MyD88 in the renal cells and renal fibroblasts from ES-62 treated mice. Such a reduction would likely perturb the responsiveness of kidneys to TLR ligands including DAMPs such as dsDNA and HGMB1 (26,462,482,483).
The biological function of the kidney is to filter the blood and thus, tight barrier formation of glomerular basement membrane is paramount to ensure that macromolecules and cells are not lost into the urine; the level of proteinuria is therefore inextricably linked to the integrity of the glomerular basement membrane (443). Modulation of MyD88 signaling in the kidneys of ES-62 treated mice may therefore explain how renal function is maintained in the presence of such strong pro-inflammatory pathology, as whilst MyD88-ARNO-ARF6 signaling increases vascular permeability MyD88-MAL signaling promotes the maintenance of epithelial tight junctions (3,5,6,9). Thus, exposure to ES-62 may promote MyD88 signaling via MAL over that coupled to ARNO-ARF6, to maintain the viability of the renal barrier function (Fig. 7.1); indeed, ES-62 has been observed to suppress PLD signaling in T cells and mast cells, which has been linked to the ARNO-ARF6 pathway, whereas it has no effect on the expression of MAL (Ball, Eason, Tay, Harnett & Harnett unpublished data). Furthermore, ES-62 suppression of C5aR expression by renal fibroblasts may also contribute to the maintenance of renal barrier function as antagonizing this receptor has been reported to promote the viability of the blood-brain barrier in the MRL/*lpr* mouse (455) (Fig. 7.1).

7.3. ES-62 modulates cell infiltration of the kidneys of MRL/*lpr* mice

Consistent with them expressing lower levels of proinflammatory receptors and signal transducing adaptor molecules, renal fibroblasts from ES-62 treated mice produced less MCP-1 in responses to TLR stimulation. MCP-1 is involved in the recruitment of effector leukocytes to sites of inflammation and is directly linked to lupus disease activity and renal involvement (14). The reduction in MCP-1 expression was perhaps surprising given that histological analysis of the kidneys from ES-62 treated mice failed to identify any substantial reduction in cellular infiltration, however, analysis of the phenotype of the cells infiltrating the kidneys of ES-62 treated mice, indicated that the parasite product modulated the composition of the leukocyte population in the kidney. Indeed, the levels of pro-inflammatory effector cells such as $CD11b^{hi}GR1^{hi}$ neutrophils, $CD11b^{hi}GR1^{int/low}$ monocytes, $CD4^{+}$ T helper cells, $CD8^{+}$ and $CD8^{low}$ cytotoxic T cells, $CD3^{+}B220^{+}CD4/8^{-}$ DN T cells, $\gamma\delta$ T cells, ILC and plasmablasts were significantly reduced in the kidneys of ES-62 treated mice. Interestingly, a common feature with the reduction of these cell populations was an apparent retention or increase of these populations at distal sites such as the spleen, LN or blood. This may suggest that ES-62 modulates leukocyte migration and although preliminary data presented throughout this thesis supports this theory, further investigation is required to confirm this theory and characterize the mechanisms involved.

7.4. ES-62 modulates the IL-17A/IL-22 axis in the MRL/*lpr* mouse

ES-62 suppression of disease in mouse models of RA and asthma is via modulation of the IL-17A:IL-22 axis (336,338); as both of these cytokines have been implicated in the pathogenesis of SLE, the effect of ES-62 on these parameters was assessed in the MRL/*lpr* mouse. Prior to the onset of disease, ES-62 suppressed the level of IL-17A and IL-22 producing lymphocytes, suggesting that these two cytokines were involved in promoting initiation of pathogenesis. ES-62 also suppressed the levels of IL-22 producing lymphocytes in the LN and kidney during established disease, the latter of which correlated with a reduction in the concentration of IL-22 in the renal interstitial fluid. Interestingly, therefore the number of IL-22 producing cells in the pLN the MRL/*lpr* mice

positively correlated with proteinuria, whilst IL-22 producing cells were undetectable in the pLN of MRL/MP mice. Thus it appeared that IL-22 was associated with both initiation of pathogenesis and development of chronic kidney pathology in the MRL/*lpr* mouse. Indeed, neutralization of IL-22 from 12-weeks of age completely ablated the development of proteinuria in the MRL/*lpr* mouse whereas the administration of rIL-22 significantly accelerated and exacerbated disease, supporting this proposal and suggesting that IL-22 was sufficient and essential for disease pathogenesis in the MRL/*lpr* mouse. Thus, suppression of IL-22 production provides a mechanism that contributes to the protection against proteinuria afforded by ES-62.

In direct contrast to its effect on IL-22-producing cells, ES-62 significantly increased the proportion of IL-17A$^+$ cells in mice during established disease. Although few IL-17A producing cells were detected in the kidney, the concentration of IL-17A was also significantly increased in the renal interstitial fluid of ES-62 treated mice, suggesting that the stimulation with PMA and ionomycin, typically used to identify cytokine-producing T cells by flow cytometry, was inducing death of cells, such as neutrophils, that may have been a predominant source of this cytokine (41,98) at this site. Nevertheless, there was an inverse correlation between the number of IL-17A producing cells in the pLN and the level of proteinuria; moreover, high levels of IL-17A producing cells were also found in the pLN of MRL/MP mice at 20 weeks, a time point at which these mice do not exhibit pathology, suggesting, perhaps controversially, that IL-17A does not play a solely pathogenic role in the MRL/*lpr* mouse. This possibility was confirmed, as whilst the neutralization of IL-17A production prior to the onset of disease, slowed the onset and reduced the severity of proteinuria, it did not block it completely, indicating that such early production of IL-17A was not sufficient for pathogenesis. Moreover, the administration of recombinant IL-17A during the established phase of disease partially mimicked the protective effects of ES-62 in suppressing development of proteinuria. Although these data suggest that IL-17A can indeed exert protective effects in the MRL/*lpr* mouse, together with recent findings that neutralization of IL-23 in MRL/*lpr* mice suppresses disease (368,369), collectively the data presented here suggest it is possible that IL-17A plays dual roles in the MRL/*lpr* mouse promoting pathogenesis prior to the onset of disease and acting to resolve

inflammation during established disease, such a precedent having been set for IL-17A in asthma (118,338).

The finding that IL-17A can protect against proteinuria in the MRL/*lpr* mouse runs contradictory to the current literature, which suggests an essential role of the cytokine in pathogenesis (103). However, closer scrutiny of previous reports examining IL-17A in SLE patients may reconcile this contradiction. Thus, IL-17A is undoubtedly elevated in SLE patients compared to healthy controls (103,373,374) however, few publications have reported a direct correlation between IL-17A and SLEDAI (114,375-378). On the other hand, considerably more groups have found that IL-17A is not associated with SLEDAI and may in fact be associated with protection in SLE patients (379-383,385,386). For example, an informative study by Szeto *et al.* measured the levels of IL-17A mRNA in the urine of SLE patients over time, and discovered that in the weeks preceding a disease flare, IL-17A mRNA levels in the urine declined significantly (388,389). Supporting evidence for a protective role for IL-17A is provided by its suppression by IFNα, a cytokine strongly implicated in the pathogenesis of SLE (38,484). Moreover, whilst in SLE models using mouse strains that are not genetically susceptible to develop the disease spontaneously, such as the FcγRIIb$^{-/-}$, Ro52$^{-/-}$ or the B6$^{lpr/lpr}$ models, there is strong evidence that IL-17A promotes pathogenesis (15,16,112), such a role for IL-17A has not been definitively demonstrated in lupus-prone strains, such as the NZB/NZW F1 or the SNF1 mouse. In the MRL/*lpr* mouse, which is genetically predisposed to develop lupus-like disease, IL-17A can demonstrably be protective during established disease. Thus, although others have demonstrated IL-17A producing cells in the MRL/*lpr* model, these may reflect the potential protective effects of the cytokine during established disease (112,369) as reported in other models of autoimmune and allergic inflammatory disease (118,485) and perhaps most pertinently, during renal host versus graft disease (98) and models of glomerular nephritis. Altogether, these data suggest that IL-17A may be important in the induction of SLE, perhaps by promoting germinal center-dependent generation of autoantibody-producing plasma or memory B cells (403): however, during established disease IL-17A may act to suppress generation of pathogenic anti-dsDNA producing plasmablasts by promoting GC formation and production of less pathogenic auto-antibodies by long-lived plasma cells. Furthermore, IL-22R

has recently been identified as a marker for GC stromal cells, tempting speculation that IL-17A and IL-22 may be involved in counter-regulation of the GC reaction and by resetting the balance of these cytokines, ES-62 may modulate GC function. Indeed, preliminary data presented in Chapter 4 suggested that IL-22 plays a role in the generation of extra-follicular plasmablasts, as the neutralization of IL-22 reduced the levels of these cells in the kidneys (Fig. 7.2).

7.5. ES-62 modulates the effector/regulatory B cell balance in the MRL/*lpr* mouse

As the producers of the pathogenic autoantibodies, B cells have long been heavily implicated in the pathogenesis of SLE; however, B cells contribute towards the pathogenesis in the MRL/*lpr* mice via both antibody dependent and independent mechanisms (162,232). Consistent with this, the onset of disease in the MRL/*lpr* mouse correlates with the expansion of MZ, Fo1 and GC effector B cell populations in the spleen. As ES-62 is known to modulate TLR, NFκB and BCR signaling; it was predicted that the parasite product might modulate the population dynamics of peripheral B cells *in vivo* (305,324,325), as all three of these signaling pathways can determine the fate of B cells (174). Although ES-62 did not seem to favor the development of a particular B cell subset, for example marginal zone over follicular, the parasite product did appear to specifically expand the MZP and Fo B cell populations. This suggested that ES-62 could block the development of these mature effector cells: however, this proved at least not entirely to be case as although the proportion of $Dump^{-}CD138^{+}B220^{low/-}CD19^{+}$ plasmablasts, was significantly reduced in the kidney, the levels of MZ, GC and plasma B cells were unaltered in the ES-62 treated mice.

Plasmablasts, rather than long-lived plasma cells, have been associated with pathogenesis in SLE as they have been predicted to be the main source of the pathogenic anti-dsDNA responses associated with disease flares (159,194-196). In this study, such anti-nuclear autoantibodies (ANA) were detected in the serum of MRL/*lpr* mice, but not MRL/MP mice, from 12-weeks of age, and treatment with ES-62 significantly suppressed the development of ANA responses, both prior to and following onset of disease. The suppression of ANA in ES-62 treated mice

was associated with a reduction of the levels of plasmablasts in the blood and kidneys and this likely contributes to the protective effect of ES-62 in MRL/*lpr* mouse. Interestingly, the serum from ES-62 treated mice stained the cell membranes of the Hep-2 cells, rather than the cyptoplasmic and nuclear antigens characteristic of lupus, and this may reflect a switch to anti-PC specificities that elicited by ES-62. As anti-PC antibodies are thought to be protective in SLE, by acting to improve clearance of apoptotic cells, this may provide another mechanism by which ES-62 protects against disease (412,486). By contrast, ES-62 did not alter the levels of plasma cells in the blood, kidney or rLN in the MRL/*lpr* mice, and this possibly explains why no alteration in serum IgG1, IgG2a and IgM antibody levels were observed; however, a reduction in pathogenic specificities of these plasma cells may be masked by the significantly elevated levels of anti-PC produced.

The reduction in effector B cell responses was associated with ES-62-mediated induction of a hypo-responsive phenotype of B cells, as indicated by reduced surface expression of activation or co-stimulatory molecules such as CD40, CD80 and CD86 and the pro-inflammatory receptor, TLR4. Interestingly the up-regulated expression of the negative feedback inhibition receptor, FcγRIIb as well as the receptor for the B cell survival factor, BAFF suggested that ES-62 promoted the survival of such hypo-responsive cells. The increase of the inhibitory antibody receptor, FcγRIIb, on B cells is of particular interest, as FcγRIIb$^{-/-}$ mice develop a lupus-like disease (15,420), and hence the up-regulation of its expression likely contributes to the reduction in ANA responses.

The success of targeting B cells in mouse models of disease has been testament to the role of these cells in pathology; however, the translation of these strategies into the clinic has only been moderately successful. Rituximab, an anti-CD20 monoclonal antibody that depletes B cells, showed promise in mouse models of SLE but failed to meet its primary end points in two independent phase III clinical trials (275,281,293). Belimumab, a monoclonal antibody that binds to and reduces the bioavailability of BAFF, the B cell activating factor, was more successful and became the first drug in 50 years to receive FDA approval for the treatment of SLE (487). The failure of rituximab, an off-label therapy favored by many

rheumatologists to treat SLE patients that do not respond to conventional therapies, can easily be put down to poor clinical trial design due to inadequate patient characterization, an issue with SLE patients that has previously likely obstructed beneficial drugs receiving FDA approval for this disease. However, the ineffectiveness of Belimumab suggests that alternative B cell therapies are required for the treatment of SLE and dictates that alternative approaches should be considered (38).

One possible reason for the low success rates of B cell depleting therapies may be that they generally do not discriminate between effector and regulatory B cells (281). Regulatory B cells (Bregs) can suppress the development of pathogenic T and B cell responses in autoimmune disease via IL-10- dependent mechanisms, with such Bregs being reported to suppress disease in mouse models of MS (EAE) (488), RA (CIA) (245) and SLE (MRL/*lpr*) (244). Similarly, populations of IL-10 producing B cells have also been identified in humans and indeed, the ability of these cells to suppress T cell responses has been reported to be reduced in SLE patients (247).

Interestingly, therefore, several studies have implicated a role for Bregs in the helminth-mediated suppression of autoimmune disease in both mouse and man (353,416). Perhaps reflecting this, it has previously reported that ES-62 induces IL-10 production by peritoneal B1 B cells (326). The effect of ES-62 on regulatory B cells in the MRL/*lpr* mouse were therefore evaluated and it was found that ES-62 increased the proportion of IL-10-producing B cells in the spleen, pLN, rLN and most importantly, the kidney. The major phenotype of these Bregs was that of the MZP B cells (244,245), a population that declined with kinetics mirroring the development of proteinuria in the MRL/*lpr* mouse. Moreover, during established disease, exposure to ES-62 increased the proportion of MZP B cells in the spleen and also circulating in the blood and interestingly, healthy 20-week old MRL/MP mice had substantially higher levels of B cells of the MZP phenotype relative to MRL/*lpr* mice.

To determine whether the suppression of proteinuria by ES-62 was B cell mediated, B2 B cells were purified from 20-week old ES-62 or PBS treated

MRL/*lpr* mice and transferred into 7-week old MRL/*lpr* recipients. Remarkably, the B cells from ES-62 treated mice suppressed the development of proteinuria whereas the mice that received B cells from PBS treated donors developed disease that was not significantly different to those receiving PBS alone. ES-62-B cell mediated suppression of disease was associated with a reduction in renal lymphadenopathy and suppression of ANA levels. Mechanistically, such protection was associated with an increase in the levels of rLN Bregs, a switch in Hep-2 staining indicative of anti-PC responses as well as elevated levels of IL-17A$^+$ and reduced IL-22$^+$ cells in the spleen and rLN. These data imply that ES-62 resets the IL-17A/IL-22 axis via B cell dependent mechanisms and suggest that transfer of ES-62-treated B cells is sufficient to mimic the protective actions of ES-62 (Fig. 7.3).

7.6. Is modulation of the effector/regulatory B cell balance a generality of the action of ES-62 in inflammatory disease?

ES-62 mediated suppression of collagen-induced arthritis, a mouse model of rheumatoid arthritis, is also associated with a modulation of the IL-17A/IL-22 axis (336,338). Unlike the MRL/*lpr* model, however, in the CIA model IL-17A drives pathogenesis whereas IL-22 plays dual pathogenic and protective roles (Pineda, Harnett & Harnett unpublished data). To address whether resetting of the effector: regulatory B cell balance is general to the anti-inflammatory action of ES-62 in IL-17A-associated autoimmune inflammation, the effect of ES-62 on the population dynamics of B cells in CIA was also investigated. As in the MRL/*lpr* mouse, the proportion of follicular B cells was elevated in the spleens of CIA mice treated with ES-62: however, the, proportions of GC B and Tfh cells were decreased, whereas in the MRL/*lpr* mouse, GC B cells were unaffected by the parasite product and the proportion of Tfh cells in the spleen was significantly increased. This may be due to the different roles of IL-17A/IL-22 and plasma cells/plasmablasts in the CIA mouse as the levels of both plasma cells and plasmablasts appeared to be affected by ES-62 being reduced in the paws of treated mice. Crucially, the levels of IL-10-producing B cells were also increased by exposure of CIA mice to ES-62 although in this case there didn't appear to be a preferential induction of a particular subset such as those of the MZP phenotype, but rather a homeostatic

resetting to the levels observed in naive mice. Interestingly, therefore, preliminary experiments in the ovalbumin-induced airway hypersensitivity model of asthma, in which the protective effects of ES-62 are also associated with suppression of pathogenic Th17/Th2 responses (338), revealed that such immunomodulation was also associated with increases in the levels of splenic IL-10-producing B cells (Rodgers, Coltherd, Harnett & Harnett, unpublished data). Collectively, therefore, it appears that the protective action of ES-62 in both autoimmune and allergic disorders involves resetting of the effector: regulatory B cell balance.

7.7. Developing drugs from ES-62

In order to facilitate translating the therapeutic potential of ES-62 to the clinic, small molecular analogues of ES-62, based around the structure of the active immunomodulatory phosphorylcholine moiety, were tested for their ability to suppress lupus-like pathology in the MRL/*lpr* mouse. Two lead compounds, S3 and S5, were selected on the basis that they suppressed TLR-mediated production of IL-6 and IL-12p40 by bone marrow derived macrophages, proinflammatory cytokines that promote IL-17A and IL-22 responses (Rzepecka, Al-Riyami, Suckling, Harnett & Harnett). The anti-inflammatory potential of these compounds was confirmed in the MRL/*lpr* mouse as both molecules suppressed the development of proteinuria. Consistent with their administration after disease had begun to be established, neither molecule seemed to affect the induction of regulatory B or T cell populations or IL-17A or IL-22 production, responses likely to have been initiated prior to the onset of pathology. However, as with ES-62, suppression of disease was associated with the modulation of MyD88 expression in effector cells at the site of inflammation. This finding is potentially exciting as there is an urgent requirement for drugs to treat lupus nephritis in SLE patients. Conventional therapies are effective but cause unacceptable collateral damage to the patient. Preliminary data suggest that S3 and S5 have good tolerability profiles and low toxicity (Rzepecka, Al-Riyami, Harnett & Harnett, unpublished data): furthermore, given that these compounds target mechanisms that have been selected through host-parasite evolution to result in safe immune modulation, it is possible that optimized derivatives of these compounds could suppress renal inflammation with limited side-effects.

In the study of parasite immunomodulatory mechanisms and host-parasite interactions, it is important to realize that these naturally evolved relationships do not solely provide material to be therapeutically manipulated, but rather and perhaps more importantly, they should also be viewed as tools that provide interesting insights into the mechanisms of the immune system in health and disease. By exploring the therapeutic potential of ES-62 in the MRL/*lpr* mouse model of SLE, several interesting discoveries have been made that may impact on our understanding of the human disease. Firstly, that IL-22 appears to pathogenic whilst IL-17A may have dual pathogenic and protective roles in disease. Secondly, that B cells may control such immune responses to regulate the development of autoimmunity. And finally, that modulation of pro-inflammatory signaling can suppress tissue inflammation, even in the presence of substantial kidney histopathology.

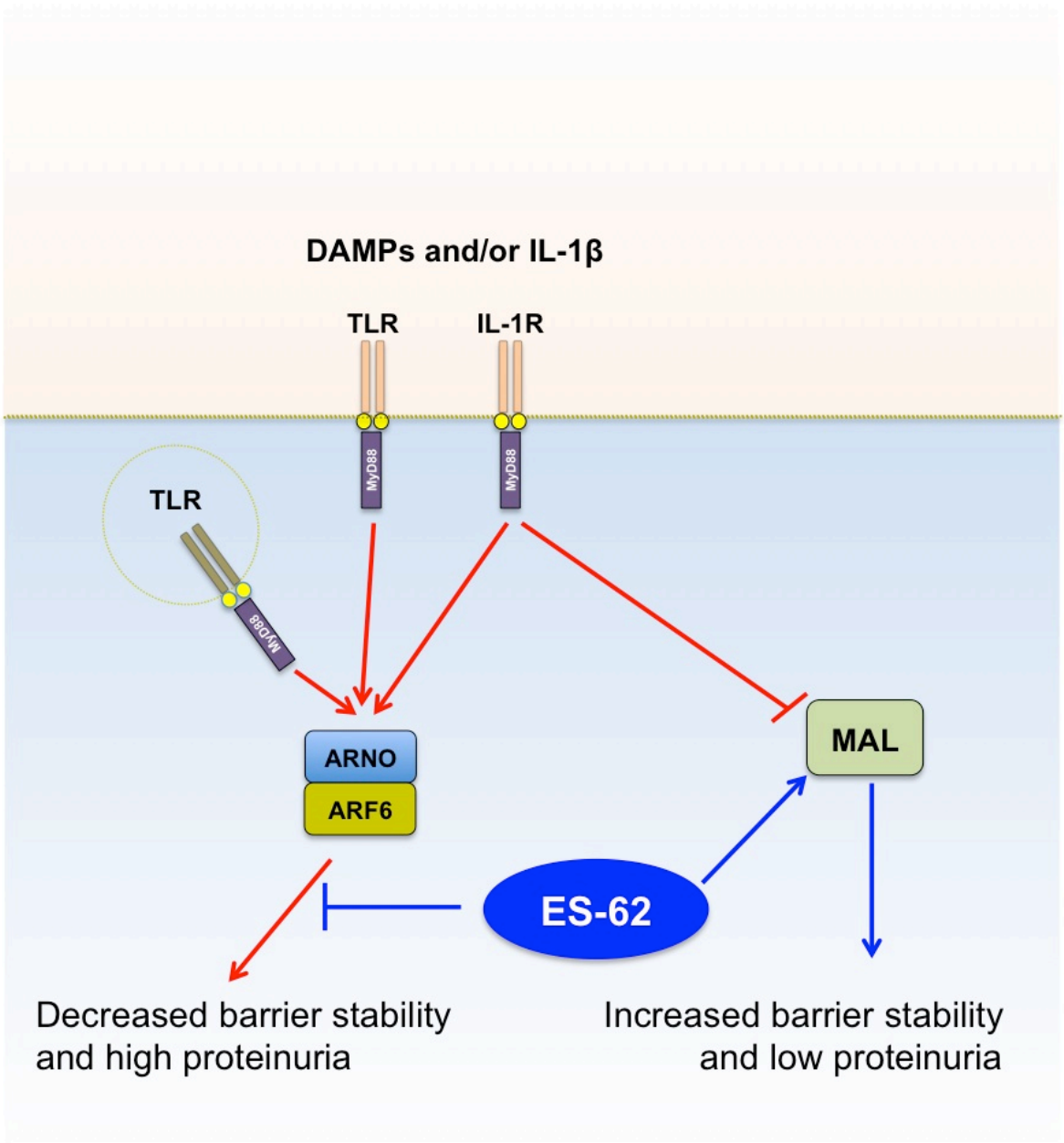
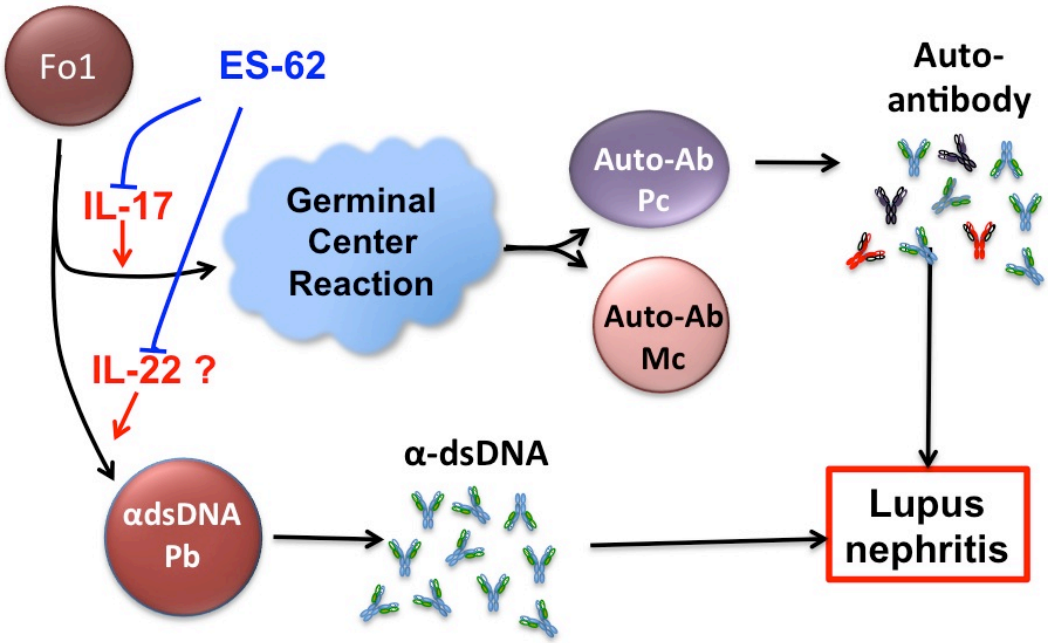

DAMPs and/or IL-1β
TLR
IL-1R
MyD88
MyD88
TLR
MyD88
ARNO
ARF6
MAL
ES-62
Decreased barrier stability
and high proteinuria
Increased barrier stability
and low proteinuria

Figure 7.1: Proinflammatory signaling in the glomerulus. MyD88 signals via ARNO-ARF6 to destabilize epithelial barriers (452) and hence as the suppression of proteinuria in ES-62 treated MRL/*lpr* mice is associated with a reduction in the levels of MyD88 expression in the kidney, this may indicate that this ARNO-ARF6 pathway is suppressed. Alternatively, ES-62 may promote signaling of this residual pool of MyD88 via the MAL-dependent pathway reportedly involved in maintaining epithelial tight barriers (454).

Prior to the onset of disease



Established disease phase

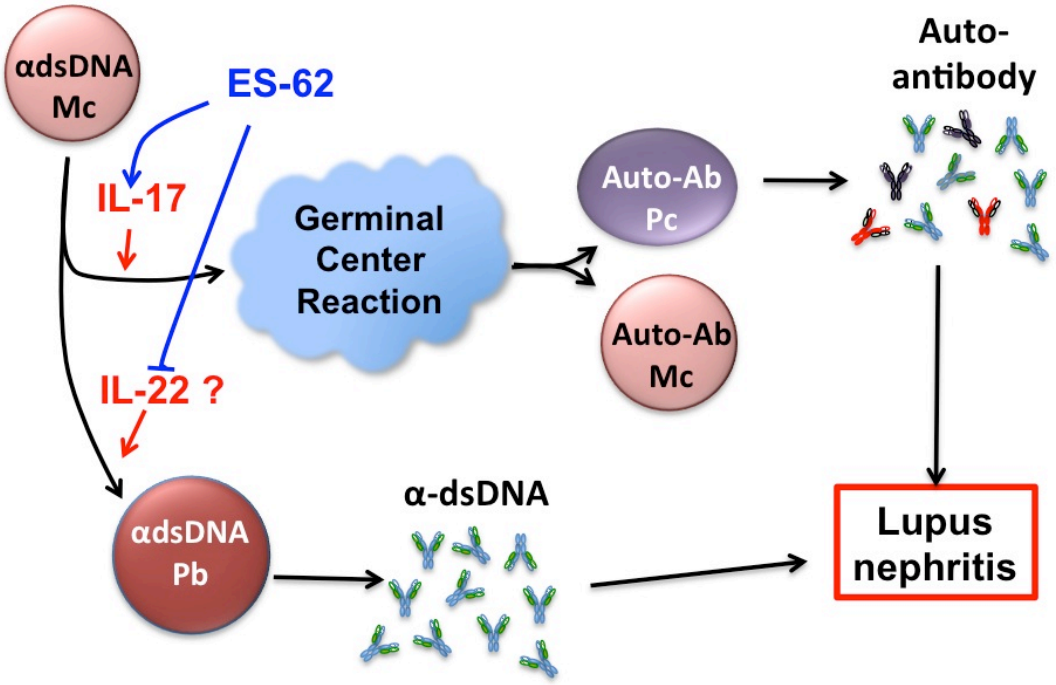


Figure 7.2: ES-62 modulation of IL-17A and IL-22 may affect the development of pathogenic B cell responses. Activated B cells can develop into long-lived plasma cells via the germinal center reaction or via extra-follicular development, into plasmablasts/short-lived plasma cells. This cell fate decision is dependent on several factors such as the affinity and signal strength of the BCR and the cytokine milieu: thus, IL-17A promotes and drives the germinal center reaction in lupus-prone mice (403). Prior to the onset of disease IL-17A appears to contribute to pathogenesis in the MRL/*lpr* mouse; this may reflect its ability to promote the break in tolerance and the early development of autoantibody-producing plasma cells (Pc) and memory B cells (Mc). IL-22 is also pathogenic prior to the onset of disease and may be involved in the activation of naturally occurring dsDNA-specific follicular B cells (Fo1) or the reactivation of dsDNA-specific memory B cells (Mc) into anti-dsDNA producing plasmablasts/short-lived plasma cells (Pb). By suppressing both IL-17A and IL-22 at this time point, ES-62 may prevent or perturb these pathogenic B cell responses. During the established disease phase, however, ES-62 suppresses IL-22 production whilst promoting that of IL-17A. This may result in the preferential entry of memory B cells into the germinal centre reaction rather than the extra-follicular development of dsDNA-specific plasmablasts. Thus by suppressing IL-22 at this time point, ES-62 may prevent the development of pathogenic plasmablast production of anti-dsDNA that is associated with the human disease (195).

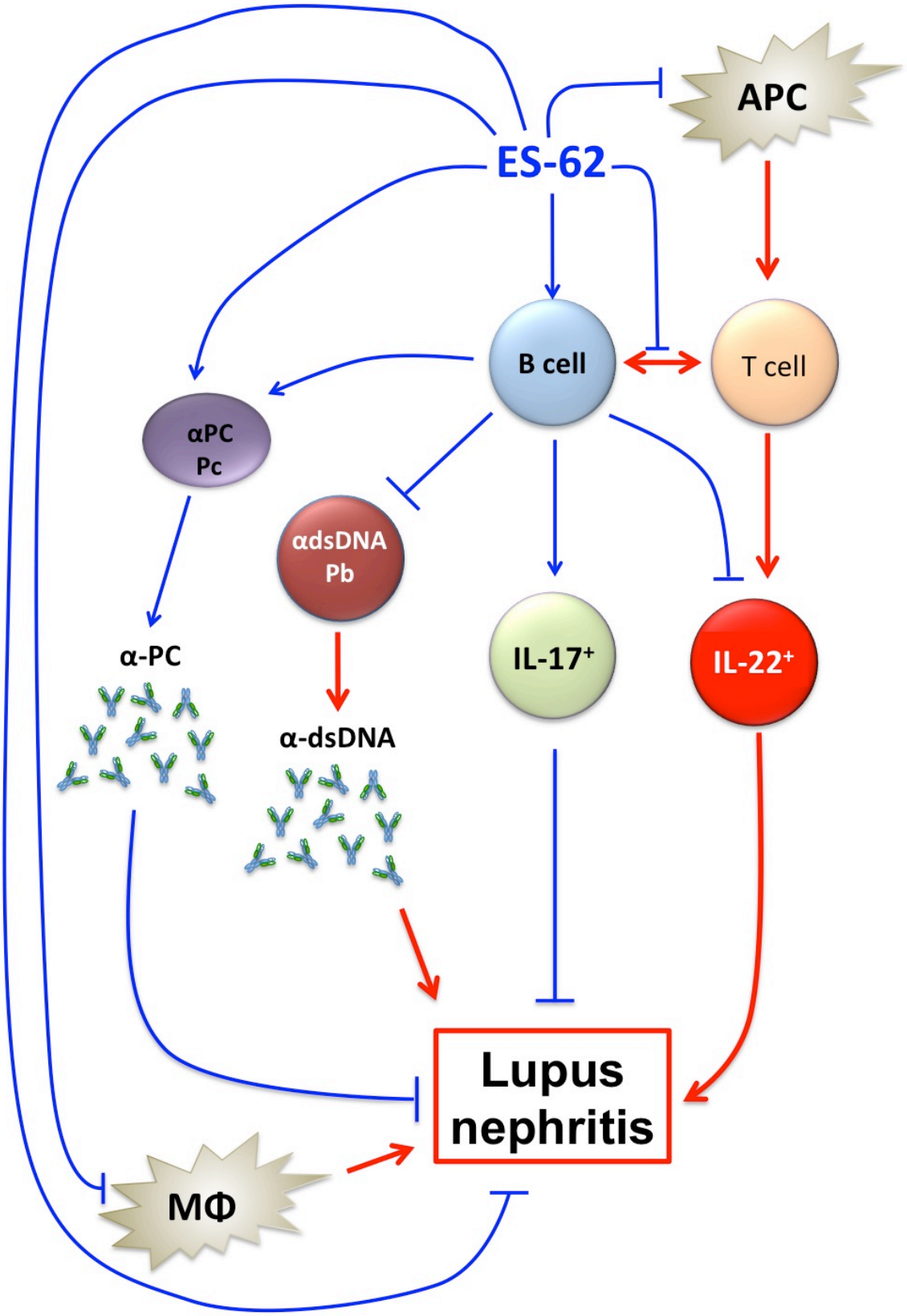

APC
ES-62
B cell
T cell
αPC
Pc
αdsDNA
Pb
α-PC
α-dsDNA
IL-17+
IL-22+
Lupus
nephritis
MΦ

Figure 7.3: ES-62 modulation of pro-inflammatory responses in the MRL/*lpr* mouse. ES-62 modulates disease in the MRL/*lpr* mouse by modulating the balance of effector and regulatory cells. Most notably, the parasite product suppresses the production of the pro-inflammatory cytokine IL-22 and the generation of pathogenic ANA producing plasmablasts, whilst inducing counter-balancing IL-17Aresponses and promoting the development of IL-10 producing regulatory B cells. ES-62 also suppresses the ability of B cells to interact with T cells, via the induction of hypo-responsive phenotype. Furthermore, the parasite product has been shown to have anti-inflammatory effects on macrophages and also renal cells. Remarkably, the majority of these effects may be B cell dependent as they are observed in recipient mice that receive B cells from ES-62 treated donors induce the reduction of ANA producing and elevation of anti-PC producing cells, suppression of IL-22 producing and elevation of IL-17Aproducing cells and a suppression of lupus nephritis.

8. List of references

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