The role of complement and granulocyte colony stimulating factor in ANCA associated vasculitis

Freeley, Simon

Awarding institution:
King's College London

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Simon Freeley

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MRC Centre for Transplantation

Division of Transplantation Immunology and Mucosal Biology

King's College London School of Medicine at Guy's, King's and St Thomas' Hospitals

Abstract

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis is a systemic disease which affects the kidneys, lungs, and other tissues. ANCA were first described in patients with focal necrotising glomerulonephritis in 1982, with myeloperoxidase (MPO) and proteinase 3 (PR3) subsequently shown to be the antigenic targets responsible for the perinuclear and cytoplasmic staining patterns, respectively. Infection is thought to exacerbate disease partially through the production of the proinflammatory cytokine TNFα which primes neutrophils for respiratory burst. In this thesis, the role of another cytokine, granulocyte-colony stimulation factor (GCSF) is examined both *in vitro* and *in vivo*. Previous reports have implicated the complement system in ANCA vasculitis. Furthermore, TNFα primed neutrophils which have been activated with ANCA *in vitro* are known to release a factor into the supernatant which causes complement activation in normal serum. This factor has yet to be identified, although many candidates such as the alternative pathway component properdin have been suggested. In this work it is shown that GCSF concentrations are elevated in patients with acute ANCA vasculitis and that GCSF can prime isolated neutrophils for anti-MPO induced respiratory burst. A passive antibody transfer mouse model of anti-MPO vasculitis was established and GCSF administration was shown to exacerbate disease. Experiments also explored other models of anti-MPO vasculitis based on MPO-deficient mice. The mouse model was also used to investigate the effect of deficiency of either properdin or MASP2 in disease. Using the passive anti-MPO passive transfer model, properdin deficiency was shown to have no effect on the extent of disease while MASP2-deficiency exacerbated disease by a mechanism which has yet to be identified. This work has established GCSF as a key cytokine and possible therapeutic target, and provided novel observations on complement in ANCA vasculitis.

ERRATA CORRIGE

Page	Line	Note	ERRATA	CORRIGE
71	14		For the anti-myeloperoxidase model, the LPS type used is not stated.	This was *Escherichia Coli R515* from Enzo Life sciences, UK – catalogue number ALX-581-007-L002.
121	10		As above	As above
120	10		As above	As above
133	22		As above	As above
73	7	Error in source of antibody used for most assays	alkaline phosphatase conjugated goat anti-mouse IgG (Southern biotechnology, Cambridge, UK).	alkaline phosphatase conjugated goat anti-mouse IgG, Fc specific (Jackson's Immunoresearch).

These minor clarifications of the methods have no effect on any of the results in this thesis.

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Signed by

Table of contents

Index of figures and tables

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Publications

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Abbreviations

ANCA	Anti-neutrophil cytoplasmic antibody
AP	Alternative pathway
BSA	Bovine serum albumin
CFA	Complete freund’s adjuvant
CIA	Collagen induced arthritis
CP	Classical pathway
CTAB	Cetyltrimethylammonium bromide
DHR 123	Dihydrorhodamine 123
DMEM	Dulbecco’s modified Eagle medium
EAV	Experimental autoimmune vasculitis
EDTA	Ethylenediaminetetraacetic acid
EGPA	Eosinophillic granulomatosis with polyangiitis
ELISA	Enzyme linked immunosorbant assay
F(ab)2	Fragment antigen binding
FBS	Fetal bovine serum
fMLP	Formyl-methionyl-leucyl-phenylalanine
GBM	Glomerular basement membrane
GCSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GPA	Granulomatosis with polyangiitis
HBSS	Hanks balanced salt solution
iCFA	incomplete freund’s adjuvant
IgAN	IgA nephropathy
IMDM	Iscove's modified dulbecco's media
I.P	Intraperitoneal
I.V	Intravenous
LP	Lectin pathway
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MPA	Microscopic polyangiitis

MPO	Myeloperoxidase
MPRO	Mouse promyelocytic
NADPH	Nicotinomide adenine dinucleotide phosphate
NOD	Non-obese diabetic
NTN	Nephrotoxic nephritis
NZB	New Zealand Black
NZW	New Zealand White
OCT	Optimal cutting temperature
OPD	O-Phenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Phosphate-lysine-periodate
PMA	Phorbol myristate acetate
PMSF	Phenylmethanesulfonyl fluoride
PNPP	p-Nitrophenyl-phosphate
RA	Rheumatoid arthritis
RPMI	Roswell Park Memorial Institute
S.C	Subcutaneous
SLE	Systemic lupus erythematosus
TBS Tween	Tris-buffered saline – tween
TE	Tris-EDTA
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
Treg	Regulatory T cell
WEHI	Walter and Eliza Hall Institute

Chapter 1 - Introduction

1.1 Overview

ANCA-associated vasculitis is a severe systemic autoimmune disease which affects the microvasculature of multiple organs including, but not restricted to, the kidneys, lungs, skin and eyes. Since the 1960's, the standard treatment regimen has been immunosuppression induced using a combination of cyclophosphamide and corticosteroids. This has proven to be a very effective treatment, leading to remission in approximately 90% of patients [1]. However, the long term use of cyclophosphamide can lead to serious adverse effects such as bladder injury, cancer, suppression of the bone marrow and an increased incidence of secondary infection. In recent years, more selective methods of immune suppression such as B cell depletion using rituximab have been introduced [2, 3]. Rituximab however, was shown to be no more effective then intravenous cyclophosphamide treatment with regard to its ability to treat severe disease[4, 5]. In these studies adverse effects were also similar in patients given rituximab or cyclophosphamide, perhaps due to the effect of coadministered corticosteroids. However, it is a useful therapy for those in whom cyclophosphamide treatment is unsuitable. Clearly there remains a need to improve our understanding of the pathogenesis of this disease in order to help identify targets for future therapeutics.

The subject of this thesis is the investigation of the role of granulocyte colony stimulating factor (GCSF) and certain aspects of the complement system in disease pathogenesis. This will be accomplished by the utilization of a murine model of ANCA-associated vasculitis.

In the first section of the introduction I will begin by introducing ANCA-associated vasculitis and what is currently known about its pathogenesis. Other types of glomerulonephritis, some of which share pathological similarities to that seen in vasculitis patients will then be described. The second section focuses on the various animal models of ANCA vasculitis and other forms of glomerulonephritis and highlights the strengths and weaknesses of each model. This information guided us to our decision on which models were most suitable to address the topics of this thesis.

This is followed by an introduction to both GCSF and the complement system and their roles in both immunity and immune regulation. The contribution of the complement system to ANCA vasculitis and various other types of glomerulonephritis, as determined by both human and animal studies is described. This section provides the basis of our hypothesis that GCSF and the alternative and lectin pathway components, properdin and MASP2 respectively, are involved in disease pathogenesis.

1.2 Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis

1.2.1 Introduction

ANCA-associated vasculitis is a severe systemic disease involving inflammation of the small blood vessel walls and primarily affects the kidneys and lungs. A defining feature is the presence of autoantibodies which target constituents of neutrophil granules, more specifically myeloperoxidase (MPO) or proteinase-3 (PR3) and are known as ANCA. Histopathologically, the disease is characterised by a pauci-immune vascular inflammation and focal segmental fibrinoid necrosis accompanied by extracapillary proliferation and subsequent glomerular crescent formation. ANCA-associated disease can vary in its clinical manifestations and can cause organ-limited disease such as in renal-limited vasculitis, or systemic disease as in the closely related granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), microscopic polyangiitis (MPA) and eosinophillic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome) [6, 7]. GPA, first described in the 1930s, is characterised by the presence of glomerulonephritis in combination with necrotising granulomas of the respiratory tract [8]. MPA possesses similar clinical characteristics to GPA including a combination of renal and pulmonary injury but lacks the presence of respiratory granulomas. EGPA, first described in 1951, is an allergic granulomatous angiitis which includes systemic vasculitis accompanied by asthma and eosinophilia[9].

ANCA-associated vasculitis is the most common form of small-vessel vasculitis to occur in adults with approximately 80% mortality if left untreated [10]. ANCA were first described in the 1980s in patients with focal necrotising glomerulonephritis and have since been used as

a diagnostic tool in the clinic [11, 12]. On immunofluorescent staining of ethanol fixed neutrophils, the ANCA present in GPA will usually appear in the cytoplasm, and are known as a cytoplasmic, or cANCA. In MPA and EGPA this staining will usually appear around the nucleus in a perinuclear or pANCA pattern. The antigens responsible for the cANCA and pANCA staining patterns were identified in the late 1980s as PR3 and MPO respectively [13, 14]. There has been much debate as to whether ANCA levels are associated with disease activity but it has been reported that many patients show a decreased ANCA titre during remission and in one study it was shown that elevated levels of ANCA preceeded relapses in about 90% of cases studied [15-17].

1.2.2 Possible links with bacterial infection

There have been reports of links between Gram positive bacterial infections and relapses of GPA. This link seems to be strongest with nasal carriage of *Staphylococcus aureus* [18, 19]. Numerous mechanisms have been suggested including the possibilities that *S. aureus* may prime neutrophils for an ANCA-induced respiratory burst or that bacterial superantigens may cause persistent lymphocyte activation leading to the development of disease [20, 21]. It has also been observed that in addition to anti-PR3 antibodies, patients with GPA also possess antibodies targeting complementary PR3 (cPR3) [22]. This is a protein which is transcribed from the anti-sense DNA strand of the PR3 encoding region. When mice were immunised with cPR3 they were shown to develop antibodies to both cPR3 and sense PR3. These anti-PR3 antibodies are thought to be produced by an immune response against the cPR3 antibodies in a process known as an anti-idiotypic response. Considering that certain peptides from cPR3 are known to be homologous to peptides from S. *aureus* a direct link to infection seems possible [23].

In addition to the presence of ANCA antigens, approximately 90% of ANCA vasculitis patients are also positive for autoantibodies targeting human lysosomal membrane protein 2 (LAMP2) [24]. LAMP2 is a membrane protein with roles in antigen presentation and the maintenance and adhesion of the lysosome. In neutrophils it is known to shuttle between the lysosome and the cell membrane and is thus found in significant quantities on the surface of the neutrophil [25]. Focal necrotizing glomerulonephritis was induced in WKY rats by intravenous injection of rabbit anti-human LAMP2 IgG which was shown to cross react

with rat LAMP2 [26]. These LAMP2 antibodies were also shown to activate neutrophils *in vitro* and to induce apoptosis of human endothelial cells. Interestingly, one of the two identified antibody binding epitopes of LAMP2 was shown to have 100% homology to a 9-amino acid portion of the bacterial adhesin protein, FimH of Gram negative bacteria. Furthermore, rats immunized with FimH went on to develop antibodies to LAMP2 and pauci-immune necrotizing crescentic glomerulonephritis. This is suggestive of a role for molecular mimicry in the development of ANCA vasculitis. This link with the Gram negative FimH is in contrast to the data reported by Stegeman et al suggesting a link between Gram positive bacteria, not Gram negative with relapse of GPA [27]. However, another study failed to find any association between LAMP2 antibodies and ANCA vasculitis and the issue remains unresolved [28].

1.2.3 Priming of neutrophils for ANCA induced respiratory burst

Neutrophils are a highly specialised phagocytic cell type which are indespensible in the battle against invading micro-organisms. They constitute up to 70% of the leukocyte population. Indispensible as they are for protecting the host from infection they are also known to cause tissue injury in the context of autoimmune inflammatory disease and have been shown to have an essential role in animal models of ANCA-associated vasculitis [29-31].

Opsonized pathogens are phagocytozed by neutrophils into the phagosome where a variety of cytotoxic agents are concentrated. The membrane of the phagosome contains a portion of the NADPH oxidase complex, a cluster of proteins responsible for the reduction of molecular oxygen (O_2) to superoxide ion (O_2^-). The increase in oxygen consumption by the phagocyte upon activation is known as the respiratory burst. In resting phagocytes, a portion of this complex, known as cytochrome b_{558} (consisting of gp91phox and p22phox) remains in the membrane while three other proteins (p47phox, p67phox and p40phox), which are linked together, remain in the cytosol. Upon phagocytosis, the cytosolic proteins become phosphorylated on p47phox and subsequently bind to p22phox thus activating the complex and allowing the transfer of electrons from NADPH to O_2 forming O_2^- which accumulates in the phagosome [32, 33]. Following shortly thereafter, the azurophilic and specific granules fuse with the phagosome and deliver a variety of pre-formed enzymes

including gelatinases and the ANCA antigens MPO and PR3 [34]. The site of reactive oxygen species (ROS) generation depends largely on the physical characteristics of the pathogen. Particulate stimuli such as opsonised bacteria are taken up into the phagosome where NADPH oxidase activity is concentrated along the phagosomal membrane. In other circumstances, where the agonist is too large to be phagoctyozed, the phagocyte will undergo what is called "frustrated phagocytosis" thereby releasing these toxic anti-microbial substances into the immediate environment [35]. It has been reported that the membrane bound cytochrome b_{558} is also located in the membranes of specific granules and in tertiary granules and upon activation of the neutrophil is known to incorporate into the surface membrane [36].

Many *in vitro* studies have demonstrated that ANCA can induce the activation of cytokine-primed isolated human neutrophils resulting in respiratory burst, degranulation into the extracellular environment, and proinflammatory cytokine production [37-40]. Subsequently these activated neutrophils adhere to and kill endothelial cells *in vivo* and *in vitro* [41-44]. These are the mechanisms which are believed to be crucial to the vascular injury seen in ANCA-associated vasculitis. Priming, which is crucial for the ANCA response, can be mediated by various stimuli including the proinflammatory cytokines TNF-α, IL-1, IL-6 and the anaphylotoxin C5a [37, 45, 46]. The principle consequences of priming are an increase in the response of these cells to an activating stimulus, delayed apoptosis and upregulation of the integrin CD11b expression [47, 48]. TNFα priming also induces the partial phosphorylation of p47phox thus contributing to the assembly of the NADPH oxidase complex at the cell membrane [49]. In addition, cytokine priming also causes the translocation of the ANCA antigens MPO and PR3 to the cell surface [40]. Resting neutrophils express PR3 on their surface and this is known to be greatly increased upon priming. In contrast, little to no MPO is found on the surface of resting cells, and its upregulation in response to priming is small when compared to that of PR3.

Experimental evidence suggests that ANCA are pathogenic *in vivo*. A case of the transplacental transfer of anti-MPO ANCA from mother to neonate resulting in pulmonary haemorrhage and renal involvement was recently reported, suggesting a pathogenic role for ANCA in a system independent of an anti-MPO cellular immune response [50]. Also, Xiao et

al showed convincing evidence for a pathogenic role of antibody alone by inducing crescentic glomerulonephritis in mice by the passive transfer of anti-MPO antibodies [51].

1.2.4 Role of T cells in ANCA vasculitis

There is evidence indicating that T cells are involved in the pathogenesis of ANCA-associated vasculitis. The pathogenic antibodies involved in disease are predominantly of the IgG1 and IgG4 subclasses suggesting that T cell dependent class switching has occurred. The granulomatous inflammation found in the airways of patients with GPA are known to contain activated T cells and disease remission can be induced in patients by the targeting of T cells by antibodies [52-54].

The T_H1/T_H2 paradigm is complicated in regard to the ANCA-associated vasculitides with generalised GPA and EGPA (which involves eosinophils) being polarized toward a T_H2 immune response [55]. However, patients with a localised GPA, and MPA, show a polarisation toward a T_H1 response [56, 57]. In recent years, the IL-17 producing T_H17 cell subset has gained notoriety for playing a role in the inflammation seen in many autoimmune disorders, including multiple sclerosis and inflammatory bowel disease [58, 59]. GPA was found to be no exception to this as PR3-specific T_H17 cells have been identified in patients with GPA [60]. In addition to this, irregularities have been reported in the T cells of patients with ANCA-associated disease. These include a reported defect in the suppressor function of regulatory T cells (Treg), which may contribute to persistent autoreactivity of T cells [61]. An upregulation of CTLA-4 on activated T cells, a phenotype which is known to induce resistance to apoptosis has also been reported in patients [62, 63]. Consistent with the idea of persistent antigenic stimulation, a decrease in circulating naïve T cells and an expansion of a subset of circulating effector memory T cells which migrate to sites of inflammation have been observed in GPA patients [64-66]. In addition, infiltrating T cells which have been found to localise to the glomeruli of patients with active disease have a memory phenotype [67]. Interestingly, these $CD4^+$ T effector memory cells may play a direct role in tissue injury as they have been shown to express the natural killer (NK) cell NKG2D protein. When stimulated by its ligand major-histocompatibility complex class I chain related molecule A (MICA), which is present on the renal endothelium of active GPA

patients, it induces the release of perforins and granzyme B, thus resulting in glomerular injury [68, 69].

A role for $CD4^+$ T cells in disease was demonstrated using an *in vivo* autoimmune mouse model of ANCA-associated vasculitis developed by Ruth et al [70]. This group immunized wildtype mice with human MPO, eliciting an immune response which cross-reacted with murine MPO. The addition of a glomerular binding antibody induced the influx of neutrohils into the glomeruli where they deposited murine MPO. This approach resulted in crescentic glomerulonephritis in the wildtype mice, and interestingly in mice which were B cell deficient, suggesting an antibody independent mechanism of injury. This study also showed that $CD4^+$ T cell depletion prior to disease induction protected from disease development despite ANCA titres remaining similar to control mice. This model suggests a role for both antibody and cellular immunity in the pathogenesis of disease.

1.2.5 Intracellular signalling in response to ANCA

The intracellular signalling cascade which is triggered upon ANCA binding has been extensively studied in recent years but it is still unclear as to how ANCA induce neutrophil activation. However, it has been shown that both the antigen binding F(ab)2 and the Fc part of the ANCA are essential for neutrophil activation as F(ab)2 binding alone was insufficient to induce activation [71]. Binding of the Fc part of the antibody to FcγRIIa was shown to be essential for ANCA induced activation [72-74]. It has also been reported that ANCA utilize the FcγRIIIb on neutrophils causing an upregulation of CD11b expression and a downregulation of L-selectin, which are consistent with neutrophil activation [75]. Despite being expressed at much lower levels on the surface of the neutrophil, the ligation of FcγRIIa is known to induce a respiratory burst much more readily than the ligation of FcγRIIIb [76].

Neither PR3 nor MPO possess transmembrane domains and they do not participate in intracellular signalling directly. Therefore they must work in cooperation with other molecule(s) in order to propagate a signal. This may be partly explained by ANCA induced activation of neutrophils through stimulation of β2 integrins. There have been reports that in addition to FcγRIIa, the ANCA must also interact with the β2 integrin CD18 in order to

induce respiratory burst in response to anti-PR3 or anti-MPO antibodies [74]. It was more recently uncovered that membrane bound PR3 can be coimmunoprecipitated with β2 integrin from phorbol myristate acetate (PMA) activated neutrophils suggesting that the proteins colocalize [77]. Furthermore, the treatment of pre-activated neutrophils with anti-CD11b negatively affected the amount of membrane bound PR3, providing further evidence of a direct interaction of the two proteins. MPO has also been shown to associate with β2 integrin on the neutrophil surface and to induce its activation [78, 79]. Despite these observations, the intricate details of ANCA signalling remain to be uncovered. Some signalling molecules have however been shown to be important. For example, p38 MAPK was shown to be required for the translocation of both PR3 and MPO to the surface of TNFα primed neutrophils [80]. Furthermore, an *in vivo* mouse model demonstrated that selective PI3Kγ inhibition protected from anti-MPO induced crescentic glomerulonephritis, and this was also confirmed *in vitro* [81].

1.3 Other types of glomerulonephritis

There are many types of glomerulonephritis seen in man. Some have little proliferative or inflammatory features. These include membranous nephropathy, focal segmental glomerulosclerosis (FSGS) and minimal change disease. Here I discuss some diseases in which proliferation or inflammation is a feature. While the pathological mechanisms of these diseases vary widely from each other and from ANCA-vasculitis, they highlight the various ways by which a disregulated immune system can cause disease in the kidney. Some features such as immune complex deposition and subsequent complement activation are a shared feature among different types of glomerulonephritis. Insight into the causes of these events is a crucial step in the understanding of pathogenesis and may provide opportunities for therapeutic intervention which may be applicable to various forms of glomerulonephritis.

1.3.1 IgA Nephropathy

IgA nephropathy (IgAN) is the most common cause of primary glomerulonephritis in man. It is characterised by the presence of IgA1 with galactose-deficient O-glycans in the hinge region of the heavy chains. The presence of such antibodies in the circulation is, on its own,

not sufficient to cause renal injury [82]. A crucial step in disease development is the production of IgA and IgG antibodies with binding specificity to the undergalactosylated hinge region [83]. The binding of these antibodies to the IgA1 forms immune complexes which bind to the renal mesangium via a receptor which remained unidentified for many years. Evidence has shown that IgA colocalizes with the transferrin receptor, CD71, in the kidneys of IgAN patients suggesting that it is a major IgA receptor in the mesangium [84]. The accumulation of these immune complexes combined with reduced clearance rates triggers the activation of the mesangial cells causing the production of inflammatory cytokines and chemokines which results in damage to renal tissue [85]. The pathogenesis of this disease is complicated, involving both a genetic predisposition toward the production of the undergalactosylated IgA1 molecule, and environmental factors such as mucosal infection [86, 87].

1.3.2 Anti-glomerular basement membrane disease

Anti-glomerular basement membrane disease is a rare autoimmune disease characterised by the presence of elevated titres of antibodies against the glomerular basement membrane (GBM). More specifically, these autoantibodies are specific for the noncollagenous domain of the α3 chain of type IV collagen (α3(IV)NC1). The lungs and kidneys express this autoantigen and thus patients often present with either kidney disease alone, known as anti-GBM glomerulonephritis, or with disease of both the kidneys and lungs, known as Goodpasture syndrome. Experimental evidence strongly suggests that these antibodies are pathogenic, and their removal from the circulation via plasmaphoresis is necessary for clinical recovery [88, 89]. Animal models have shown a pathogenic role for autoreactive T cells in direct kidney injury but there currently is a lack of evidence for this in human studies [90, 91]. Evidence derived from animal studies suggests that, through the process of molecular mimicry epitopes from antigens derived from Clostridium botulinum are sufficient to induce anti-GBM disease [92]. It has been speculated that viral infections may precede the onset of disease but this has yet to be proven [93]. The antibodies targeting the GBM are known to target two conformational epitopes of a3(IV)NC1 which are structurally hidden under normal conditions. It is hypothesized that damage to the lungs or kidneys by infection or pre-existing autoimmune disease leads to the exposure of a3(IV)NC1 on the basement

membrane allowing it to act as an autoantigen. However, this hypothesis has yet to be proven.

1.3.3 Lupus Nephritis

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder which is responsible for immune mediated injury affecting multiple organs such as the skin, joints, central nervous system and kidneys. Lupus nephritis, a major cause of morbidity and mortality, is present in approximately 50% of lupus patients. The heterogeneity in disease manifestation has complicated the process of elucidating the precise mechanisms of disease. The severity and progression of SLE varies greatly between individuals but the production of autoantibodies is common to all. The disease progresses through four broad stages, the first being the detectable presence of autoantibodies targeting numerous self antigens such as double stranded DNA and nucleosomes. Following this is the deposition of autoantibody and the formation of immune complexes in the tissues which leads to the third stage, tissue inflammation. Finally, tissue damage and fibrosis lead to end organ damage. There are known to be genetic factors involved in the predisposition to the development of lupus. In rare cases this can be traced to the deficiency of a single gene such as the complement components C4 or C1q. C4 deficiency has been linked to the impaired elimination of auto-reactive B cells while deficiency in C1q results in an impaired clearance of immune complexes, necrotic material and apoptotic bodies [94, 95]. The exposure of these normally sequestered antigens may be an important factor in the breakdown of tolerance and subsequent autoimmunity [95, 96]. However, in most cases it is the cumulative effect of multiple genetic abnormalities which leads to SLE. Much of what is known about the pathogenesis of human lupus nephritis has been derived from animal studies using mice which have been bred with a predisposition to the development of lupus-like disease. For example, the crucial role of B cells in disease was demonstrated in B cell depletion studies using the lupus prone MRL/lpr mouse [97]. This strategy markedly ameliorated histological disease in these mice. As a result of this, B cell depletion has emerged as a possible therapy in humans and has resulted in clinical remission in patients [98, 99]. A pathogenic role for B cells, independent of autoantibody production, was also demonstrated using mice of an MRL/lpr background which were unable to produce antibody. These mice developed less

severe nephritis suggesting that B cells have other functions in lupus nephritis such as antigen presentation to autoreactive T cells and proinflammatory cytokine production [100]. Lupus nephritis has been considered to be a T_H2 driven disease with high concentrations of the T_H2 associataed cytokines IL-6 and IL-10 reported in the serum of patients with active disease [101]. However, there are also reports of high concentrations of the T_H1 associated cytokines IL-12 and IL-18 in the glomeruli of patients and in mouse models [102, 103]. This reflects the complex nature of SLE and suggests that it is driven by different lymphocyte subsets within the nephritic kidney. Studies have reported imbalances in $CD4^+$ and $CD8^+$ T cell populations in disease as well as an increase in a circulating CD3+ CD4-/CD8- subset [104]. This subset of T cells was shown to induce the production of pathogenic anti-DNA autoantibodies. This has also been demonstrated in mice and is considered to be a major inducer of renal failure in mouse models of SLE [105-107]. Much of what is known about the pathogenic role of cytokines in lupus nephritis comes from murine models. Antibody depletion of IL-6 was shown to inhibit lupus nephritis in the NZB/W while IL-6 administration exacerbated it [108, 109]. Meanwhile in the MRL/lpr strain, genetic deficiency in either IL-12 or IFNγ resulted in protection from nephritis [110, 111]. In addition MRL/lpr mice decficient in the IFNγ receptor were shown to be protected from glomerulonephritis [112]. Studies have also revealed a role for the type I interferon, IFNα, in the pathogenesis of SLE [113]. Elevated levels of IFNα in the serum of SLE patients were shown to correlate with the level of immune complexes and disease activity [114]. IFNα is produced primarily by a subset of DCs called plasmacytoid DCs (pDCs) and its production was shown to be triggered by immune complexes [115]. Mouse studies using a lupus prone NZB strain deficient in the type I interferon receptor revealed that these mice exhibited reduced disease severity and mortality rates [116]. Interestingly, studies showed that the administration of IFNα induced lupus in NZB/NZW F1 mice but not in the BALB/c strain, highlighting the importance of genetic background in SLE [117]. It has been suggested that IFNα may induce the differentiation of monocytes into DCs which take up apoptotic cells and nucleosomes and present fragments of them via MHC II to CD4+ T cells, thus initiating the autoimmune response [118].

It has also been suggested that a defective Treg response may contribute to the persistent renal inflammation. Reduced numbers of peripheral $CD4^+/CD25^+$ Tregs have been reported

in patients with active lupus nephritis which may result in the hyperactivity of auto reactive T and B cells [119]. The current data suggest that lupus nephritis is complex disease involving many contributing factors.

1.3.4 Henoch-Schonlein purpura

Henoch-Schonlein purpura (HSP) is a vasculitic disease predominantly of children which affects the skin, causing small haemorrhages (palpable purpura), joints and in some instances the kidneys. It is characterised by the presence of IgA1 dominant immune complex deposits in the microvasculature [120, 121]. Approximately 40% of pediatric patients will go on to develop Henoch-Schonlein purpura nephritis (HSPN) within approximately 6 weeks of initial presentation. At this stage the clinical phenotype is almost identical to that of IgAN, hence it has therefore often been hypothesized that HSPN and IgAN share common pathogenic mechanism and may represent different spectrums of the same disease [122]. All patients with HSP possess circulating IgA1 containing immune complexes but only those with accompanying nephritis possess IgA1-IgG immune complexes [123]. As in patients with IgAN, patients with HSPN present with elevated levels of galactose-deficient IgA1 (Gd-IgA1) while those without kidney involvement have Gd-IgA1 titres similar to those of healthy controls, suggestive of a pathogenic role for Gd-IgA1 in HSPN [124, 125]. It is hypothesized that Gd-IgA1 molecules are recognised and targeted by naturally occurring circulating IgG resulting in the formation of the circulating immune complexes. These complexes then become deposited in the renal mesangium causing their activation, proliferation and the production of cytokines and chemokines resulting in renal injury [126, 127].

1.3.5 Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis (MPGN) is a term used to describe a variety of diseases which share similarities in their pathogenesis. It is characterised by the presence of structural changes in the glomeruli due in large part to mesangial cell proliferation in response to injury caused by immune complexes. It is a disease which primarily affects young adults and children. It is divided into three types but a common characteristic of all types is hypocomplementemia due to disregulated activation of the complement system. However, there are differences in the mechanism of complement activation.

Histopathologically, type I MPGN is characterised by the diffuse thickening of the GBM due to the presence of subendothelial immune deposits. It is defined as either secondary to chronic infection or autoimmune disease (eg, SLE, hepatitis C) or idiopathic (from unknown cause). The deposited immune complexes chronically activate the complement system resulting in complement mediated injury accompanied by leukocyte recruitment and Fc receptor-mediated damage to the glomeruli. These infiltrating mononuclear cells and neutrophils contribute to the glomerular hypercellularity seen with type I disease. Cytokines and growth factors subsequently released by glomerular cells induce mesangial cell proliferation [128]. Instead of the subendothelial immune complex deposits, type II MPGN has additional intramembrane dense deposits which appear as areas of highly electron dense material in the GBM and mesangium on electron microscopy. Type III MPGN shares clinical features with type I disease with the presence of subendothelial immune deposits, but is also characterised by the presence of subepithelial deposits associated with spike formation [129]. However, hypercellularity is less prominent in type III when compared with type I. Patients of all three types of MPGN can suffer from proteinuria, haematuria, hypertension and some degree of loss of renal function.

1.4 Animal models of glomerulonephritis

1.4.1 Animal models of ANCA-associated vasculitis

1.4.1.1 Models of ANCA vasculitis based on immunity to MPO

Several rodent models of ANCA-associated vasculitis have emerged over the years using both rats and mice. Early attempts in rats showed that treatment with mercury chloride led to anti-MPO antibodies in association with gut and cutaneous vasculitis [130]. However, this was accompanied by polyclonal lymphocyte activation and the presence of other autoantibodies which could have contributed to pathology. Further work in the rat suggested that immunity to MPO could exacerbate nephrotoxic nephritis, both through passive heterologous anti-MPO transfer and active immunisation with human MPO [131, 132]. In the last decade, various newer models of anti-MPO vasculitis have been developed in mice. Xiao et al showed that anti-MPO antibodies, which had been raised in MPO-deficient mice, injected intravenously into wildtype mice caused a pauci-immune necrotising

crescentic glomerulonephritis affecting approximately 5% of glomeruli [51]. This model will be referred to as the “passive transfer model” in the remainder of this thesis. Despite the mild nature of the disease in these mice, this was a major breakthrough in the field as it demonstrated the pathogenicity of anti-MPO antibodies alone and without a cellular immunity to MPO. Further work went on to show that the severity of disease induced in this way could be exacerbated by the administration of LPS which caused elevated levels of circulating TNFα [133]. Neutrophils are primarily responsible for the vascular damage in this model as their depletion using a monoclonal antibody completely protected mice from renal injury [31]. The administration of anti-MPO splenocytes into recombinase activating gene 2 (Rag2) deficient mice, which lack both T and B cells, caused a severe renal failure with cresecents and necrosis reported in approximately 80% of glomeruli [51]. This model will be referred to as the “splenocyte transfer model” in the remainder of this thesis. However, substantial immune deposits were reported in the kidneys of these mice, most likely due to the homeostatic proliferation of the injected splenocytes. This does not model the pauci-immune nature of the human disease and is thus a significant drawback of this model.

Schreiber et al developed an alternative approach which also involved the immunization of MPO-deficient mice [134]. However, instead of transferring this antibody into wildtype mice, the immunized MPO-deficient mice are lethally irradiated and reconstituted with wildtype bone marrow. The antibody producing plasma cells survive this dose of radiation and continue to produce antibody, including anti-MPO. The neutrophils of these mice are now MPO sufficient and available for interaction with anti-MPO antibody. These mice were reported to develop glomerular crescents/necrosis with urine abnormalities by eight weeks post bone marrow transplant. This model will be referred to as “The BM transplant model” in the remainder of this thesis. Using this model and the passive antibody transfer model it was shown that MPO positive bone marrow cells and not tissue cells are the pathogenic target for anti-MPO antibodies [134]. An advantage of this approach is that disease is induced by actively produced circulating antibody and no exogenous LPS is required. However, a drawback is that it permits the study of molecules in the BM compartment only.

1.4.1.2 Spontaneous development of vasculitis

A criticism of the above models is that they depend on antibody which has been generated in MPO-deficient animals, therefore not being autoimmune in nature. However, a strain of mice does exist that spontaneously produces anti-MPO antibodies. The spontaneous crescentic glomerulonephritis/Kinjoh (SCG/Kinjoh) mouse is derived from the selective mating of siblings of (BXSB/Mp x MRL/Mp-lpr/lpr) F1 hybrid mice with the highest degree of glomerular crescent formation. These mice are known to produce high titres of circulating anti-MPO antibody [135]. However, this autoimmune-prone strain is not pauci-immune and also produces a range of other auto-antibodies which contribute to disease. Due to the glomerular immune deposits seen in these mice they are not suitable as a model of ANCA vasculitis but may still be useful in the study of autoimmunity to MPO as demonstrated in several studies [136-138].

1.4.1.3 Induction of autoimmune vasculitis

Ruth et al have developed a mouse model which involves both cellular and humoral immunity to MPO [70]. This involves the immunization of wildtype mice with human MPO and the subsequent cellular and humoral immune response cross-reacts with mouse MPO. However, antibody levels are relatively low in comparison to those of immunized MPO-deficient mice and thus an additional stimulus is required for disease induction. The addition of a sheep anti-mouse GBM antibody induces the trafficking of neutrophils into the kidney. These neutrophils deposit MPO in the glomerulus which acts as a planted antigen for MPO specific $CD4^+$ T cells. This model has demonstrated the importance of MPO specific $CD4^+$ T cells in this disease as their depletion prior to the triggering of the neutrophil influx resulted in impaired crescent formation despite having similar anti-MPO titres as control mice. Interestingly, severe crescentic glomerulonephritis can be induced using this model in B-cell deficient mice, highlighting the importance of the cellular immune response in glomerular injury. Furthermore, it was reported that spleen cells from MPO-immunised wildtypes produced significant amounts of IL-17A and low amounts of IFNγ suggesting a predominant T_H17 anti-MPO response [139]. More recently, this group have identified an immunodominant MPO T cell epitope [140]. This was discovered using a peptide encompassing amino acids 409-428 of the MPO heavy chain ($MPO_{409-428}$). Using the model

of disease described above, this group showed that mice immunized with $MPO_{409-428}$ developed disease of a similar extent to those immunized with native MPO. Furthermore $MPO_{409-428}$ was implanted in the glomeruli of Rag1-deficient mice, thus acting as a planted antigen, and disease was induced by the injection of $MPO_{409-428}$ specific $CD4^+$ T cell clones. This study not only identified an important MPO T cell epitope but also provided further evidence for a pathogenic role for T cells in ANCA disease.

A model of experimental autoimmune vasculitis (EAV) was developed by Little et al in Wistar-Kyoto (WKY) rats. This is an inbred strain of rat that is known to be susceptible to induction of glomerulonephritis. Vasculitis is induced by immunising WKY rats with purified human MPO (hMPO) with complete freunds adjuvant (CFA) [141]. These rats elicit an immune response to hMPO. The anti-MPO antibodies were shown to cross-react with rat MPO and crescentic glomerulonephritis develops by 6-8 weeks after immunisation. A significant drawback to this approach is that only approximately 60% of the immunised animals develop crescentic glomerulonephritis and disease is mild in those that do, with crescents reported in 6-15% of glomeruli. It was later reported that disease could be induced in all animals by using a higher dose of hMPO combined with a modified CFA containing killed M *tuberculosi* and pertussis toxin [142]. Interestingly, only WKY rats are susceptible to the induction of vasculitis in this way, as Wistar Furth and Brown Norway rat strains are not despite having similar anti-MPO antibody titres after immunisation. This suggests a strong genetic component for susceptibility/resistance to this EAV.

1.4.1.4 Models of ANCA vasculitis based on immunity to PR3

Attempts to develop models of PR3 ANCA-induced vasculitis, and thus provide evidence of a pathogenic role for anti-PR3 antibodies in disease development have remained largely unsuccessful. This may be due to differences in both the structure and expression patterns of human and rodent PR3, or may be linked to the observation that PR3 is undetectable on the surface of isolated murine neutrophils thus being unavailable for antibody binding [143]. More recently, Van der Geld et al showed that the immunisation of mice and rats with chimeric human/mouse PR3 led to the development of mouse and rat PR3 autoantibodies respectively [144]. However, despite high titres of anti-PR3 antibodies being produced, there was no evidence of vascular lesions in either the lungs or kidneys of the mice and rats

tested. In another study, immunising PR3/neutrophil elastase double deficient mice with recombinant mouse PR3 generated mouse PR3 specific autoantibodies [143]. These PR3 autoantibodies however, failed to induce disease when transferred into LPS primed wildtype recipients.

More recently, it was shown that non-obese diabetic (NOD) mice immunized with recombinant mouse PR3 developed specific anti-PR3 autoantibodies but failed to develop disease [145]. However, the transfer of splenocytes from these mice into immunodeficient NOD/SCID mice resulted in vasculitis and severe segmental and necrotising glomerulonephritis leading to acute kidney failure and death. It is likely however that these mice would have had immune complexes in their kidneys thus complicating the model.

1.4.1.5 Model of ANCA vasculitis using human immune system mice

Reports of immunodeficient mouse strains with a deletion of the IL-2 receptor having improved engraftment of human haematopoietic cells paved the way for the development of human immune system mice [146]. In regards to ANCA vasculitis, the advantage of using a mouse with a functioning human immune system is that in theory, disease can be induced using ANCA which have been purified from patients. A recent study using NOD/SCID/IL2rγ$^{-/-}$ which had been reconstituted with human haematopoietic cells reported human neutrophils in the circulation [147]. These neutrophils stained with a cANCA pattern in response to patient serum containing anti-PR3. When given purified anti-PR3 IgG isolated from patients these mice developed mild pauci-immune glomerulonephritis with a small number of mice also showing mild lung injury.

1.4.2 Other animal models of glomerulonephritis

Various other models have been developed to investigate the pathogenesis of glomerulonephritis. These models vary in the ways by which disease is induced and include the injections of heterologous antibody or foreign proteins, resulting in the formation of immune complexes. Other models have been developed by the generation of various strains of mice bred for their susceptibility to the spontaneous development of glomerulonephritis.

Although these models do not mirror the paucy immune features of ANCA-vasculitis they provide important insight into mechanisms of glomerular injury common the various forms of glomerulonpehritis. These include injury caused by Fc-receptor mediated activation of granulocytes and important roles for complement in various forms of glomerulonephritis which may also be important in ANCA-vasculitis.

1.4.2.1 Nephrotoxic nephritis

Many forms of glomerulonephritis in humans are associated with the deposition of immune complexes in the glomeruli. These immune complexes are associated with glomerular injury as they drive inflammation in the kidney through Fc receptor mediated leukocyte activation, cell mediated immune responses and complement activation. In order to investigate the contribution of different immune cells/molecules in the induction of the injury seen in immune complex glomerulonephritis suitable animal models are a necessary requirement. Nephrotoxic nephritis (NTN) is a commonly used model of antibody mediated glomerular injury that is induced by the binding of a foreign antibody to the GBM. It was first developed in rabbits by intravenous injection of guinea-pig anti-rabbit GBM antibodies and has since been used in other species [148]. It is most commonly used in rats and mice which are injected with anti-GBM antibodies which have been raised in rabbits or sheep. The development of NTN can be divided into two distinct phases, the heterologous and autologous. The heterologous phase peaks approximately 2 hours after the injection of the GBM binding antibody (nephrotoxic antibody) and usually resolves by 24 hours. It is associated with the influx of neutrophils into the glomeruli and the development of proteinuria, the extent of which was shown to be dependent on the amount of antibody injected [149, 150]. Bacterial LPS and the proinflammatory cytokines IL-1 and TNFα were shown to exacerbate the heterologous phase of NTN induced in rats by increasing the numbers of glomerular infiltrating neutrophils up to six-fold [151, 152]. These cytokines are known to be produced in response to LPS stimulation. This observation helps explain the fact that glomerulonephritis is often exacerbated by infection. Furthermore, it was shown that TLR2 and TLR4 present on both BM derived cells and renal cells play an important role in glomerular injury in response to stimulation [153, 154]. TLR4 deficiency on either BM cells or renal cells caused a decrease in neutrophil influx into the kidney, but this was not the

case with TLR2 deficiency. Albuminuria was decreased when TLR2 or TLR4 was absent from either BM derived cells or renal cells but the effect was more profound when the deficiency was on the BM cells. In response to stimulation with either TLR2 or TLR4 agonists, renal mesangial cells were shown to produce CXC chemokines which have an important role in reutrophil chemotaxis.

The autologous phase of NTN occurs approximately one week after disease induction with the heterologous antibody [155]. It occurs as the result of an adaptive immune response by the host against the foreign anti-GBM antibody. It has also been speculated that sequestered host antigens which have been exposed during the heterologous phase may play a role. The NTN model can be accelerated by the presensitization of the animal with heterologous antibody before disease induction. The host immune system will mount both a humoral and cell mediated response to the foreign antibody. The antibody mediated injury occurs primarily through Fc receptor mediated mechanisms. Fc receptor involvement was shown to be crucial to the development of the autologous phase of disease as γ chain deficient (thus Fcγ-deficient) mice were protected from glomerulonephritis induced in this way [156]. Furthermore, the activatory Fcγ receptors, FcγRI and FcγRIII, were shown to be of particular importance [157, 158]. Fc receptors are also known to be expressed on renal mesangial cells. However, their presence on BM derived circulating leukocytes plays the predominant role in disease [159]. The important contribution of the cellular immune response was demonstrated by the observation that μ chain deficient mice, which lack mature B cells, are not protected from disease [160]. Further investigation showed that $CD4^+$ T cell depletion protected from the autologous phase whereas $CD8^+$ cell depletion did not [161]. $CD4^+$ T cells are thought to mediate tissue injury through the activation of macrophages. T cell depletion studies showed a reduced macrophage infiltration into the kidney and an accompanying reduction in proteinuria. This suggests that macrophages are directly involved in driving tissue damage and proteinuria. Further evidence for this was provided with the report of a reduction in proteinuria due to macrophage depletion with no effect on glomerular T cell infiltration [162]. Interestingly, it was shown in the accelerated NTN model that TLR2 ligand given at the same time as immunization with the heterologous antibody could exacerbate disease, accompanied by greater amounts of antigen specific IgG and an increase in the numbers of glomerular $CD4^+$ T cells. This effect was shown to be TLR2

dependent as mice deficient in TLR2 did not have exacerbated disease [163]. The above reports suggest a combination of both humoral and cell mediated immune responses contribute to the tissue injury seen in the autologous phase of NTN.

1.4.2.2 Serum sickness

The serum sickness models of antibody mediated glomerulonephritis are the result of antibody production, and immune complex formation, in response to the injection of a foreign protein. This causes a transient haematuria and proteinuria which resolves after a short period of time. In some of the first models of serum sickness, rabbits were injected with bovine serum albumin (BSA), which becomes deposited in the glomerular filtration system. The subsequently produced rabbit anti-BSA antibodies target the albumin and this can result in the formation of immune complexes in the glomeruli. A single dose of antigen is not sufficient to cause glomerulonephritis. This requires several injections of antigen which may result in glomerulonephritis which resembles membranous nephropathy [164]. These models have also been developed in mice with disease being induced in the BALB/c strain by injection of horse spleen apoferritin (HAF). Daily injections of HAF result in the development of necrotizing glomerulonephritis in these mice [165].

1.4.2.3 Lupus nephritis

Numerous mouse models have been developed over the years in order to study the mechanisms of SLE. These models can be grouped into being either spontaneous or induced.

1.4.2.3.1 Spontaneous models

The oldest spontaneous model was developed as the F1 hybrid of the New Zealand Black (NZB) and the New Zealand White (NZW) autoimmune mice and is known as the NZB/W F1 [166]. The severe disease which these mice develop by approximately 6 months of age is remarkably similar to human lupus including phenotypes such as splenomegaly and the

presence of anti-dsDNA antibodies. As in human disease there was also a gender bias toward females which is thought to be related to oestrogen levels [167].

The MRL/lpr strain was generated by crossing several strains of inbred mice and selecting for a phenotype resembling human lupus. These mice possess elevated levels of autoreactive antibodies targeting both single stranded and double stranded DNA and large amounts of immune complexes as a result. In contrast to the NZB/W F1 strain the MRL/lpr showed no gender bias in the development of disease. One of the mutations responsible for the observed phenotype was identified as the Fas receptor. Fas is involved in the induction of apoptosis of cells and plays an important role in homeostasis. T and B lymphocytes of the MRL/lpr mouse do not express a functional Fas receptor and therefore cannot undergo Fas induced apoptosis, a trait that is generally accepted as a factor in the development of lupus. However, MRL mice which are sufficient in Fas still develop nephritis although at a delayed rate, highlighting the influence of the genetic background of the MRL/lpr mouse [168].

The BXSB/Yaa strain develops an early onset severe lupus-like disease with mean survival rates of 5 and 14 months for males and females respectively [169, 170]. A susceptibility locus on the male Y chromosome is thought to be responsible for the more severe disease seen in males and is termed the Y-linked autoimmune accelerator (Yaa). This is known to be due to the duplication of multiple genes caused by the translocation of part of the X chromosome onto the male Y chromosome. Toll-like receptor 7, an innate pattern recognition receptor, responsible for detecting viral ssRNA, is one of these genes and is thought to be crucial to the Yaa phenotype [171].

1.4.2.3.2 Induced models

In contrast to the spontaneous models of lupus in which genetic backgrounds play an important role, there are methods by which lupus-like disease can be induced. Autoreactive antibodies targeting molecules such as DNA and histones can be induced in BALB/c mice by intraperitoneal injections of pristane [172]. The BALB/c strain is not known to be susceptible to the development of spontaneous autoimmune conditions. Nonetheless, these mice go on

to develop immune complexes and nephritis characteristic of lupus. However, they also develop plasmacytomas and erosive arthritis which resembles rheumatoid arthritis [172]. An SLE-like syndrome was also induced in the BALB/c strain by the intradermal injection of active chromatin. This resulted in the production of anti-dsDNA, anti-ssDNA and anti-histone antibodies which were detectable by day 44 after immunisation [173]. These mice were shown to develop severe renal Ig deposition and segmental or diffuse glomerular lesions.

1.5 Granulocyte colony stimulating factor (GCSF)

1.5.1 Introduction

GCSF is a 19kDa secreted colony stimulating factor hormone that functions as an important regulator of haemopoiesis. It is produced by a number of different cell types such as stromal cells, fibroblasts and endothelial cells [174]. It is also produced and secreted by a number of immune cells such as monocytes, macrophages and neutrophils after activation. Its primary role is the stimulation of the bone marrow to release granulocytes and stem cells into the circulation [175]. This is an essential function in order to meet the increased demand for phagocytic cells during infection. GCSF signals through the GCSF receptor (GCSFR) which is known to be present on the surface of myeloid progenitors, mature granulocytes, endothelial cells, monocytes/macrophages and also on both B and T lymphocytes [174, 176]. Its importance in granulocyte maturation/mobilisation is highlighted by the neutropenia present in GCSF-deficient mice. However, it is worth noting that GCSF is not absolutely essential for the production and release of granulocytes, as GCSF-deficient mice produce approximately 25% of normal neutrophil numbers and are capable of producing and mobilizing mature neutrophils in response to *Candida Albicans* infection [177].

Basal concentrations of GCSF in humans are extremely low, usually at the very limit of detection by conventional ELISA [178]. During infection, proinflammatory cytokines including IL-1β, and TNFα are produced in considerable quantities and these induce the production of GCSF by various cell types including macrophages, endothelial cells and fibroblasts [179]. IL-17A, a proinflammatory cytokine produced by activated neutrophils and some subsets of T cells is also known to be a potent stimulus for GCSF production [180]. Its

importance in GCSF production was demonstrated by the *in vivo* blockade of IL-17 which resulted in the reduction of GCSF serum levels [181].

1.5.2 GCSF and innate immunity

GCSF is known to play a role in the regulation of the innate immune system. It has been shown to prolong the survival of neutrophils, to enhance neutrophil superoxide production and degranulation and to prime neutrophils for oxidative burst [182-185]. However, there are conflicting reports in regard to the ability of GCSF to prime isolated human neutrophils for an oxidative burst in response to formyl-methionyl-leucyl-phenylalanine (fMLP). Some reports clearly show that GCSF concentrations in the range of 50ng/ml are sufficient to prime human neutrophils for an fMLP response in a temperature dependent manner [186, 187]. This effect was not due to an increase in the numbers of fMLP receptors on the surface of the human cells after priming as they were comparable to those of unprimed cells. However, another study reports the inability of GCSF to prime for an fMLP response as measured by a luminol oxidation assay [188]. Neutrophil adhesion to the endothelium is enhanced in response to GCSF treatment *in vitro*, and this is reflected by an upregulation of both Mac-1 and LFA-1 on the neutrophil surface [189, 190]. This enhanced binding is almost completely abrogated by the blockade of CD18, a common subunit of both Mac-1 and LFA-1[190]. This data predicts that GCSF may exacerbate diseases which are driven by disregulated inflammation. This has shown to be the case in inflammatory arthritis as elevated GCSF concentrations have been found both in the serum and synovial fluid of rheumatoid arthritis patients [191]. Interestingly, there has been a report that GCSF administration in the treatment of neutropenia in a patient with Felty's syndrome, an inflammatory disease characterised by a combination of rheumatoid arthritis and neutropenia, was reported to have exacerbated the patient's arthritis [192]. This has also been reported in rheumatoid arthritis patients [193]. Animal models of inflammatory arthritis have corroborated this data with GCSF administration exacerbating disease in a murine collagen induced arthritis (CIA) model and in a rat model induced by the passive transfer of anti-type II collagen antibody [194, 195]. Furthermore, GCSF-deficient mice were reported to be profoundly resistant to CIA and the blockade of endogenous GCSF prior to

CIA onset reduced the severity of disease [196]. There has also been a report of disease exacerbation by GCSF in patients with ANCA vasculitis [197].

1.5.3 GCSF and adaptive immunity

In contrast to the above data implicating GCSF in the exacerbation of ANCA-associated vasculitis and rheumatoid arthritis, it is known to have an immunomodulatory effect on the adaptive immune response and in other autoimmune diseases. GCSF is widely used in the clinic to mobilise haematopoietic stem cells from the bone marrow into the circulation for the purposes of stem cell transplantation in the treatment of some malignant diseases. In recent years this method has largely replaced the bone marrow as the primary source of haematopoietic stem cells. Interestingly, it has been reported that despite the presence of a significantly (approximately x10) larger number of activated T cells in the GCSF treated donor graft, there was both a reduced incidence and severity of graft versus host disease (GVHD) [198, 199]. This is most likely attributable to a variety of effects of GCSF on T cells, including suppression of proliferation, skewing toward a T_H2 phenotype and the induction of regulatory T cells [199-202]. It has been suggested that the reported suppression of T cell proliferation in response to mitogenic stimulation may be an indirect effect of GCSF on monocytes. Certain subsets of monocytes are a major source of IL-10, which is a potent anti-inflammatory cytokine which is also known to inhibit T cell proliferation and IL-2 production [203, 204]. This suppression may be due to the presence of elevated levels of monocyte derived IL-10 resulting from the approximately 50-fold increase in circulating monocyte numbers in GCSF treated individuals [205, 206]. The exact mechanism by which GCSF protects against GVHD is not fully understood but a shift toward a T_H2 phenotype is thought to play a significant role. Franzke et al reported the presence of the GCSFR on the surface of T cells treated with GCSF both *in vivo* and *in vitro* [207]. In addition, these cells also demonstrated the induction of the T_H2 transcription factor GATA-3 and subsequent IL-4 production upon GCSF treatment *in vivo*. Sloand et al reported similar findings in regard to IL-4 production by $CD4^+$ T cells after GCSF treatment *in vitro* and *in vivo* [201]. This was accompanied by a decrease in IFN-γ production, suggesting the downregulation of a proinflammatory T_H1 response. In addition to this direct effect on T cell polarization, an increase in the mobilisation of plasmacytoid dendritic cells which could promote T_H2

responses has been reported in GCSF stimulated stem cell donors [208]. This production of T_H2 cytokines in combination with IL-10 and a simultaneous reduction in T_H1 cytokines is thought to be one of the primary protective roles for GCSF in GVHD [209, 210]. In contrast to the exacerbating effect of GCSF in the CIA murine model of arthritis, several groups have shown a protective effect of GCSF administration in models of other inflammatory diseases. In a rat model of hapten-induced colitis, pre-treatment with GCSF before disease induction reduced the degree of colitis through the suppression of T_H1 cytokine production [211]. In a murine model of multiple sclerosis, treatment of mice with GCSF at the onset of disease provided a strong degree of protection from injury to the central nervous system [212].

Interestingly, it has been reported that GCSF treatment induces the production and secretion of the B cell regulator, B lymphocyte stimulator (BLyS) by neutrophils both *in vivo* and *in vitro* [213]. Furthermore, serum BLys concentrations were shown to be significantly higher in rheumatoid arthritis (RA) patients than in healthy controls [214]. BLyS is known to be an important factor in the mounting of a normal humoral immune response. This is evident from studies showing that mice lacking BLyS have a profound deficiency in B cell development and maturation and are impaired in both T cell dependent and T cell independent antibody responses [215, 216]. BLyS production was significantly enhanced when GCSF treated neutrophils were stimulated with an inflammatory stimulus such as C5a, LPS or fMLP [217]. This may have implications for antibody mediated autoimmune diseases which are exacerbated by GCSF administration such as RA. The administration of GCSF may prime the neutrophils of patients for elevated BLyS production in response to a proinflammatory stimulus. These elevated BLyS levels may then induce the proliferation and maturation of autoreactive B cells.

1.6 Complement

1.6.1 Introduction

Building on the early work in microbiology by Pasteur and Koch in the 19th century, numerous scientists have contributed to the discovery of what we now know as

complement. Its discovery more than 100 years ago was initiated by the observation of the bactericidal activity inherent in normal serum [218-220]. In 1885 Jules Bordot first noted the heat sensitive, or heat-labile property of complement by demonstrating that in a mixture of *Vibrio choleriae* and heat treated serum, lysis of the bacterium only occurred after the addition of normal serum [221]. This was the first direct evidence for the requirement of a heat-sensitive serum component for bacteriolysis in addition to the heat stable antibody. This serum component, which complemented the effects of specific antibody was first referred to as alexin by Buchner and Bordet but it was Paul Erlich who coined the term complement. The most important observation of the time was reported by Bordet and Gengou in 1901, who were the first to show complement fixation [222]. They showed that combinations of antigen and antibody removed complement activity from normal serum. This showed that complement exists as a substance which can be removed from the serum and is not an anti-microbial property of the serum itself. Much research on the complement system has been done since these seminal experiments and much has been learned. It is now known to be an extremely complex system comprising approximately 40 proteins and cell-surface receptors. One of its primary functions is in the recognition and subsequent destruction of invading pathogens through direct cytolysis and/or the labelling of pathogens for phagocytosis [223, 224]. However, in recent years additional functions of the complement system have been identified such as the disposal of immune complexes and in immunoregulation where it plays a role in both humoral and T cell immunity [225-227].

Complement is known to be activated through three distinct pathways, the classical, lectin and alternative. All three pathways share many common components but they differ in the nature of initiation due to differences in microbial recognition. Each pathway results in the activation first of a C3 convertase, then a C5 convertase and finally the assembly of the membrane attack complex (MAC) from five hydrophilic precursor proteins, which binds to lipid membranes and destabilizes them resulting in cytolysis. A simplified representation of all three complement cascades is shown in figure 1.1.

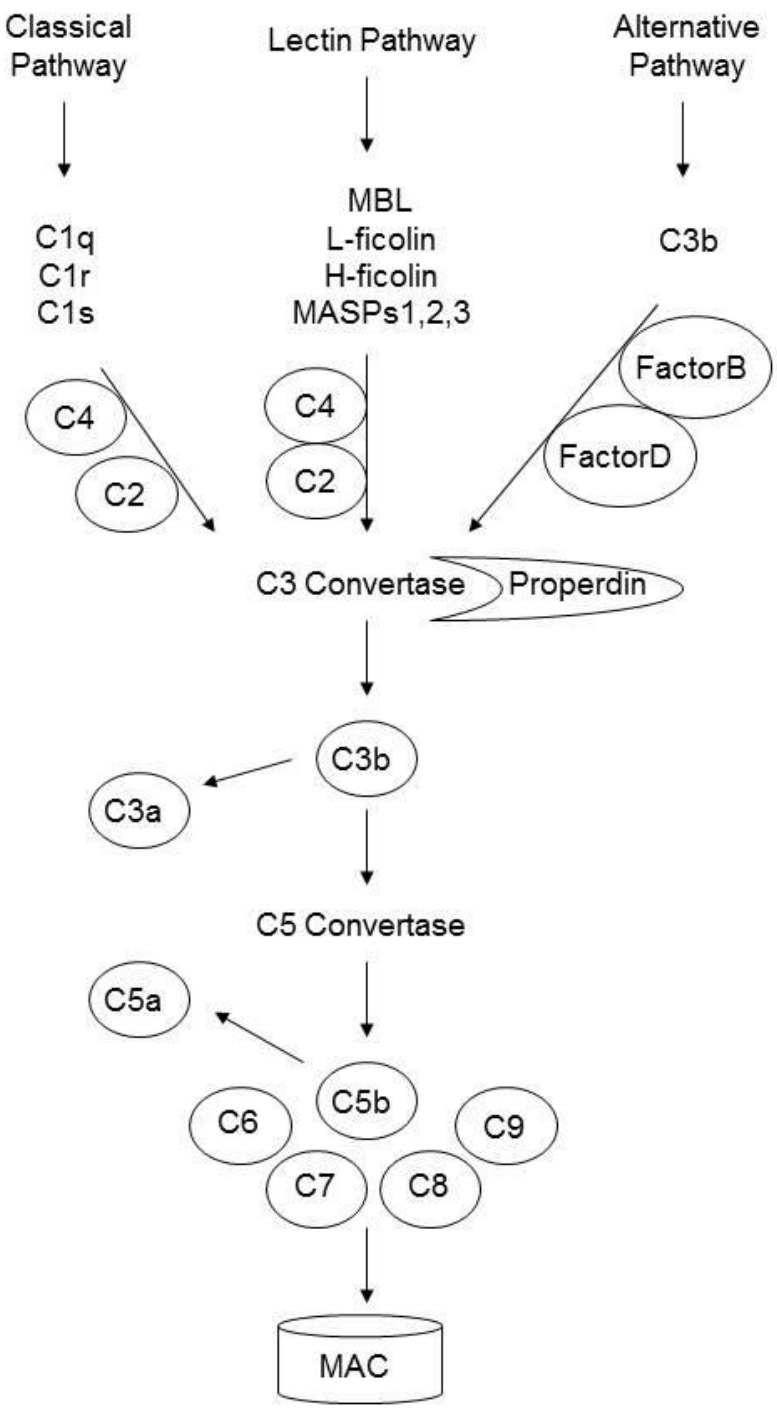


Figure 1.1 Overview of the three known complement cascades, the classical, lectin and alternative pathways and the means by which each pathway leads to the formation of the C3 and C5 convertases.

1.6.2 The classical pathway

The classical pathway (CP) of complement activation was the first to be discovered and studied. It is initiated by the binding of the complement fixing antibodies IgM and IgG to the surface of pathogens. The serum protein C1, known as the C1 complex, which is composed of the subunits C1q and two copies of each of C1r and C1s circulates in an inactive precursor state. It is now well known that C1q is the subunit responsible for the recognition of a variety of targets encompassing microbes, immune complexes and apoptotic/necrotic cells. Upon binding by C1q, the complex undergoes a conformational change leading to the activation of the serine poteases C1r and C1s. C1r cleaves and thus activates C1s resulting in the formation of an active complex which sets into motion a series of protein cleavages resulting in the activation of other important components. Active C1s cleaves the circulating component C4 into two fragments, C4a and C4b. C4b binds to the pathogen (or immune complex) surface via a reactive thioester bond. C2 then binds to C4b and is also cleaved by C1s. This results in the formation of the classical pathway C3 convertase C4b2a. This complex is responsible for the cleavage of C3 into C3a and C3b. C3b forms a complex with C4b2a to form the classical C5 convertase C4b2a3b which cleaves C5 into C5a and C5b. Following this, the MAC is formed on the cell surface resulting in cell death. Once the C3 convertase has been formed, each of the three complement pathways follows a common sequence of events resulting in the assembly of the MAC.

1.6.3 The lectin pathway

The lectin pathway (LP) is very similar to the CP but does not require antibody for complement fixation. Instead, the plasma proteins mannose-binding lectin (MBL), L-ficolin and H-ficolin recognise different carbohydrate moieties on the surfaces of pathogens ranging from bacteria, fungi and viruses [228-230]. Both MBL and the ficolins circulate in an inactive form in a complex with three structurally related proteins called mannose-binding protein-associated serine proteases (MASPs) 1, 2 and 3. Upon the recognition and subsequent binding to pathogen derived oligosaccharides by either MBL or the ficolins, conformational changes result in the autoactivation of MASP2 which cleaves C4 into C4a and C4b [231]. MASP2 is homologous to the CP C1s and they are closely related. As in the CP, C4b will bind to the surface of the pathogen thus inducing the binding of C2. C2 is

subsequently cleaved and activated by MASP2 to form C2b and C2a. The C4b2a complex forms the LP C3 convertase. The exact function of all of the MASP proteins is not known. MASP1 is known to cleave C2 (but not C4) and thus may play a role in propagating the LP activation [232]. There is data to suggest that MASP1 can directly cleave and activate C3, independently of C4 and C2 [233]. However, this MASP1 mediated C3 cleavage has been shown to be inefficient which has led to speculation as to the relevance of such a mechanism [234]. The sequence of events downstream of C3b generation in the LP is identical to that of the CP.

1.6.4 The alternative pathway

The alternative pathway (AP) is an antibody independent humoral defence mechanism which is the most ancient of the complement pathways. It is responsible for approximately 90% of all complement activity. The AP is triggered by a variety of activators including LPS from bacterial membranes, zymosan from fungal cells walls, as well as many polysaccharides, viruses, tumour cells and parasites. It remains unclear what each of these activatory structures have in common but it is known that recognition involves C3b [235]. The AP C3b is generated by a constant low rate of hydrolysis of the thioester group of C3, referred to as “tick over”. In conjunction with other proteins, the AP is amplified and regulated resulting in the formation of the AP C5 convertase. Upon covalently binding to the pathogenic surface, C3b is accompanied by the binding of the proenzyme factor B. Upon binding of factor B, C3b initiates the activation of factor D which cleaves factor B into two fragments, the noncatalytic Ba and the catalytic serine protease Bb. The active Bb associates with C3b to form the AP C3 convertase C3bBb which amplifies the AP response allowing additional C3b molecules to bind to target cells in the immediate environment. Through C3b generated in this way the AP also amplifies the CP and LP [236]. The C3bBb complex can be bound by the serum glycoprotein properdin, the only known positive regulator of complement activation [237]. Properdin is known to increase the stability of the AP C3 convertase by 10-fold and to inhibit C3b cleavage and inactivation by complement regulators [238, 239]. It is composed of multiple identical subunits, each having a separate ligand binding site. It has been shown that properdin can bind to surface bound C3b using one subunit, and can, through its other subunits, provide receptors for bystander C3b

molecules, thus aiding in the assembly of the C5 convertase [240]. The binding of the second C3b molecule to the C3 convertase results in the formation of the AP C5 convertase C3bBb3b. The new C3b molecule is required for the binding and modification of C5 for cleavage by Bb into C5a and C5b, leadin to the eventual formation of the MAC[241].

1.6.5 The membrane attack complex

The cellular death which is caused by the activation of the complement system is mediated by the MAC, a supramolecular complex containing one each of the circulating C5b, C6, C7, C8 molecules and numerous molecules of C9. It forms a tubular structure which spans the membrane, disrupting the lipid bilayer leading to cell death. C5b contains a binding site for C6, and the two molecules readily interact forming a loosely bound link with the cell membrane. Upon interaction with C7 the complex firmly anchors itself in the membrane [242]. The inserted C5b-7 forms a receptor for the next component, C8, which penetrates deep into the lipid bilayer [243]. Binding of the first C9 molecule to the C5-8 complex results in activation and conformational changes, exposing hydrophobic regions which insert themselves into the lipid bilayer. Subsequent C9 molecules bind to activated C9 and undergo the same conformational change independently of C5-8 and insert themselves into the membrane [244]. As more C9 molecules are incorporated the transmembrane pore gets larger. With numerous MACs on the cell surface, the cell will die primarily due to osmotic leakage.

1.6.6 Anaphylatoxins

During complement activation both C3 and C5 are each cleaved into two fragments, namely C3a/C3b and C5a/C5b respectively. As stated above, both C3b and C5b have crucial roles in the events leading to the assembly of the MAC. However, both C3a and C5a are anything but inactive biproducts of complement activation. They are both potent anaphylatoxins with various roles in the promotion of inflammation. They act through their surface receptors, C3aR and C5aR, which are both G-protein coupled receptors (GPCR), but differ in both ligand specificity and function. C3aR is expressed on the surface of myeloid cells including neutrophils, monocytes/macrophages, DCs, eosinophils and basophils and on both B and T lymphocytes [245-250]. It is also known to be expressed on the surface of some non-

myeloid derived cells such as endothelial cells [251]. The expression of C3aR on the surface of B and T lymphocytes suggests that C3a may play a role an important role in the modulation of the adaptive immune responses. Other functions of C3a include the chemotaxis of mast cells and eosinophils and the contraction of smooth muscle cells [248, 252, 253]. C5a is produced by the cleavage of C5 by the complement C5 convertase and is also a potent anaphylatoxin. It mediates a variety of biological effects including smooth muscle contraction, increasing vascular permeability, chemotaxis of neutrophils and monocytes and in the priming of neutrophils for respiratory burst [46, 254]. C5aR is ubiquitously expressed on a variety of cells but particularly on immune cells including macrophages/monocytes, neutrophils and on some T cells [255-257].

The complement system is also known to play a role in the mobilisation of hematopoietic stem/progenitor cells (HSPCs) from the bone marrow to the peripheral blood especially in the context of GCSF infusion. During HSPC mobilisation the bone marrow becomes a somewhat proteolytic microenvironment due to the release of proteolytic enzymes. The enzymes degrade stromal derived growth factor 1 (SDF1) and the CXCR4 receptor, two important molecules involved in retaining HSPCs in the bone marrow. This process exposes a neo-epitope on the surface of damaged bone marrow cells to which naturally occurring antibodies may bind. This is thought to result in activation of the classical pathway of complement, causing C3a and C5a formation. Mouse studies have shown that C3a-deficiency results in accelerated mobilisation after GCSF infusion [258]. The mechanism behind this observation is thought to be a C3a mediated increase in the responsiveness of HSPCs to an SDF1 gradient by causing an increase in CXCR4 incorporation into the HSPC membrane. C5-deficient mice were shown to be poor mobilisers of HSPCs, thus suggesting that C5 is important for HSPC mobilisation [259]. The mechanism for this is unclear but the potent chemotactic properties of C5a are thought to be a contributing factor.

1.6.7 Regulation of the complement system

Complement is a crucial component of innate immunity as it plays an important role in anti-microbial defence and in enhancing inflammation. Therefore it is required that the complement system be regulated in order to prevent collateral damage to healthy host tissues. This may be due to activation in response to pathogens, immune complexes or the

spontaneous activation of the alternative pathway. Regulation is mediated by an array of both membrane bound and plasma proteins. Due to the spontaneous nature of activation of the alternative pathway, its regulation is of particular importance. Regulatory molecules work by destabilising activation complexes and encouraging their dissociation, and by mediating the cleavage and thus deactivation of reactive fragments. The soluble complement regulators C4 binding protein (C4bp) and factor H act as decay accelerating factors for the CP and AP respectively. C4bp irreversibly displaces C2a from the CP C3 convertase C4b2a, thus deactivating it. C4bp also acts as a cofactor for another complement regulator, the serine protease factor I, which cleaves C4b into an inactive form called inactive C4b (iC4b) which can no longer function in the C3 convertase [260]. Further cleavage of iC4b results in the formation of the two fragments C4c and C4d. The exact function of C4d is not known but it is used as an indicator of antibody mediated rejection in organ transplantation as it binds to tissue close to the site of initial activation [261]. The AP analogue of C4bp is the fluid phase inhibitor factor H. Factor H also acts as a decay accelerating factor, this time for the AP C3 convertase C3bBb by displacing Bb from the convertase. It is also known to bind to C3b and to compete with factor B [261, 262]. Furthermore, like C4bp, it acts as a cofactor for factor I in the cleavage of C3b to iC3b. The classical pathway proteases C1s and C1r as well as the lectin pathway MASP2 are inhibited by the C1 inhibitor (C1 INH) in order to prevent auto-activation. Upon complement activation, C1 INH is cleaved and dissociates allowing C1s/MASP2 to cleave C4 [263].

Host cells express membrane bound complement regulators such as complement receptor 1 (CR1, CD35), membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55) and protectin (CD59). CR1 is present on the surface of many cell types including neutrophils, monocytes, B cells, erythrocytes and on some T cells [264]. CR1 expressed on erythrocytes is crucially important in the clearance of C3b expressing immune complexes by liver macrophages via their CR1 and iC3b receptor CR3. MCP, which is present on numerous cell types including all circulating cells (except erythrocytes), haematopoietic cells and endothelial cells, acts as a cofactor in the factor I mediated cleavage of C3b/C4b into iC3b/iC4b [265]. In contrast to CR1, MCP does not possess a decay accelerating factor activity. This function is carried out by DAF which is also expressed on a variety of cells including most circulating cells and endothelial cells. CD59 is an inhibitor of the later stage

of complement activity, more specifically, an inhibitor of the assembly of the MAC. CD59 binds to C5b-8 thus preventing the binding, activation and subsequent polymerisation of C9. CD59 is widely expressed on both erythrocytes and leukocytes as well as on endothelial cells and extravascular tissue [266].

The potent anaphylatoxins C3a and C5a are regulated by plasma carboxypeptidases which cleave their C-terminal arginine residues to form C3a des-Arg and C5a des-Arg respectively. This modification causes both C3a and C5a to lose approximately 90% of their activity [267].

1.6.8 Complement and adaptive immunity

The discovery that B cells could bind C3 and the subsequent finding of both CR1 and CR2 on their surface was the first clue to a link between complement and adaptive immunity. Later studies reported that C3 deficiency resulted in impaired humoral immune responses [268]. It has since been reported that the coexpression of CR1 and CR2 occurs on B cells, FDCs, and on a subset of T cells [269, 270]. On B cells, CR2 is known to form a complex with the B cell signalling molecule CD19 and the transmembrane receptor CD81 [271]. The ligation of this receptor complex by antigen which has been coated in complement component C3d substantially lowers the activation threshold of the B cell in response to B cell receptor (BCR) stimulation [272]. Most circulating C3 is synthesized by the liver, but it has been shown that local production of C3 by haematopoietic cells plays an important role in the induction of an antibody response as C3-deficient mice that have been reconstituted with C3 sufficient BM are rescued from this impaired antibody response [273]. The complement system has also been speculated to be involved in the negative selection of self-reactive B cells in the BM and periphery. Complement coated self-antigens are known to bind to developing B cells via CR1 and CR2 which enhances their negative selection [274].

In recent years there have been reports of other complement components exerting effects on T cell responses. It was shown that coengagement of CD3 and CD46 on $CD4^+$ T cells, in the presence of IL-2, induces a regulatory T cell phenotype with the production of IL-10 [275]. It has also been reported that locally produced C5a, acting through its receptor on T cells, plays an important role in naïve T cell survival through the suppression of apoptosis [276].

1.7 The role of complement in glomerulonephritis

The following section details some of what is currently known about the contributory role of complement in the pathogenesis of both human and animal models of glomerulonephritis. The first part of the section covers what has been learned from human studies of various forms of glomerulonephritis. The second part relates to what has been gathered from the various animal models. These models provide an insight into disease pathogenesis at the molecular level, which is often impossible in human studies.

1.7.1 Evidence from clinical observations

1.7.1.1 Complement in ANCA-associated vasculitis

Due to the pauci-immune nature of the disease, the contribution of complement was initially somewhat overlooked. This was partly due to the fact that there is less complement deposition in the vessel walls of patients with ANCA vasculitis when compared to those with diseases involving immune complexes such as anti-GBM disease [277]. However, considering the involvement of complement in many other forms of kidney disease it seemed likely that it played a contributory role. One study reported the presence of the complement components C3d, factor B, properdin and the MAC in renal tissue from ANCA vasculitis patients. Neither MBL nor C4d were detected suggesting a possible role for the AP but not the CP or LP in disease [278]. A recent study showed that circulating levels of the complement components C3a, C5a, soluble C5b-9 and Bb were significantly higher in patients with active disease than in those in remission [279]. Furthermore, levels of properdin were lower in the active disease group than in the remission group suggesting that it is being utilized. Plasma levels of Bb correlated with the extent of renal injury suggesting that the alternative pathway is responsible. Plasma levels of the classical pathway component C4b were comparable between the groups, lending support to the dominant role of the AP in disease.

1.7.1.2 Complement in IgA nephropathy

In the mesangial deposits typically found in IgA nephropathy, the complement components C3, C5, properdin and the MAC are often seen, while the CP components C1q and C4 are not [280]. This suggests a role for the AP and terminal pathways in disease. Furthermore, serum concentrations of the breakdown products of activated C3, iC3b and C3d are often found in patients and correlate with disease severity [280]. There is also evidence of deposits of the LP component MBL in the mesangial deposits suggesting the involvement of the LP [281]. MBL is known to bind to polymeric IgA. The presence of MBL in the glomeruli was reported in 25% of the patients studied and was associated with the colocalisation of L-ficolin, MASPs and C4d. Furthermore, MBL and L-ficolin deposition in the glomeruli of patients was associated with more severe histologic disease and increased proteinuria.

1.7.1.3 Complement in MPGN

All three types of MPGN are associated with hypocomplementemia due to the disregulated activation of complement. However, there are some differences between the types of MPGN with regard to the pathways of complement activation. Type I, the most common form of the disease, is associated with subendothelial immune deposits and CP activation. Patients are known to have low or normal levels of C3 and low C4 levels [282]. Chronic activation of the CP results in the production of C3a and C5a which leads to leukocyte infiltration and tissue injury. Renal biopsies show strong immunofluorescence staining for C3 along the capillaries. C1q, C4 and properdin have also been shown to colocalize here. Type II MPGN, in contrast to type I, is characterised by uncontrolled activation of the AP. It is a less common form of MPGN accounting for up to 35% of total cases. A common feature of type II disease is the presence of an IgG autoantibody which binds to and prevents the deactivation of the AP C3 convertase (C3bBb). This autoantibody is known as the C3 nephritic factor (C3Nef). However, C3Nef is also known to be present in healthy individuals and its presence in MPGN patients does not always correlate with disease progression [283]. This suggests that other factors are also involved. Mutations in the complement regulatory molecule factor H which affect its ability to curb AP activation have been identified. Mice lacking factor H have uncontrolled activation of the AP and develop a disease similar to type I MPGN [284]. Furthermore, autoantibodies targeting factor H have been identified in others

[283]. Immunofluorescence staining of kidney sections from patients with type II MPGN shows the deposition of C3 along the glomerular capillaries and in the mesangium. Patients also have low levels of C3 and normal levels of C4, further implicating the AP [282]. Type III MPGN is considered by some to be a morphological variant of type I disease. In addition to subendothelial deposits, type III also has subepithelial deposits. Chronic complement activation is thought to be related to another nephritic factor which stabilises the AP properdin stabilised C3 convertase (C3bBb3bP), thus leading to activation of the terminal pathway and MAC formation. In contrast to the C3Nef, this terminal pathway nephritic factor (NeFt) has not been reported in healthy subjects.

1.7.1.4 Complement in human SLE

SLE is a complex autoimmune disease with the involvement of numerous susceptibility genes playing a role in its development. Inherited deficiencies of the classical pathway predispose to SLE providing strong evidence for a protective role [285]. Support for the pathogenic effect of complement comes from histopathological correlations of immune complex and complement deposition with human disease. Patients with SLE show substantial glomerular complement deposition which is thought to contribute to tissue injury. Complement is known to have an important role in the removal of immune complexes and this has led to a popular theory in which deficiency in these components leads to ineffective clearance of these complexes [225]. In response to the immune complexes which have become deposited in tissues, complement becomes chronically activated and induces tissue injury in conjunction with Fc receptor mediated damage by phagocytes. Furthermore, CR1 downregulation in SLE also leads to impairment in the clearance of immune complexes by erythrocytes. CR1 and CR2 expression by B cells of SLE patients is known to be decreased by as much as 50% [225]. Considering the link between CR1/CR2 and B cell regulation it is possible that this deficiency may lead to an impairment of B cell tolerance. Apoptotic cells are known to be an important source of autoantigen involved in SLE and C1q is involved in their recognition and subsequent removal by phagocytes [286].

1.7.2 Role of complement in animal models of glomerulonephritis

1.7.2.1 Complement in murine models of ANCA-associated vasculitis

Much of what is known about the involvement of the complement system in the pathogenesis of ANCA-associated vasculitis has been derived from murine models of disease. The first direct evidence in favour of a role for complement was reported in 2007 with the observation that mice that had been depleted of the complement component C3 by cobra venom factor (CVF) were protected from crescentic glomerulonephritis induced by anti-MPO antibodies [287]. They were also protected from urine abnormalities despite having similar levels of anti-MPO antibodies in the circulation as control treated mice. C5-deficient mice were also completely protected from disease induced in this way. To further investigate the involvement of complement, mice which were deficient in either the AP factor B or the CP and LP C4 were used. Disease severity was reduced in the factor B-deficient animals, while the C4 knockouts were not protected and developed disease comparable to wild types. C5 is a potent mediator of inflammation and C5a has an important role in neutrophil chemotaxis and activation. Subsequent work showed that treatment with an anti-C5 monoclonal antibody protected from disease in the same passive transfer model [288]. It was reported that the supernatant taken from isolated human neutrophils which had been TNFα-primed and stimulated with ANCA had the ability to generate C5a in normal serum [46]. C5a was shown to be crucial for neutrophil priming for respiratory burst as C5aR blockade during priming caused an inhibition of respiratory burst in response to ANCA. Furthermore, the importance of this priming effect was shown *in vivo* using the BM transplant model in which C5aR deficient mice show very mild glomerulonephritis and an absence of urine abnormalities in response to anti-MPO IgG [46]. The factors released by activated neutrophils which cause the generation of C3a and C5a in normal serum have yet to be identified and this remains a key question. Many candidates have been suggested including oxygen radicals and MPO products [289, 290]. Activated neutrophils have been reported to release not only C3 but also the AP components factor B and properdin. In addition they are also known to produce the late stage components C6 and C7 [291]. Properdin is known to be produced by neutrophils, stored in their secondary granules and is released upon neutrophil activation [292]. C5a and LPS are known to

stimulate the release of properdin from neutrophils and this could potentiate or even initiate the activation of the AP [293, 294].

1.7.2.2 Complement in nephrotoxic nephritis models

Complement activation is implicated in the pathogenesis of many immune-complex mediated autoimmune diseases. As a model of antibody mediated glomerular inflammation, the NTN model has been used to investigate the role of complement in disease progression. The heterologous phase of the NTN model has been used to assess the role of complement in acute antibody-mediated inflammation. The published data are somewhat conflicting, with some reports but not others showing a role for complement [295-297]. These differences may be, at least in part, the result of differences in genetic background.

Other studies have examined the role of complement in the autologous phase of the NTN model. C1q deficiency led to an increased susceptibility to glomerular injury in response to nephrotoxic antibody [297]. Another study suggested that C3 deficient mice had increased disease compared to wild types [298]. These results may be due to a role for complement in clearing or preventing deposition of immune complexes.

The alternative pathway was shown to have a role in renal injury in the autologous phase of disease. Factor B deficient mice, which were not significantly protected from injury in the heterologous phase, showed both reduced glomerular C3 deposition and albuminuria 5 months post injection of nephrotoxic serum when compared to control mice [299]. The role of complement in the glomerular injury induced in response to nephrotoxic serum is therefore complex, with evidence for both protective and injury related roles.

1.7.2.3 Complement in animal models of SLE

Much of the evidence regarding the involvement of complement in glomerulonephritis has come from the various inbred lupus prone mouse strains. The observations in human

studies of CP deficiencies leading to SLE were confirmed using animal models. It was shown that C1q and C4 deficiency in mice leads to the development of an autoimmune disease with all the hallmarks of human SLE [300, 301]. C1q deficient mice were shown to have higher titres of autoantibodies and increased mortality compared with strain-matched controls. They were also shown to have a significantly raised number of apoptotic bodies in their glomeruli [95]. It has subsequently been shown that C1q binds to apoptotic cells and that C1q deficient mice possess an impaired ability to clear them [302, 303].

It has been reported that MRL/lpr mice which were deficient in C3 had significantly greater glomerular IgG deposition and albuminuria than control mice. This suggests that C3 is not required for the full expression of renal disease in these mice and may even play a protective role in the clearance of immune complexes [304]. The pathogenesis of SLE is very complex and involves a number of factors, many of which involve the complement system. There are however data that suggest complement can play a proinflammatory role in lupus. MRL/lpr mice deficient in DAF have increased disease [305]. In addition, treatment of lupus-prone mice with C5 blockade was protective [306]. Consistent with this, more recent data has shown that the C5a receptor can enhance disease perhaps through T cell mediated effects [307] whereas C3a receptor appears to promote a degree of protection [308]. With further investigation, this may provide opportunities for the development of therapeutics which target the complement system.

1.8 Conclusions

A great deal of progress has been made over the years in the understanding and treatment of ANCA-associated vasculitis. Despite this, a number of key issues remain to be resolved. As discussed in section 1.2, evidence from *in vivo* studies point to a pathogenic role for ANCA [51]. *In vitro* studies have demonstrated that TNFα primed neutrophils undergo respiratory burst in response to ANCA [37-40]. It is thought that an inflammatory stimulus such as bacterial infection induces the production of TNFα which leads to this neutrophil priming. While this may be true, it may not be the entire picture. An interesting report published in 2004 details the exacerbation of disease in a patient with ANCA-associated vasculitis who had been given GCSF [197]. Considering many reports of the inflammatory effects of GCSF in the context of joint disease in both humans and animal models [191, 193-195], and its

priming effects on isolated neutrophils [186, 187], it is likely that GCSF plays an adverse role in ANCA vasculitis.

Evidence from mouse models of anti-MPO vasculitis point to the complement system playing a significant role in pathogenesis of ANCA-associated disease [287]. More specifically, the alternative pathway but not the classical or lectin pathways, was implicated. One particular study showed that isolated neutrophils which had been primed and activated with ANCA released a factor which had the ability to activate complement in normal serum [46]. This factor remains to be identified and several candidates have been suggested in the literature ranging from properdin to proteases. In light of recent advances in complement research, and the identification of new routes of activation, its role in ANCA disease warrants further investigation.

1.9 Aims of thesis

- To compare serum concentrations of GCSF in patients with active disease to those of age matched healthy controls.

- To investigate whether GCSF has a priming effect on isolated human neutrophils in response to anti-MPO stimulation.

- To set up a robust and reproducible mouse model of anti-MPO vasculitis.

- To investigate the *in vivo* effects of GCSF administration using the murine model of disease.

- To investigate the roles of properdin and MASP2 in ANCA disease using the mouse model.

Chapter 2 – Materials & Methods

2.1 Buffers and reagents

All reagents were purchased from Sigma Aldrich (Poole, UK) unless stated.

Phosphate buffered saline (PBS)

3.2mM Na_2HPO_4, 0.5mM KH_2PO_4, 1.3mM KCl, 135mM NaCl, pH 7.4.

500mM Carbonate-Bicarbonate buffer

160mM Na_2CO_3, 330mM $NaHCO_3$, pH 9.5.

MPO Purification buffer A

6.7mM sodium phosphate, pH 6.0, 1mM $MgCl_2$, 3mM NaCl, 0.5mM phenylmethanesulfonyl fluoride (PMSF)

MPO Purification buffer B

100mM sodium acetate, pH 6.3, 100mM NaCl

MPO Purification elution buffer

100mM sodium acetate, pH 6.3, 100mM NaCl, 750mM methyl-D-mannopyranoside, 1mM $CaCl_2$, 1mM $MgCl_2$, 1mM $MnCl_2$

0.1M Gylcine, pH2.7

A 0.1 M glycine solution was prepared by dissolving 3.75g glycine in 500ml tissue culture grade water. The pH was adjusted to 2.7 using 1M HCl.

1M Tris, pH9

A 1M Tris solution was prepared by dissolving 24.23g of Tris in 200ml tissue culture grade water. The pH was adjusted to 9 using 1M HCl.

Formal Saline

1 in 4 dilution of 39% formaldehyde in PBS

Bouin's Solution

Solution A. Saturated 1.2% w/v picric acid

Solution B. Formaldehyde 40% w/v

Bouin's stock solution is 3 volumes of A and 1 volume of B. 1ml/20ml of acetic acid is added immediately prior to use.

Phosphate-Lysine-Periodate fixative

A lysine stock solution was prepared by adding 3.65g/100ml (0.2M) lysine monohydrochloride to an equal volume of disodium hydrogen orthophosphate (3.58g/100ml). The solution was adjusted to pH 7.4 and stored at 4°C. Prior to use, 4g of paraformaldehyde dissolved in 100ml distilled water while stirred at 60°C in fume hood. This milky solution was cleared using a few drops of 1M NaOH. Immediately prior to use, 1 volume of paraformaldehyde was added to 3 volumes of lysine solution and 0.214g/100ml sodium metaperiodate was added giving a final concentration of 10mM.

1% Periodic Acid

5g of Periodic Acid

500ml of sterile water

Sucrose Solution

38mM sucrose dissolved in PBS (13g/100ml).

Anaesthetics

1.36ml vetalar - ketamine hydrochloride, 100mg/ml (Pharmacia, Animal Health Ltd, Northamptonshire, UK) was added to 2ml of domitor - medetomidine, 1mg/ml (Pfizer Ltd, Kent, UK) and 15.7ml of PBS. Mice were anaesthetized by IP injection of 200µl of this solution.

Red cell lysis buffer

4.17g NH_4Cl

0.00185g EDTA

0.5g $NaHCO_3$

500ml sterile water

Filter sterlizied and stored in aliquots at -20°C

1X DNA extraction buffer

50mM Tris-HCl, pH 8 (5ml 1M/100ml)

100mM EDTA pH 8 (20ml 0.5M/100ml)

100mM NaCl (2ml 5M/100ml)

1% SDS (5ml 20%/100ml)

Immediately prior to use proteinase K was added to a final concentration of 0.5mg/ml

1X Tris-EDTA (TE) Buffer

10mM Tris-HCl, pH8

1mM EDTA

Saturated ammonium sulphate

A saturated ammonium sulphate solution was prepared by dissolving 300g of ammonium sulphate in 500ml of tissue culture grade water. The solution was heated to ~56-60°C with frequent shaking. It was then cooled overnight with crystal formation indicating saturation.

Preparation of dialysis tubing

Dialysis tubing (Medicell, London, UK) was cut to appropriate length (always handled with gloves) and submerged in 200ml of tissue culture grade water with 2% sodium bicarbonate (Sigma, Poole, UK) and 1mM EDTA (Sigma, Poole, UK). It was incubated at 80°C for 30 minutes. It was then washed with 200ml tissue culture grade water. The tubing was kept wet until ready to use. Two knots were tied at the bottom end of the tubing and a clip was attached between them.

Antibodies used for flow cytometry

Target	Isotype	Clone	Fluorochrome	Dilution	Supplier
CD62L	Rat IgG2a, κ	MEL-14	Pacific Blue	1 in 80	Biolegend
CD11b	Rat (LEW) IgG2b, κ	MI/70	FITC	1 in 100	Becton Dickinson
CD11c	Armenian Hamster IgG	N418	PE	1 in 62.5	Biolegend
Ly6G	Rat (LEW) IgG2b, κ	1A8	Alexafluor 700	1 in 200	Becton Dickinson
Thy1.2	Rat (LOU) IgG2a, κ	53-2.1	FITC	1 in 2000	Becton Dickinson
CD3	Rat (SD) IgG2b, κ	17A2	PE	1 in 200	Becton Dickinson

Table 2.1 Antibodies used for flow cytometry.

Antibodies used for immunohistochemistry

Target	Isotype	Clone	Fluorochrome	Dilution	Supplier
CD68	Rat anti-mouse IgG2a	FA11	-	1 in 200	Serotec
IgG	Mouse anti-rat IgG (H+L)	-	DyLight488	1 in 200	Jackson Immunoresearch
IgG	Goat anti-mouse IgG Fcγ	-	FITC	1 in 200	Jackson Immunoresearch

Table 2.2 Antibodies used for immunohistochemistry

2.2 Cell Culture

2.2.1 Mouse promyelocytic cells

Mouse promyelocytic (MPRO) cells are a murine granulocyte cell line which in the presence of GM-CSF differentiate into neutrophil granulocytes and display promyelocyte characteristics such as azurophilic granules [309]. MPRO cells (ATCC, CRL-11422, LGC standards, London, UK) were grown in IMDM (PAA, Somerset UK) containing 20% FBS (Sigma Aldrich, Poole, UK), 1% Pen/Strep (Thermo Fisher Scientific, Loughborough, UK), and 10ng/ml GM-CSF (Peprotech, London, UK) and grown to a density of 2 x 10^6cells/ml. Upon harvesting, the cell pellets were resuspended in buffer A with 100mM PMSF and stored at -80°C.

2.2.2 WEHI-3B cells

Walter and Eliza Hall Institute-3B (WEHI-3B, ATCC, TIB-68, LGC standards, London, UK) cells are a macrophage-like myelomonocytic leukaemia cell line. They were initially derived from BALB/c mice which were subject to mineral oil injections in order to induce plasma cell tumour development [310]. The resulting tumour cells had characteristics of human myelomonocytic leukaemia. Cells were grown in IMDM supplemented with 10% FBS and 1% Pen/Strep to a concentration of 2 x 10^6 cells/ml.

2.2.3 32Dcl3 cells

The 32Dcl3 cell line (ATCC, CRL-11346, LGC standards, London, UK) is an immortalized IL-3 dependent cell line derived from long term cultures of bone marrow from C3H/HeJ mice [311]. Once removed from IL-3 containing medium and resuspended in GCSF containing medium, the cells differentiate into mature neutrophil granulocytes over 12 days. Growth medium consists of RPMI containing 2mM L-glutamine (Life Technologies, Paisley, UK), supplemented with 10% FBS, 1% Penn/strep and 10% WEHI-3B conditioned medium as a source of IL-3. When the cells had reached a concentration of 2 x 10^6 cells/ml they were

resuspended in RPMI containing 2mM L-glutamine, supplemented with 10% FBS, 1% Penn/strep and a final concentration of 2ng/ml murine GCSF (Peprotech) and allowed to differentiate for 12 days.

2.3 Cytospin

To prepare cell suspensions for staining, 100µl of cells, resuspended at a concentration of 2 x 10^5 per ml, were loaded into a cytopsin cassette and spun at 100xg for 5 minutes onto a microscope slide using a Shandon cytospin 3 centrifuge.

2.4 Cell Counting

An aliquot of cell preparation was diluted 1 in 10 in trypan blue viability stain (Sigma, Poole, UK) and counted on a haemocytometer (Thermo Fisher Scientific, Loughborough, UK) using an Olympus BX51 microscope (Olympus microscopy, Southend-on-sea, UK). Dead cells take up the dye and were excluded from the counts. The number of viable cells in the centre square of the field of view were counted. The cell concentration was calculated using the following formula.

$$\text{Number of cells in centre square x dilution factor x } 10^4 = \text{number of cells/ml}$$

Peripheral white blood cell counts were performed using a 1 in 10 dilution of heparin treated blood in Turks solution (VWR, Lutterworth, Leicestershire, UK,). Turks solution contains acetic acid which lyses erythrocytes and gentian violet which stains purple the nuclei of leukocytes. The total number of leukocytes was determined using the method above.

2.5 Murine MPO Purification.

MPRO cell pellets were defrosted and centrifuged at 167xg for 10 minutes. The pellets were pooled in buffer A (see section 2.1) containing 100mM PMSF. The cells, while kept on ice, were homogenized using a probe sonicator. The cell lysate was then centrifuged at 20,000xg for 30 minutes using a J2-21MIE ultracentrifuge (Beckman Coulter, High Wycombe, UK) with

a JA-14 rotor. The cell pellet was resuspended in buffer A (with 100mM PMSF) at a ratio of 10ml of buffer per 1ml of cell pellet. Cetyltrimethylammonium bromide (Sigma, Poole, UK) was added to a final concentration of 1% and the mixture was stirred vigorously for 2 hours at 4°C. The solution was then centrifuged at 20,000xg for 20 minutes at 4°C and the pellet was discarded. The sample has a distinct green colour by this stage. The sample was concentrated to approximately 40mls and buffer exchanged into buffer B using a Vivaflow 50 filtration device (Sartorius Stedim, Epsom, Surrey, UK) with a 30kDa molecular weight cut off, and stored at 4°C overnight. $CaCl_2$, $MgCl_2$ and $MnCl_2$ were added then added to a final concentration of 1mM of each.

A 1ml Concanavalin A Sepharose (GE Healthcare, Chalfont St Giles, UK) column was equilibrated with 50mls of 0.2µm filter sterilized buffer B (see section 2.1) containing 1mM of each of the ions $CaCl_2$, $MgCl_2$ and $MnCl_2$ at a flow rate of 60mls/hr. The sample was diluted up to 50mls in buffer B, containing ions, filter sterilized, and run through the column at 12mls/hr. The column was then washed with 7mls of buffer B containing ions at 12mls/hr. The MPO was eluted using elution buffer (750mM mannopyranoside (Sigma, Poole, UK) diluted in buffer B with ions) at 60mls/hr and 1ml fractions were collected. The absorbance was measured at 280nm and 430nm using a spectrophotometer (Beckman Coulter, DU-530). MPO containing fractions have a distinct green colour. The pooled aliquots containing MPO, as determined by absorbance measurements at 430nm, were dialysed against PBS at 4°C (dialysis tubing was prepare as described in section 2.1). After 4 hours the PBS was changed and the sample was left to dialyse overnight at 4°C.

At this stage of the procedure, a 40 litre culture of cells will yield approximately 10mg of MPO (RZ approx 0.3). The purity of the MPO preparation in relation to total protein was determined by its rheinheitzahl (RZ) value, ie., the ratio of 430nm to 280nm absorbance (RZ = A430/A280) [312]. An RZ>0.84 is considered to be of high purity.

The MPO used in some experiments contained in this thesis was not further purified beyond this stage. It was adjusted to a concentration of 1mg/ml and stored in 100µl aliquots at -20°C. The purity of the MPO used in each experiment is stated in the results sections.

A 1ml HiTrap SP FF ion exchange column (GE Healthcare, Chalfont St Giles, UK) was equilibrated with 10mls of PBS at a flow rate of 60mls/hr. It was then washed with 10mls of 1M NaCl dissolved in PBS at 60mls/hr before being put through a final wash of 10mls PBS at 60mls/hr. The sample was then run through the column at 30mls/hr. The MPO, which is bound to the column, was eluted using stepwise increases of NaCl in PBS. A starting concentration of 0.075M NaCl was used to elute the majority of the MPO. The remainder of the MPO was eluted using 0.1M NaCl. 1 ml fractions were collected and analyzed at 280nm and 430nm using a spectrophotometer. The MPO containing fractions were pooled and buffer exchanged into PBS using PD-10 columns (GE Healthcare, Chalfont St Giles, UK). Each PD-10 column was first calibrated with 50ml PBS. After calibration, 2.5ml of sample was added to each column and the runoff was discarded. 3 ml of PBS was then added and the runoff which contained the MPO was collected. The absorbance was then measured at 280 and 430nm and the concentration calculated using the following information.

Absorbance at 430nm

0.19 = 125µg/ml MPO

The sample was then adjusted to a final concentration of 1mg/ml using Vivaspin 20 ultrafiltration device with 30kDa molecular weight cut off (Sartorius Stedim, Epsom, Surrey, UK). After determination of the RZ value the MPO was stored in 100µl aliquots at -20°C.

At this stage a 40 litre culture of cells will yield approximately 5mg of MPO (RZ approx 0.6).

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were diluted to the appropriate concentration using PBS. For non-reducing gels, 5µl of LDS 4x sample buffer (Life Technologies, Paisley, UK) was added to 15µl of the diluted sample and the mixture was boiled at 70°C for 10 minutes. In the case of reducing gels, the LDS 4x sample buffer contained 2.5% beta-mercaptoethanol and the samples were boiled as for non-reducing gels. A volume of 12µl of SeeBlue Plus 2 prestained standard (Life Technologies, Paisley, UK) was added to the first well of a NuPAGE Novex 4-12% Bis Tris gel (Life Technologies, Paisley, UK). 12µl of protein sample was carefully added to each of the

remaining wells. Both chambers of the Surelock tank (Life Technologies, Paisley, UK) were filled with 1x MES running buffer (Life Technologies, Paisley, UK) and In the case of reducing gels, 500µl of antioxidant (Life Technologies, Paisley, UK) was added to the middle chamber immediately prior to running the gel. The gel was run using a PowerEase500 power supply (Life Technologies, Paisley, UK) at 200V for 35 minutes. The gel was removed from the cassette and washed 3 times in 100ml deionized water for 5 minutes each time on a shaker plate. The gel was then stained with Simply blue (Life Technologies, Paisley, UK) for 1 hour before being washed in 100ml of deionized water for a further hour. The gel was then dried using the DryEase system (Life Technologies, Paisley, UK) by soaking it in drying solution for 5 minutes. It was then placed onto cellophane which had been pre-soaked in drying solution (20 seconds – 2 minutes maximum soaking time). Another cellophane sheet was placed over the gel and it was mounted in the frame provided overnight.

2.7 Western Blot

Murine MPO samples were run on NuPAGE gels, either under reducing or non-reducing conditions as described above. Concentrations of 1µg/ml and 2.5µg/ml MPO were used.

Western Blots were carried out using the iBlot gel transfer device as per the manufacturer's instructions (Life Technologies, Paisley, UK). The iBlot NC anode stack bottom (Life Technologies, Paisley, UK) was placed onto the blotting surface of the iBlot machine. After completion, the SDS-PAGE gel was removed from the cassette and placed directly onto the transfer membrane of the anode stack. iBlot filter paper that had been pre-soaked in deionized water was placed over the gel and air bubbles were removed using the iBlot roller. The iBlot cathode stack top (Life Technologies, Paisley, UK) was placed over the filter paper with the electrode side facing up and aligned to the right edge. Air bubbles were again removed using the iBlot roller. The disposable sponge was placed with the metal contact on the upper right corner of the lid. The lid was closed and the gel was transferred in seven minutes. The completed blot was blocked for 1 hour in 1xTBS tween/5% powdered milk (shaking vigorously) after which it was washed in 1xTBS tween three times for 15 minutes each time whilst being shaken vigorously.

A volume of 15ml of the appropriately diluted (in 1xTBS tween/5% powdered milk) primary antibody was added to the blot and incubated at 4°C overnight (shaking gently). The blot was then washed as before. A horseradish peroxidase (HRP) secondary antibody was diluted appropriately (in 1xTBS tween/5% powdered milk) and 15ml was added to the blot for 2 hours at room temperature (with gentle shaking). The blot was washed as before. The HRP substrate was prepared by mixing equal volumes of Supersignal west pico chemiluminescent substrates (Thermo Fisher Scientific, Loughborough, UK) (7.5ml + 7.5ml) and was added to the blot (shaking gently) for 5 minutes. The blot was then put into a polythene pocket and secured into a Kodak BioMax cassette (Anachem, Bedfordshire, UK). In the dark room the Kodak BioMax Light film (Anachem, Bedfordshire, UK) was placed inside and the cassette was closed for the appropriate exposure time. The film was then developed.

2.8 Mice

2.8.1 Mouse strains

MPO-deficient mice [313] were purchased from Jackson Laboratories (Bar Arbour, Maine, USA) and bred in house.

Thy1.1 mice [314] were obtained from Jackson Laboratories (Bar Arbour, Maine, USA) and bred in house.

Properdin-deficient mice were obtained from Dr. Cordula Stover at the University of Leicester, UK.

MASP2-deficient mice [315] were initially obtained from Professor Wilhelm Schwaeble at the University of Leicester, UK.

C57BL/6 mice were purchased from Harlan (Bicester, Oxon, UK).

All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

2.8.2 DNA isolation

MPO-deficient and properdin deficient mice were genotyped by PCR using DNA acquired from ear clippings. The clippings were incubated overnight at 55°C in 375µl of DNA extraction buffer (50mM Tris-Hcl, 100mM EDTA, 100mM NaCl and 1% SDS, pH 8) with 20mg/ml proteinase K (Sigma, Poole, UK). The degraded ear tissue was then vortexed thoroughly and 125µl of saturated NaCl was added. The solution was again mixed thoroughly and centrifuged at 9000xg for 10 minutes. 375µl of this solution (taken from the top without disturbing the pellet) was placed into an empty eppendorf and 250µl of isopropanol was added. The DNA was precipitated out of solution by inverting the tube. It was then centrifuged at 9000xg for 4 minutes using a tabletop microcentrifuge and the supernatant was discarded. The pellet was then washed by adding 750µl of 70% ethanol. Then ethanol was discarded and the pellet allowed to air dry. Finally it was resuspended in 150µl of TE buffer (10mM Tris, 10mM EDTA, pH8) and stored at -20°C.

2.8.3 Genotyping of mice

The genotype of the MPO-deficient and properdin-deficient mice was confirmed by PCR analysis of DNA extracted from ear clippings.

Primers for PCRs were as follows (sense, antisense)

The following sequences were obtained from the Jackson Laboratory

MPO wild type: 5'- CTG GAC GCC AGG AGT CAA TCG - 3', 5' – GTG AAG AAG GAG AAG CGG GTA G – 3'

MPO deficient: 5' – TCC TCG TGC TTT ACG GTA TCG – 3', 5' – GTG AAG AAG GAG AAG CGG GTA G – 3'

The following sequences were obtained from Dr. Cordula Stover at the University of Leicester, UK.

Properdin wild type: 5' – GGA TTA TCA CAT ACT CGT TGA CGG – 3', 5' – CTC TTG AGT GGC AGC TAC AG – 3'

Properdin deficient: 5' – CAT CTG CAC GAG ACT AGT GAG A – 3', 5' – CAA TGG TTG AAG GTC AGG GTG – 3'

Primers were diluted in sterile water to a concentration of 2µM. For each PCR reaction 1µl of template DNA was added to 11.5µl of primers and 12.5µl of HotStarTaq (Qiagen, Crawley, West Sussex, UK) to give a final volume of 25µl and a final primer concentration of 1µM. PCR reactions were run on a PE Apllied Biosystems GeneAmp PCR System 9700 under the following conditions.

MPO reaction conditions: 94°C for 15 minutes, then 35 cycles at 55°C for 30 seconds, 72°C for 1 minute, and then 72°C for 10 minutes
Properdin reaction conditions: 94°C for 15 minutes, then 30 cycles at 56°C for 30 seconds, 72°C for 1 minute, and then 72°C for 10 minutes.

2.9 Construction of bone marrow chimeras

Donor BM cells were isolated from the tibia and femurs of mice. The muscle and tissue was stripped away and the bones were flushed with PBS using a 27G needle. Clumps of cells were dispersed by repeated aspiration and expulsion from a syringe using a 21G needle. The cell suspension was then centrifuged as 150xg for 10 minutes and the pellet resuspended in 1 ml PBS. The total number of viable cells was counted using trypan blue viability stain (Sigma, Poole, UK) and a haemocytometer. The cells were then resuspended at a final concentration of 25 x 10^6 cells/ml. Recipient mice were lethally irradiated with 9 Gy in a single dose using a caesium source irradiator. They were then injected with 200µl of the cell suspension (5x10^6 cells) I.V immediately after irradiation.

2.10 IgG Purification

IgG was purified from a pooled mouse serum by ammonium sulphate precipitation and protein G chromatography. An equal volume of saturated ammonium sulphate was added to the serum sample in a dropwise fashion while gently shaking the tube, to a final concentration of 50%. The sample was left at 4°C overnight for the antibody to precipitate out of solution. The sample was centrifuged at 1500xg for 20 minutes and the pellet resuspended in PBS and aspirated until completely dissolved. The sample was then dialysed against PBS overnight, with the PBS being replaced after 4 hours.

The dialysed sample was diluted 1 in 3 in PBS in preparation for protein G chromatography. A 5ml HiTrap protein G column (GE Healthcare, Chalfont St Giles, UK) was equilibrated with 50ml of PBS at a flow rate of 60ml/hr. The sample was then filter sterilized through a 0.2µm filter (Sartorius Stedim, Epsom, Surrey, UK) and run through the column at a flow rate of 10ml/hr. The column was washed with 40ml of PBS at 60ml/hr. The IgG was eluted using 0.1M glycine-HCl solution, pH 2.7, into eppendorfs containing 150µl of Tris pH 9. 1.5ml fractions were collected. The absorbance at 280nm of each fraction was measured using a spectrophotometer (Beckmann Coulter DU-530). Selected fractions were pooled and buffer exchanged into PBS using PD-10 desalting columns (GE Healthcare, Chalfont St Giles, UK). The absorbance at 280nm of the pooled sample was determined using the following information.

Absorbance at 280nm

1.4 = 1mg/ml

The concentration of the antibody was adjusted to 10mg/ml using a Vivaspin 20 ultrafiltration device with 30kDa molecular weight cut off (Sartorius Stedim, Epsom, Surrey, UK). The antibody was stored in 1 ml aliquots at -20°C.

2.11 Induction of anti-MPO crescentic glomerulonephritis in mice

2.11.1 Preparation of MPO for immunisation

MPO for immunization was prepared by mixing 50µl of MPO (1mg/ml) with 450µl PBS and 500µl of complete freund's adjuvant (CFA) (Sigma, Poole UK) for first immunisation or 500µl incomplete freund's adjuvant (iCFA) (Sigma, Poole, UK) for subsequent boosts. The mixture was kept on ice and homogenized using a T-10 ultra-turrax homogenizer (Sigma, Poole, UK). The extent of homogenization was tested by aspirating a small drop of the solution onto water. If the sample remained intact and did not disperse in the water it was deemed ready for injection.

2.11.2 Bone marrow transplant model

MPO-deficient mice were immunized via S.C injection with 10µg murine MPO in CFA on day 0. They were boosted at days 21 and 35 by I.P injection of 10µg MPO in iCFA. On day 41 the

mice were bled from the saphenous vein and anti-MPO titres were measured by ELISA (as described in section 2.12.1). On day 42 the mice were lethally irradiated with 9 Gy in a single dose and immediately injected I.V with $5x10^6$ BM cells from wild type C57BL/6 donor mice. They were then bled from the saphenous vein at two week intervals for anti-MPO titre measurement. The mice were sacrificed 8 weeks post BM transplant and their kidneys were analysed for disease as described in sections 2.15 and 2.16.

2.11.3 Passive transfer model

MPO-deficient mice approximately 6-8 weeks old were immunized with 10µg (200µl of MPO preparation) of murine MPO (purity stated in results sections) via S.C injection on day 0 in CFA (Sigma, Poole, UK). They received approximately 100µl of this solution on either side of the flank. On days 21 and 35 they were boosted with 10µg MPO by I.P injection in iCFA. On day 42 the mice were exsanguinated and the serum was pooled. Anti-MPO titres were measured by ELISA (as described in section 2.12.1).

This anti-MPO serum, or purified IgG, was injected I.V directly into wild type C57BL/6 mice on day 0. They also received 10µg of LPS (Enzo Life Sciences, Exeter, UK) (200µl of 50µg/ml LPS) via I.P injection after 1 hour on day 0 and on day 3. The mice were placed in metabolic cages overnight on day 6 and sacrificed on day 7.

The effectiveness of the serum pool to induce disease in mice was tested by experiment using small groups of mice. In later experiments involving mice with congenital complement deficiencies the anti-MPO IgG was purified by ammonium sulphate precipitation and protein G chromatography and this is stated in the relevant results sections.

2.11.4 Splenocyte transfer model

MPO-deficient mice were immunized S.C with 10µg murine MPO in CFA on day 0. They were boosted at days 21 and 35 by I.P injection of 10µg MPO in iCFA. The mice were bled from the sapenous vein at day 40 and anti-MPO titres were measured by ELISA. The mice were sacrificed on day 42 and the spleens were harvested. The spleens were placed in a 70µm filter and mashed using a 10ml syringe plunger into a 50ml tube containing 10ml PBS. The cells were centrifuged at 280xg for 5 minutes. The supernatant was discarded and the pellet

was resuspended in 1ml red cell lysis buffer for 5 minutes on ice. 15ml of PBS was then added and the cells were centrifuged at 280xg for 5 minutes. The cells were then resuspended in PBS at a concentration of 25 x 10^7 cells/ml.

Recipient mice were sublethally irradiated with 6.5 Gy using a caesium source irradiator and immediately injected I.V with 200µl (5 x 10^7 ThY1.2 cells) of anti-MPO spleen cells. On day 77 the mice were bled from the saphenous vein into heparin coated tubes. In order to assess the level of chimerism the blood was stained with ThY1.2-FITC (Beckton Dickinson, Oxford, UK) and CD3-PE (Becton Dickinson, Oxford, UK) antibodies (see table 2.1). Neutrophils were also stained using a Ly6G-Alexafluor700 (Beckton Dickinson) antibody. On day 78 the mice were boosted by I.P injection of 10µg MPO in iCFA. On day 91 the mice were placed in metabolic cages overnight to collect urine and were sacrificed on day 92. Kidneys were processed and analysed for disease as described in section 2.15 and 2.16.

2.11.5 Transplacental transfer model

Female MPO-deficient mice of approximately 8-10 weeks old were immunised with 10µg MPO S.C in CFA on day 0. They were subsequently boosted with a further 10µg MPO by S.C injection on days 21 and 35. These mice were not boosted by I.P injection as they would be carrying litters shortly thereafter. The female mice were bled for serum on day 42 in order to measure anti-MPO titres and mated with wildtype males. Pups of weaning age were placed in metabolic cages overnight to collect urine and sacrificed the following day. Serum samples were taken for anti-MPO titre analysis and kidney samples were processed and analysed for disease by periodic acid Schiff staining.

2.12 ELISA

2.12.1 Murine anti-MPO ELISA

All anti-MPO antibody titres were determined by ELISA. 96 well maxisorb ELISA plates (SLS, Hessle, Yorkshire, UK) were coated with 50µl per well of 10µg/ml murine MPO (RZ=0.62), which was diluted in 500mM carbonate-bicarbonate buffer pH 9.5. The plate was coated

overnight at 4°C and then washed four times using PBS/0.05% Tween20. 100µl of blocking solution (1% BSA in PBS) was added to each well for 1 hour at room temperature. The plate was washed four times using PBS/0.05% Tween20. A standard curve was produced from an anti-MPO positive serum pool. Serum samples were diluted 1 in 12,800 in 1% BSA (Sigma, Poole, UK). 50µl of samples and standard were added in duplicate to the appropriate wells and incubated for 1 hour at room temperature. The plate was then washed as before. 50µl of diluted (1 in 500 in 1% BSA) alkaline phosphate conjugated goat anti-mouse IgG (Southern biotechnology, Cambridge, UK) was added to each well for 1 hour at room temperature. The plate was then washed as before. The substrate for the alkaline phosphate reaction, p-nitrophenyl phosphate (PNPP) (Sigma, Poole, UK) was prepared by dissolving 1 of each of two tablets in 5ml of distilled water. 50µl of PNPP solution was added to each well and allowed to develop in the dark for approximately 1 hour before being analyzed at 405nm using a SpectraMax Plus384 microplate reader (Molecular Devices, Wokingham, UK).

2.12.2 Murine albuminuria measurement

Murine albuminuria was measured using a commercially available ELISA kit (Bethyl laboratories, Montgomery, TX, USA). ELISA plates were coated with 100µl/well of a 1 in 100 dilution of capture antibody diluted in 500mM bicarbonate buffer pH 9.5. Plates were coated for 1 hour at 37°C and then washed four times using PBS/0.05% Tween20. Plates were blocked using 100µl/well 1% BSA for 30 minutes. A standard curve ranging from 1000 – 7.8ng/ml was prepared from a murine albumin stock rather than the albumin standard that was part of the kit. This albumin was purchased from Sigma and stored -20°c in aliquots at 2.8mg/ml. Urines were diluted 1 in 200 in 1% BSA. After blocking for 30 minutes, 100µl of each standard and sample were added in duplicate to the plate for 1 hour at room temperature. The plate was then washed four times in PBS/0.05% Tween20. The HRP conjugated detection antibody was diluted 1 in 50,000 in 1% BSA and 100µl/well was added to the plate for 1 hour in the dark. The plate was then washed as before. The HRP substrate, O-phenylenediamine dihydrochloride (OPD) (Sigma, Poole, UK) was prepared by dissolving each of two tablets in 20mls of deionized water. 100µl of OPD solution was added to each well and was allowed to develop in the dark for approximately 20 minutes. The reaction was then stopped by the addition of 100µl 1M H_2SO_4. The reaction was then analysed at 490nm

using a SpectraMax Plus384 microplate reader (Molecular Devices). The total albumin content of the urine was calculated from albumin concentration and the urine volume.

2.13 Serum and urine creatinine measurements

Serum and urine creatinine concentrations were measured by Dr N Dalton and Dr C Turner by electrospray mass spectrometry in the Department of Paediatric Biochemistry at the Evelina Children's Hospital, London. A volume of 5µl of neat serum was diluted into 250µl of D3 creatinine isotope and 250µl of acetonitrile containing 0.05% formic acid. Urine samples were diluted 1 in 10 in PBS before dilution in creatinine isotope and acetonitrile. Creatinine concentrations were measured using Applied Biosystems MDS Sciex API 4000 (Applied Biosystems, Warrington, UK) and Applied Biosystems Analysis Version 1.4 software.

Urine albumin : creatinine ratios (ACR) were calculated by dividing the albumin concentration (in mg/ml) by the urine creatinine concentration (in mg/ml). The resulting ratio was multiplied by 1,000 in order to express the ratio in milligrams of albumin per gram of creatinine excreted.

2.14 Haematuria

Haematuria was measured by dipstick (Combur-Test® from Roche, Sussex, UK). Severity was measured on a scale ranging from 0 (none) to 4 (severe).

2.15 Histological sample processing

2.15.1 Paraffin sections

Mouse kidneys were immediately removed from the animal and cut into halves along the longitudinal axis. The kidney sections were prepared for light microscopy by fixation in Bouin's solution (prepared as described in section 2.1) for 4 hours before being transferred to formal saline. The kidneys were then processed overnight using Leica ASP 300 S processor and embedded in hot wax.

2.15.2 Phosphate-lysine-periodate fixation

Phosphate-lysine-periodate (PLP) solution was prepared as described in section 2.1. Kidney samples were fixed in PLP at 4°C for 4 hours before being transferred to 13% sucrose overnight (prepared as described in section 2.1). The samples were then frozen in pre-cooled isopentane and fixed onto cork boards using OCT (Thermo Fisher Scientific, Loughborough, UK) and stored at -80°C.

2.15.3 Snap frozen

Kidney samples were placed onto cork boards using OCT and immediately frozen in pre-cooled isopentane. The samples were then stored at -80°C. Kidney sections of 5µm thickness were cut and placed onto microscope slides. The sections were allowed to air dry at room temperature overnight. For prolonged storage the slides were stored in airtight bags containing silica crystals (VWR, Lutterworth, Leicestershire, UK) to prevent moisture build up and stored at -80°C.

2.16 Histological staining

2.16.1 Periodic acid Schiff stain and Mayer's Haematoxylin

Paraffin embedded sections of approximately 1µm thickness were cut using a microtome and placed onto microscope slides. The sections were baked onto the slides at 60°C for a minimum of 30 minutes before staining. The slides were placed in xylene for 10 minutes in order to dissolve the wax. They were then rehydrated by placing them in 100%, 90% and 70% alcohol for 10 seconds each. They were then washed in running distilled water for 3 minutes before being put into 1% periodic acid (Sigma, Poole, UK) (prepared as described in section 2.1) for 10 minutes. They were again washed for 3 minutes in running distilled water. Using a pasteur pipette each slide was then covered in Schiff's reagent (Thermo Fisher Scientific, Loughborough, UK) for 20 minutes in the fume hood and then again washed for 3 minutes. The slides were then treated with mayer's hematoxylin (VWR, Lutterworth, Leicestershire, UK) for 5 minutes and then washed for a further 5 minutes. They were then dehydrated by placing them in 70%, 90% and 100% ethanol for 10 seconds each. They were then mounted in DPX (Thermo Fisher Scientific, Loughborough, UK).

2.16.2 Immunofluorescent staining for glomerular macrophages

Sections of frozen kidney (PLP fixed) were cut to 5µm thickness using the cryostat and placed onto multispot slides (C.A Hendley, Essex, UK) The slides were allowed to air dry overnight at room temperature. A 1 in 200 dilution (in PBS) of a 1mg/ml stock rat anti-mouse CD68 antibody, clone FA11 (Serotec, Abingdon, Oxon, UK) (see table 2.2) was prepared. 100µl of this staining solution was added to each kidney section and incubate in the dark in a humidity tray for 2 hours. The slides were then washed in PBS for 5 minutes. 100µl of a 1 in 200 dilution (in PBS) of a 1.5mg/ml stock of a DyLight488 mouse-anti rat IgG (Jackson Immunoresearch, Westgrove, PA, USA) was added to each section and incubated for 2 hours in the humidity tray. The slides were washed as before. The slides were then mounted using permafluor (Thermo Fisher Scientific, Loughborough, UK) and scored within 3 days.

2.16.3 Immunofluorescent staining for glomerular IgG deposition

Sections of snap frozen kidneys were cut as described above. They were then fixed in acetone for 5 minutes and left on the bench to air dry. A FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch) (see table 2.2) was diluted 1 in 100 in PBS. 100µl of this staining solution was added to each section and incubated in the dark in a humidity tray for 1 hour. The slides were then washed in PBS for 5 minutes and mounted in permafluor (Thermo Fisher Scientific, Loughborough, UK). Slides were analyzed within 3 days.

2.16.4 Peroxidase Staining

MPRO, WEHI-3b and CD32Cl3 cells were stained for peroxidase activity using chloronaphthol (Thermo Scientific, Hemel Hempstead, UK), a peroxidase substrate used for chromogenic detection of peroxidases. A stock solution (3mg/ml) of chloronaphthol was prepared by dissolving one tablet in 10ml of ethanol. A working solution was prepared by adding 1ml of stock to 10ml PBS. Immediately before use 0.1ml of 3% hydrogen peroxide (Sigma, Poole, UK) was added. Cells were prepared for peroxidase staining by cytospining 100µl of a $2x10^5$ cells/ml cell suspension at 100xg for 5 minutes using a Shandon Cytospin 3. The cells were then fixed in leucognost (VWR, Lutterworth, Leicestershire, UK) for 1 minute and washed in running distilled water for 10 seconds. They were then immersed in chloronaphthol for 10 minutes and again washed for 10 seconds. Finally they were

immersed in mayer's haematoxylin (VWR, Lutterworth, Leicestershire, UK) for 2 minutes and washed. The slides were allowed to air dry and mounted in permafluor (Thermo Fisher Scientific, Loughborough, UK).

2.17 Scoring of histological sections

2.17.1 Glomerular crescent scores

The number of glomerular crescents was scored on paraffin embedded kidney sections stained with periodic acid Schiff stain and mayer's haematoxylin. Glomerular crescents were defined as having two or more layers of cell in Bowman's space. The number of crescents identified was expressed as a percentage of a total of 100 glomeruli per animal.

2.17.2 Glomerular macrophage scores

Glomerular macrophages were scored on PLP fixed kidney tissue stained with a rat anti-mouse CD68 antibody and a fluorescent secondary, as described above. The extent of glomerular macrophage staining was determined using one of two methods. The first method was used when glomerular macrophage infiltration was mild and involved counting individual macrophages in the glomeruli. A mean number was calculated from a minimum of 20 glomeruli per section. The second method was used when glomerular macrophage infiltration was more extensive. This involved using ImageJ software [316] to determine the total area of each glomerulus. The area of each glomerulus which stained positive for macrophages was then expressed as a percentage of the total area.

2.18 Human GCSF electro-chemiluminescence immunoassay

The human GCSF ultra-sensitive assay was purchased from Meso Scale Discovery (Meso-Scale Discovery, Gaithersburg, Maryland USA). The provided 96 well plate was pre-coated with an anti-human GCSF capture antibody. Prior to use, all reagents were brought to room temperature. 25µl of diluent 2 was added to each well and the plate was shaken vigorously for 30 minutes at room temperature. A standard curve ranging from 2500 – 0 pg/ml was prepared using a 1 in 4 dilution series. 25µl of standard or samples (neat serum) was added directly to each well and the plate was incubated at room temperature, with vigorous shaking for 2 hours. The plate was washed using PBS/Tween20. The SULFO-TAG™ detection

antibody was diluted 1 in 60 in diluent 3 and 25µl was added to each well. The plate was incubated for 2 hours in the dark at room temperature with vigorous shaking. A 1 in 2 dilution of read buffer was prepared in deionized water. The plate was washed as before and 150µl of diluted read buffer was added to each well. The plate was read immediately using MSD SECTOR Imager (Meso-Scale Discovery, Gaithersburg, Maryland USA) which had been pre-cooled to -37°C.

2.19 Human neutrophil isolation

Heparin treated venous blood from both patients and healthy controls, was diluted 1:1 in Hanks buffered salt solution (HBSS). A total of 8ml of this was carefully layered on top of 4ml Ficoll-Paque™ PLUS (GE Healthcare, Chalfont St Giles, UK) in a 15ml tube. This was centrifuged at 380xg at 20°C with no brake. The upper layer of cells, which contains lymphocytes was aspirated using a Pasteur pipette and discarded. The pellet was loosened by gentle tapping and 10ml of red cell lysis buffer was added for 5 minutes on ice with gentle shaking. This was then centrifuged at 380xg for 5 minutes at 20°C. The supernatant was aspirated and the pellet resuspended in 10ml red cell lysis buffer for a further 5 minutes on ice. This was centrifuged at 380xg for minutes at 20°C. The supernatant was discarded and the cells were washed in 20ml of ice cold HBSS and centrifuged at 200xg for 5 minutes. The cells were resuspended in HBH (500ml Hanks balanced salt solution + 5ml 1M Hepes) and counted using Turks solution. The neutrophils were resuspended at a concentration of 2.5 x 10^{6} cells/ml.

2.20 Flow Cytometry

2.20.1 Staining and analysis of peripheral blood neutrophils

A volume of 100µl of heparin treated whole blood was added to each tube. 25µl of a 1 in 125 pre-diluted Fc block (Becton Dickinson, Oxford, UK) was added for 20 minutes at room temperature. The appropriate antibodies (see table 2.1) were then added and the blood was incubated at room temperature for 20 minutes in the dark. Erythroyctes were lysed using 2mls of a 1 in 10 dilution BD FACs Lysing solution (Becton Dickinson, Oxford, UK) in deionized water. The blood was then centrifuged at 280xg for 5 minutes. The supernatant was discarded and the cells resuspended in 2mls of PBS and centrifuged at 280xg for 5

minutes. Finally the cells were resuspended in 500µl of PBS with 0.1% sodium azide. FACs analysis was carried out immediately after staining using a FACS Canto flow cytometer (Becton Dickinson, Oxford, UK) and FACsDIVA software (Becton Dickinson, Oxford, UK). Neutrophils were identified based on their forward and side scatter profiles. A miniumum of 10,000 events were collected for each sample and data was anlaysed using FlowJo software (Treestar, Ashland, OR, USA). Total circulating neutrophil counts were determined from the percentage of Ly6G positive cells and total leukocyte numbers.

2.20.2 Dihydrorhodamine 123 assay of neutrophil respiratory burst in response to GCSF priming

Neutrophil respiratory burst was measured using the dihydrorhodamine (DHR) 123 assay. DHR is a cell permeable non-fluorescent probe that is oxidised in the presence of H_2O_2, which is produced during the respiratory burst, to the fluorescent molecule rhodamine 123 which fluoresces at approximately 534nm and can be detected in the FITC channel using flow cytometry. Neutrophils were isolated from the venous blood of healthy volunteers as described in section 2.19. They were resuspended in HBH at a concentration of 2.5×10^6 cells/ml. The cells were then loaded with 17µg/ml DHR 123 (Calbiochem, Nottingham, UK), 5µg/ml cytochalasin B and 2mM sodium azide (Sigma, Poole, UK) for 10 minutes at 37°C in the dark.

Cells were primed with 50ng/ml GCSF (Peprotech, London UK), or 2ng/ml TNFα (Peprotech, London, UK) for 15 minutes at 37°C in the dark with gentle mixing of the cells at 5 minute intervals. Before stimulation of the cells, all antibodies were centrifuged at 16,000xg at 4°C for 15 minutes in order to remove antibody aggregates which may give a false positive result. 5×10^5 cells (200µl of the cell suspension) were added to each FACs tube and stimulated with 5µg/ml of either of the monoclonal antibodies anti-PR3 clone WGM2 (Hycult, Biotech, Uden, Netherlands), anti-MPO clone 266.6K2 (IQ products, Groningen, Netherlands) or 2.5µg/ml of monoclonal istoype control IgG1 clone 107.3 (Becton Dickinson, Oxford, UK) (see table 2.1). Cells were stimulated for 60 minutes at 37°C in the dark. As a control, neutrophils were also stimulated with 1.25 or 5µg/ml fMLP (Sigma, Poole, UK) for 10 minutes. The reaction was stopped by the addition of a 30-fold volume of ice cold HBSS containing 1% BSA. Cells were centrifuged at 150xg at 4°C for 5 minutes and resuspended in

300µl of HBSS. Cells were kept on ice in the dark for immediate analysis. Samples were analysed by flow cytometry using a FACs Canto (Becton Dickinson, Oxford, UK) with FACsDiva software (Becton Dickinson, Oxford, UK). A minimum of 10,000 events were collected per sample and data was analysed using FlowJo software.

2.21 Statistics

Data was analysed using Graphpad Prism software (Graphpad Software Inc, La Jolla, CA, USA). The specific tests used in each experiment are stated in the results sections. If two groups of independent variables, with an assumed normal distribution were compared, an umpaired t test was used. When three or more groups were compared, a one-way ANOVA was used, followed by a Tukey's post test to compare significance between the groups. When data was compared in the same group both before and after treatment, a paired t test was used. If variances were significantly different between the groups being compared, analysis was performed after a logarithmic transformation of the data. Significance levels are represented as follows *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Chapter 3

GCSF concentration in acute ANCA-vasculitis and its role in neutrophil priming

3.1 Introduction

As stated in section 1.5 GCSF is an important regulator of haematopoiesis and is involved in the mobilisation and maturation of granulocytes. It also exerts a range of complex immunomodulatory effects on cells of both the innate and adaptive immune system. In the context of haematopoietic stem cell transplantation GCSF treatment of donors leads to a skewing of T cell responses toward T_H2 and Treg, and thus an anti-inflammatory phenotype [199-202]. It may also indirectly induce the maturation and proliferation of B cells by inducing the production and secretion of BLyS from neutrophils [213]. However, as stated in section 1.5.2, it is also known to exacerbate some autoimmune diseases. Its levels are known to be elevated in the serum of RA patients, and its administration has been known to exacerbate disease [191, 193]. This has been corroborated in animal studies using both rats and mice [194, 195]. Furthermore, endogenously produced GCSF was shown to be crucial in animal model of inflammatory arthritis, as GCSF deficient mice, and mice in which endogenous GCSF was blocked, were profoundly resistant to disease induction [196]. It was recently reported using a murine model of ANCA-associated vasculitis that anti-MPO immunity is predominantly a T_H17 associated phenomena and that experimental glomerulonephritis was markedly attenuated in IL-17A deficient mice [139]. It is also known that GPA patients have higher proportions of circulating T_H17 cells than healthy controls [60]. In view of these exacerbatory effects and given that IL-17 is an important stimulator of GCSF production, it is likely that GCSF concentrations would be elevated in patients with active ANCA-vasculitis. It was therefore decided to measure the serum concentrations of GCSF in a group of patients with active disease and compare it to those of age matched healthy controls.

There are various studies showing a role for GCSF in the enhancement of neutrophil effector functions such as superoxide production and degranulation and its ability to prime for

oxidative burst [182-185]. We considered whether GCSF could prime isolated human neutrophils for respiratory burst in response to a monoclonal anti-MPO or anti-PR3 antibody. This would demonstrate a proinflammatory effect of GCSF in ANCA vasculitis and could be a potential future therapeutic target.

Aims

- To measure the concentrations of GCSF in the serum of ANCA vasculitis patients with active disease and compare them to those of an age matched healthy control group.

- To show a priming effect for GCSF on isolated human neutrohphils in response to anti-MPO and anti-PR3 antibody stimulation.

3.2 Methods

3.2.1 Measurement of GCSF serum concentrations

Both patient and control serum samples were stored at -80°C until analysis. GCSF concentrations were determined by ultrasensitive electrochemiluminescence assay as described in section 2.18. All blood samples were taken with informed consent and ethical approval (NRES committee London – London Bridge 09/H084/72). Patient samples were collected from Kent and Canterbury, Royal Sussex County, King's College, St Helier and Guy's and St Thomas' hospitals. Control samples were provided by Dr Deborah Dunn Walters which were collected as part of a study investigating immune responses in older people.

3.2.2 Human neutrophil respiratory burst assays

Human neutrophils were isolated from the venous blood of healthy volunteers by ficoll separation as described in section 2.19. Isolated neutrophils (2.5 x 10^6 cells/ml) were loaded with 17µg/ml DHR 123, treated with 5µg/ml cytochalasin B and 2mM sodium azide for 10 minutes at 37°C and subsequently primed with 50ng/ml GCSF, or 2ng/ml TNFα for 15 minutes at 37°C, as described in section 2.20.2. The cells were then stimulated with a monoclonal anti-MPO or anti-PR3 antibody (clone details in section 2.20.2). Cells were also

stimulated with 2.5µg/ml of an isotype control IgG1 antibody to control for any non-specific rhodamine-123 production. The stimulated cells were then analysed by flow cytometry as described in section 2.20.2. Because not all donors were analysed in a single experiment, it was necessary to standardise the results in order to accurately compare data points. This was done by subtracting the median fluorescence intensity (MFI) of unprimed/GCSF primed isotype control stimulated cells from the MFI of unprimed/GCSF primed anti-MPO/anti-PR3 stimulated cells. This served to compensate for non-specific DHR 123 production.

3.2.3 Statistics

Statistical analysis was performed using Graphpad Prism software (Graphpad software Inc, La Jolla, CA, USA). Data was analysed using unpaired or paired t tests as stated in the appropriate figures. Data analysis to determine correlations were done using Pearsons correlation tests. Analysis of GCSF concentration versus treatment status was carried out using a one-way ANOVA and a Tukey's multiple comparison post test.

3.3 Results:

3.3.1 GCSF levels are elevated in patients with active disease

We measured serum levels of GCSF in 38 patients with active disease and compared this to levels in 31 age matched healthy controls. In line with the reports of elevated GCSF levels in the serum of RA patients we found that serum GCSF levels of patients with active ANCA vasculitis were significantly elevated when compared to age matched controls, with mean levels of 38.04 and 18.3pg/ml respectively, ($p<0.001$) as shown in figure 3.1. The patient group consisted of 23 males and 15 females with a mean age of 66.6 years. All patients had renal disease with clinical details as shown in table 3.1 below. As most ANCA vasculitis patients are over 60 years old we thought it necessary to age match the controls to rule out the possibility of an age related difference between the groups. The control group consisted of 18 females, 12 males and one of unknown sex with a mean age of 69.2 years (details in table 3.2). These samples were kindly provided by Dr. Deborah Dunn-Walters from a study of the immune response to vaccines in older people. There was a significant correlation between GCSF concentration and age, with younger subjects having higher levels in both the control group ($P<0.05$) and the patient group ($P<0.05$). There was no correlation

between GCSF concentration and treatment status. Patients who received no treatment had GCSF levels that did not significantly differ from those who received either high doses or prolonged low doses of oral prednisolone ($P>0.05$), or those who received short treatments of methylprednisolone ($P>0.05$).

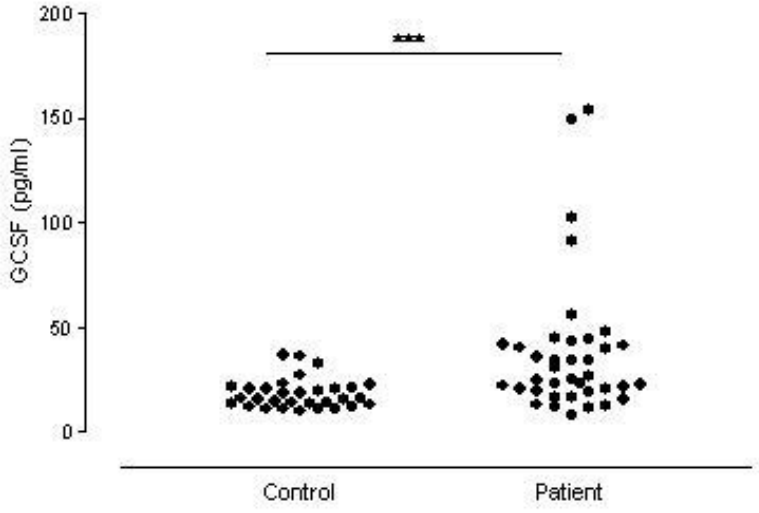


Figure 3.1 Serum concentrations of GCSF in patients with active disease (mean age 66.6 years) compared to those of age matched healthy controls (mean age 69.2 years). Levels were measured using an ultra sensitive electrochemiluminescence assay. There was a significantly raised serum concentration of GCSF in the patient group (P<0.001). Data was analysed using an unpaired t test.

Age	Sex	ANCA	ANCA IIF pattern	renal BVAS	Total BVAS	renal biopsy	features besides kidneys	Treatment at time of sample	GCSF concentration (pg/ml)
59	m	PR3	C	12	27	yes	ent/chest/eyes	mtx	41.8
72	f	PR3	C	12	33	yes	ent/chest/eyes/ns	1d HDOP	22.2
73	f	PR3	C	12	21	yes	ent	Nil	21.6
57	m	PR3	C	12	15	yes	no	MP 2d	20.6
62	m	MPO	P	12	19	yes	ent	sz, HCq	19.6
75	m	PR3	C	12	19	yes	skin	Nil	34.2
74	m	MPO	C	12	12	yes	no	MP 2d	56.2
66	m	MPO	P	12	18	no	chest	Nil	19.2
68	f	MPO	P	12	15	yes	no	1d HDOP +CyP	91.6
82	f	MPO	P	12	12	yes	no	Nil	16.8
82	m	MPO	P	17	12	yes	ent	Nil	19.8
68	f	DP	P	12	22	yes	ent/chest	MP 3d	15
58	f	MPO	P	12	15	yes	no	MP 1d	154
63	f	MPO	P	5	11	yes	skin/eyes/ent	MP 1d HDOP 1d	48.2
72	f	MPO	P	12	18	no	chest	MP 1d	44.8
88	f	PR3	C	12	15	yes	no	Nil	23.2
55	f	MPO	P	12	15	yes	no	Nil	26.8
77	f	MPO	P	12	18	yes	ent/chest	LDOP>6 mths	43.2
67	f	PR3	P	12	24	yes	ent/eyes	Nil	25
38	m	MPO	atypical	6	10	yes	eyes/skin	Nil	12.8
77	f*	PR3	C	5	11	yes *	chest	LDOP>6 mths	13.4
82	f	MPO	positive	12	15	yes	no	LDOP>6 months	8
63	m	MPO	P	12	25	No	skin/eyes/ent	Nil	34.6
86	m	PR3	C	12	21	yes	ent	Nil	36
76	f	PR3	C	12	27	yes	ent/chest	MP 1d	34.6
88	f	MPO	P	12	15	yes	no	Nil	23.2
64	m	MPO	P	12	19	yes	ent	HDOP 1d	12
74	f	MPO	P	12	12	yes	no	MP 3d	11.8
51	m	PR3	C	10	14	yes	ent/eyes	Nil	25.4
51	m	PR3	C	12	24	yes	ns	LDOP > 6 mths	40.6
51	m	PR3	C	12	19	yes	skin/eyes	MP 2d	44.6
72	f	MPO	equivocal	12	12	yes	no	Nil	22.8
73	m	PR3	C	12	30	yes	eyes/ent/chest	MP 1d	20.8
23	f	MPO	P	12	12	yes	no	MP 1d	149.2
67	f	MPO	equivocal	12	12	yes	no	Nil	30.6
71	f	PR3	C	12	18	yes	ent	Nil	41.6
34	f*	MPO	P	12	12	yes	no	HDOP 2d LDOP>6 mths	102.6
73	m	MPO	C	12	20	yes**	skin/eyes	Nil	16.8

Table 3.1. Clinical features of patients studied. Samples were obtained from patients in a number of renal units in SE England (see acknowledgements). All patients had active ANCA

vasculitis. All were new presentations with the exception of 2 (denoted f*) who had relapses. All patients had evidence of active renal involvement which was confirmed by renal biopsy in most cases (as indicated. DP= double positive (MPO and PR3). yes* indicates that the patient did not have a renal biopsy on this relapse, but did have one previously at initial presentation. yes** denotes that the patient had a renal biopsy but there was only fibrosis with no evidence of active disease. We have indicated where patients had clinical evidence of other tissue involvement (ent= ear, nose or throat: ns = nervous system). We have also indicated treatment received before the blood sample was taken: (mtx= methotrexate; HDOP = high dose oral prednisalone; LDOP=low dose oral prednisalone; Cyp= cyclophosphamide; MP= methylprednisalone (0.5 or 1g); sz= sulphasalazine; hydroxychloroquine; 1-3d= 1-3 days). There was a significant correlation between GCSF concentrations and age with younger patients having higher levels ($P<0.05$). Data was analysed using a Pearson correlation test.

Age	Sex	GCSF Concentration (pg/ml)
89	f	13.6
87	m	14.2
86	m	16.2
82	f	11.2
81	f	21
80	m	14.6
78	m	10.2
76	m	11.2
76	f	13
74	f	15.6
74	m	19.8
73	f	16.2
73	f	27.4
73	f	21.2
71	f	16.6
71	f	15.6
71	m	21.6
70	m	11
68	f	32.8
66	f	23.4
44	m	13.8
66	unknown	21
65	m	11
46	f	37
41	f	12.2
45	f	19
66	f	14.4
49	m	36.4
43	f	22.8
82	f	12
79	f	20.8

Table 3.2 Control sample data. Control serum samples were obtained as part of a study investigating immune responses in older people. GCSF concentrations were also significantly correlated with age, with younger subjects expressing higher levels ($P<0.05$). Data was analysed using a Pearson correlation test.

3.3.2 GCSF primes isolated human neutrophils for respiratory burst

We assessed the ability of GCSF to prime human neutrophils, isolated from a total of eight donors, for a respiratory burst in response to a monoclonal anti-MPO and anti-PR3 antibody. This was measured using a dihydrorhodamine 123 assay. Figure 3.2A shows data from isolated neutrophils from all eight donors which had been either unprimed, or primed with GCSF or TNFα and stimulated with a monoclonal anti-MPO IgG. Each symbol represents a single donor. Five of the eight donors primed with GCSF showed a significant increase in rhodamine-123 production ($p<0.05$) compared to unprimed cells. As expected, all TNFα primed cells showed increased rhodamine-123 production. Arbitrary units were obtained as described in the methods. A representative histogram is shown in figure 3.2B. An identical experiment was carried out to determine if GCSF could prime isolated neutrophils for a monoclonal anti-PR3 IgG induced respiratory burst. As shown in figure 3.3A only three of the eight donors showed an increase in rhodamine-123 production in response to GCSF priming and anti-PR3 stimulation. A representative histogram is shown in figure 3.3B.

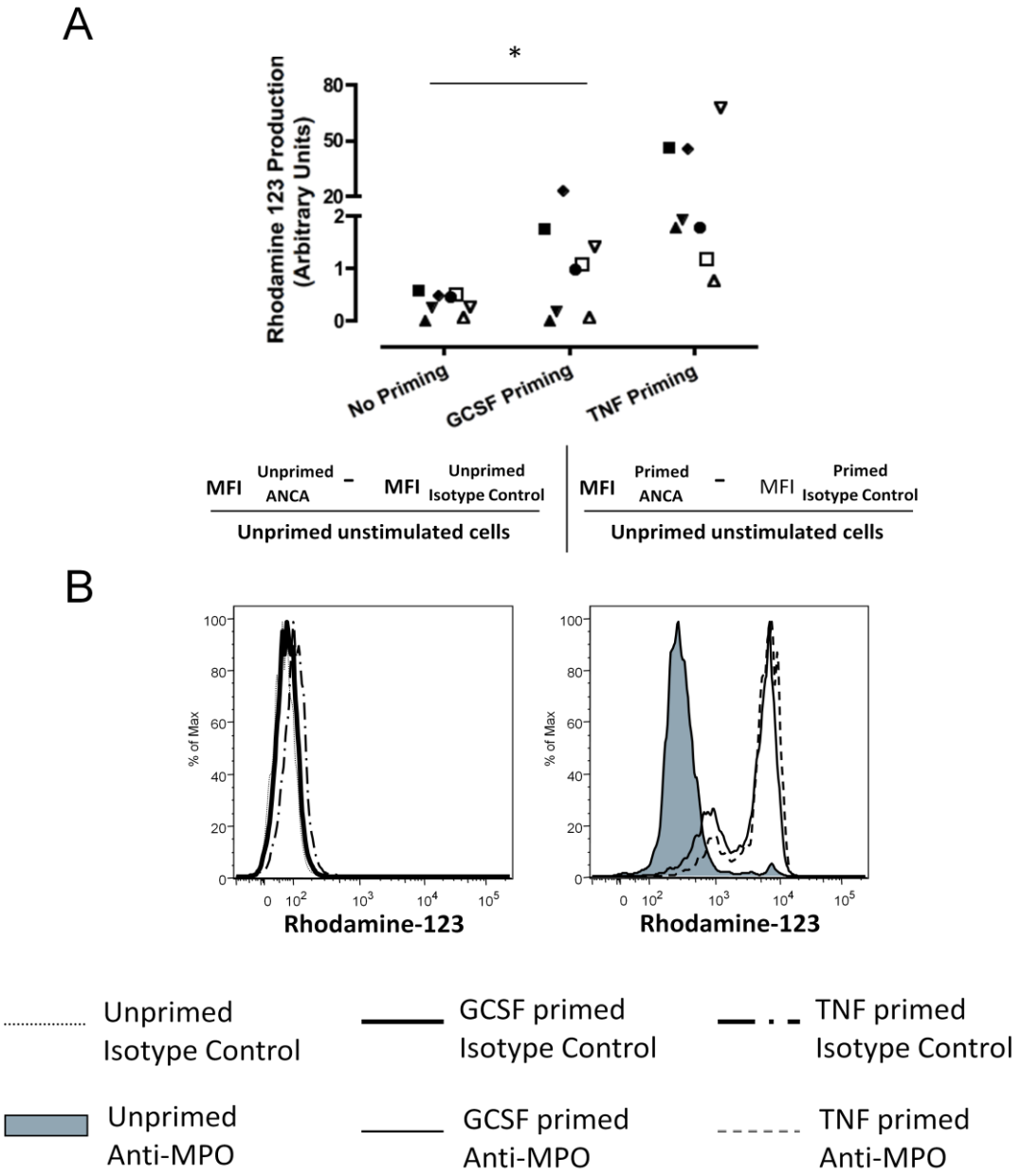


Figure 3.2. The role of GCSF and TNFα in the priming of the ANCA induced respiratory burst as measured by the conversion of dihydrorhodamine (DHR)-123 into fluorescent rhodamine-123. Unprimed, GCSF primed and TNFα primed neutrophils isolated from heparinised blood using Ficoll and red cell lysis were stimulated with 1.25 μg/ml of monoclonal human anti-MPO IgG or a relevant control. (A) shows the results from 3 experiments with eight donors while (B) shows a representative graph. Arbitrary units were obtained from the Mean fluorescence intensities (MFI) as indicated in the figure, with data normalised for the MFI of unstained unstimulated cells in each experiment.Each symbol is an individual blood donor, with a specific symbol denoting the same blood donor in each data set in this figure. *$p<0.05$, **$p<0.01$. Data was analysed using a paired t test.

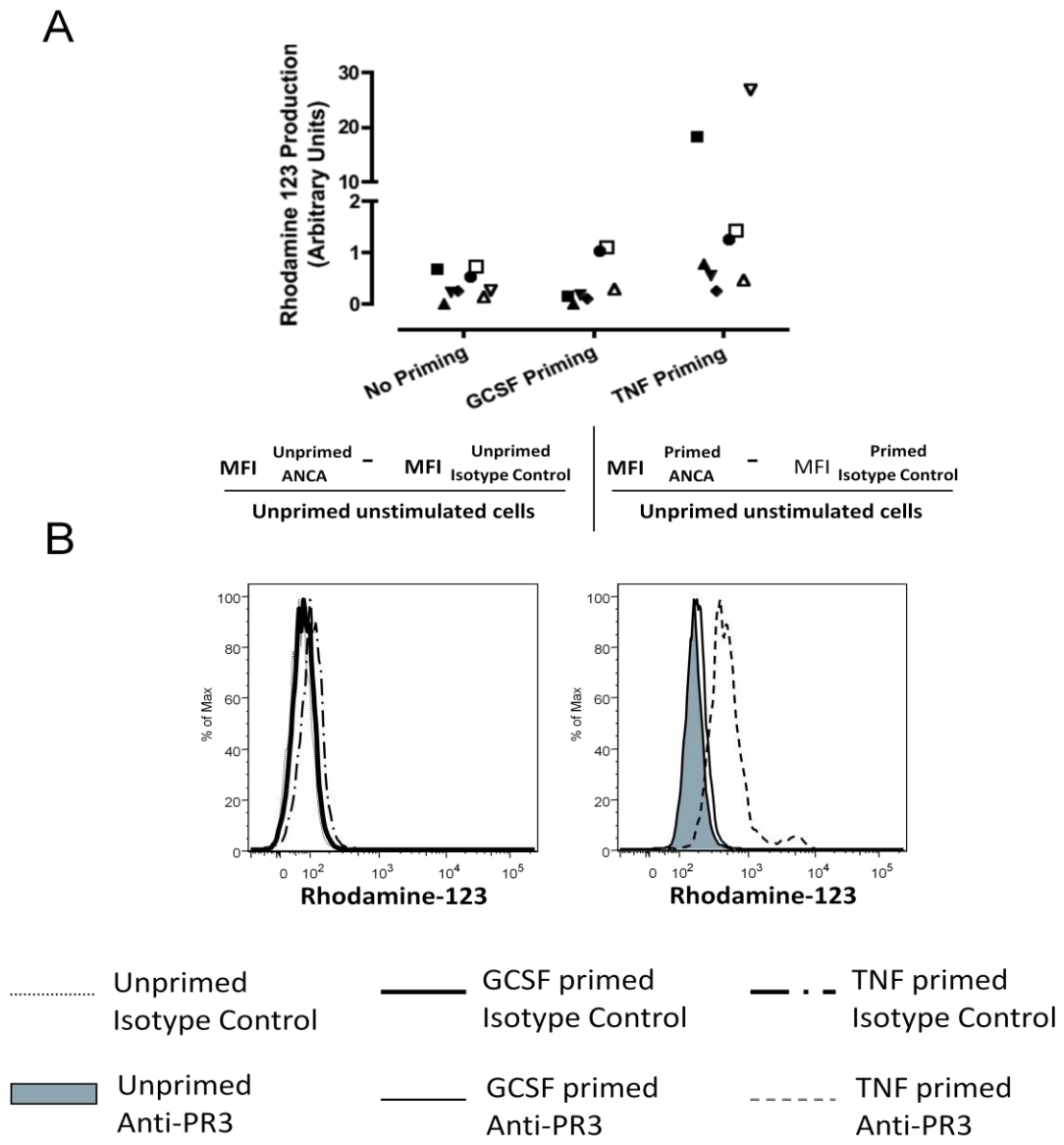


Figure 3.3. The role of GCSF and TNFα in the priming of the ANCA induced respiratory burst as measured by the conversion of dihydrorhodamine (DHR)-123 into fluorescent rhodamine-123. Unprimed, GCSF primed and TNFα primed neutrophils isolated from heparinised blood using Ficoll and red cell lysis were stimulated with 5μg/ml of monoclonal human anti-PR3 IgG or a relevant control. (A) shows the results from 3 experiments with eight donors while (B) shows a representative graph. Arbitrary units were obtained from the Mean fluorescence intensities (MFI) as indicated in the figure, with data normalised for the MFI of unstained unstimulated cells in each experiment. Each symbol is an individual blood donor, with a specific symbol denoting the same blood donor in each data set in this figure.

3.4 Discussion

In this chapter a potential role of GCSF in the pathogenesis of ANCA vasculitis is investigated. This hypothesis was generated from observations in both human cases of rheumatoid arthritis and in rodent models of experimental arthritis. Considering that GCSF administration has been known to exacerbate joint disease in both humans and rodents, and that GCSF levels are elevated in arthritis patients, we hypothesized that this may also be the case in ANCA vasculitis [193-195]. In support of this hypothesis there has been a reported case of disease flares in two patients with ANCA vasculitis after GCSF administration [197].

It was shown in figure 3.1 that GCSF concentrations are significantly raised in ANCA vasculitis patients with active disease when compared to age matched healthy controls. It was important to age match the control samples in this experiment as GCSF concentrations were shown to correlate with age, with younger people having higher levels. This correlation is in contrast to a study which showd no association between serum GCSF levels and age in a healthy volunteer population [317]. These elevated GCSF levels are also in contrast to a report of reduced levels of GCSF in the serum of GPA patients compared to healthy controls [318]. This study however, measured cytokine levels by ELISA, a method which we found lacked the sensitivity needed to accurately measure GCSF. This may go some way to explain the different results. The treatment status of these patients is an important consideration for the relevance of this study, as immunosuppressive therapy or plasma exchange would affect circulating GCSF concentrations. Serum was collected from these patients within 48 hours of presentation and therefore they would have received immunosuppression for no longer than two days at this stage. On close examination of the clinical data we saw no correlation between GCSF concentrations, and prior steroid treatment, Birmingham vasculitis activity score (BVAS), anti-MPO or PR3 status, or the presence of extra renal disease. We did not obtain longitudinal data in these patients, and it would be of interest to know how quickly levels returned to normal, and if they might correlate with disease activity or predict relapse, at least in some patients. It would also be interesting to examine whether elevated GCSF levels in patients is associated with an increased circulating neutrophil count.

Published reports in rodent models of arthritis showing a deleterious effect of GCSF administration could be argued to be due to an increase in the numbers of circulating neutrophils [194, 195]. Conversely, the protective status of GCSF-deficient animals from developing experimental arthritis is most likely due to a reduction in the numbers of circulating neutrophils [196]. However, in light of previous studies showing a priming effect for GCSF on isolated human neutrophils, it was hypothesized that GCSF may exacerbate ANCA vasculitis by augmenting the activaton status of neutrophils [182-185]. A significant priming effect for GCSF was shown in response to stimulation with a monoclonal anti-MPO IgG but this was not the case for stimulation with a monoclonal anti-PR3. The intricate mechanisms of neutrophil priming remain to be detailed, but a factor which is widely believed to be important is the translocation of the active cytochrome b_{558} subunit of the NADPH oxidase to the plasma membrane. As stated in section 1.2.3, cytochrome b_{558} is known to be contained in the membranes of both the specific and tertiary granules. Upon neutrophil activation and degranulation this cytochrome b_{558} becomes incorporated into the plasma membrane. Phospholipase D (PLD) is known to be required for both oxidase activity and degranulation. Interestingly, one study found that GCSF stimulation of neutrophils resulted in a 75-100% increase in PLD activity and a 60-100% increase in gelatinase release from the tertiary granules when compared to unstimulated cells, suggesting that degranulation had occurred [319].This was accompanied by an approximately 20% increase in the amount of cytochrome b_{558} found in the plasma membrane. This is thought to be a one of the main mechanisms by which GCSF primes neutrophils for respiratory burst.

In addition to the priming of neutrophils, GCSF may also play an important yet indirect role in the development of autoimmunity. As stated in section 1.5.2 GCSF was shown to prime human neutrophils for increased release of BLyS in response to stimulation with inflammatory stimuli such as C5a, LPS or fMLP [217]. BLyS is known to play an important role in the development of humoral immune responses as mice lacking BLyS have profound deficiencies in B cell development. Conversely, transgenic mice that over express soluble BLyS are known to develop a disease similar to human SLE [215, 216]. As stated in section 1.2.4, it has been reported in a murine model of ANCA vasculitis that anti-MPO immunity is mediated predominantly by T_H17 cells and that GPA patients have higher proportions of circulating T_H17 cells than healthy controls [60, 139]. In the context of ANCA vasculitis, GCSF

may not only prime neutrophils for respiratory burst in response to ANCA, but also for enhanced BLyS release in response to inflammatory stimuli. C5a, which is known to be generated by ANCA activated neutrophils, and LPS which may be present due to pre-existing infection, are known to be capable of inducing this BLyS release [217]. This GCSF priming effect for BLyS release has been shown in both *in vitro* and *in vivo* and may have implications for the survival of autoreactive B cells and the development of autoimmune disorders associated with autoantibodies [213]. Interestingly, elevated BLyS concentrations have been found in the serum and synovial fluid of RA patients and patients with degenerative articular diseases [214, 320, 321]. It is likely that patients with acute phase ANCA vasculitis will also have elevated BLyS concentrations.

Chapter 4

Setting up of various murine models of anti-MPO vasculitis

4.1 Introduction

In order to further investigate the effect of GCSF in ANCA vasculitis it was decided to explore its relevance in the context of an *in vivo* mouse model. Furthermore, as is addressed later in this thesis the murine model will be utilized to investigate the roles of the complement alternative pathway positive regulator properdin and the lectin pathway protease MASP2 in the pathogenesis of disease. We decided to attempt to set up four mouse models of ANCA vasculitis in the laboratory. These are (1) the passive antibody transfer model, (2) the BM transplant model, (3) the splenocyte transfer model and (4) a new model based on the transplacental transfer of anti-MPO IgG from immunized mothers to the pups.

The passive transfer model, developed by Xiao et al involves the direct I.V injection of anti-MPO IgG into wild type mice resulting in the development of crescentic glomerulonephritis affecting approximately 5% of glomeruli [51]. This was shown to be increased to approximately 20% by the administration of LPS concurrently with the passive antibody. This is an appealing model as it utilizes wild type mice as opposed to genetically altered mice such as the Rag2-deficient strain. Another appealing aspect of this model is the fact that glomerulonephritis develops within 1 week of anti-MPO administration. However, the use of an exogenous stimulus in this model to exacerbate the extent of disease is a considerable drawback as it complicates the issue of investigating the potential role of endogenous TLR4 ligands.

The BM transplant model involves the irradiation and reconstitution of MPO immunized MPO-deficient mice with wild type BM [134]. These mice develop crescentic glomerulonephritis over an eight week period, affecting approximately 30% of glomeruli. A significant advantage of this model is that it does not require the administration of LPS in order to develop robust disease. However, a considerable drawback relates to the time frame over which disease develops. Considering the time required to immunize and boost the MPO-deficient mice, combined with the eight weeks post BM transplant, this equates to 14 weeks from beginning to end.

The splenocyte transfer model involves the transfer of anti-MPO splenocytes into Rag2-deficient mice which lack the lymphocyte compartment [51]. These mice develop the most severe disease seen, with crescents/necrosis affecting approximately 83% and 63% of glomeruli respectively. However these mice develop substantial immune complex deposits in their glomeruli. This is most likely due to the homeostatic proliferation of the anti-MPO splenocytes in order to fill the vacant lymphocyte compartment of these mice. Since this feature does not reflect the pauci-immune nature of human ANCA vasculitis, this substantially hinders the use of this model. However, we hypothesized that this homeostatic proliferation may be avoided by the sublethal irradiation of wild type recipient mice. This would create enough room for the anti-MPO splenocytes but they would not undergo homeostatic proliferation and this may prevent the development and deposition of immune complexes in the glomeruli.

In view of a report of a new born baby developing crescentic glomerulonephritis after being born to a mother with anti-MPO antibodies, we considered if this could be performed in mice. By immunizing breeding age MPO-deficient female mice and then mating them, we hypothesized that the anti-MPO IgG would pass across the placenta into the neonates and thus lead to crescentic glomerulonephritis. In order to use this model to study the role of a particular molecule, complicated breeding of the mice is necessary in order to generate pups which would be deficient in the molecule of interest. However, if proven to be a successful model, it could be developed further by implanting the embryos of genetically altered mice into MPO immunized female mice.

Due to the requirement of murine MPO for all of these models the first step is the identification of a murine cell line which produces sufficient quantities of MPO, and its subsequent purification. The standard cell line used for this purpose in this field of research has been the WEHI-3B cell line used by Xiao et al [51]. This is a macrophage-like myelomonocytic leukaemia cell line which has been derived from inbred BALB/c mice. It is known to differentiate into mature granulocytes and has been used extensively as a source of MPO. We also investigated two other cell lines for MPO production. The IL-3 dependent 32Dcl3 cell line is known to differentiate into a neutrophil-like phenotype when the IL-3 in the growth medium is replaced with GCSF. The differentiated cells adopt a variety of

neutrophil functions and characteristics including, phagocytosis and granule formation. They are also known to release MPO upon degranulation and have been used as a source of murine MPO by some groups [70, 322].

A knowledge of the molecular structure of MPO is necessary in order to assess its purity during the various stages of the purification process. It is a heavily glycosylated heme containing protein enzyme and is a major constituent of the azurophilic granules [323]. It is targeted to and released into the phagosome where its primary role is the conversion of hydrogen peroxide (H_20_2, which is generated by the enzyme superoxide dismutase (SOD) using two O_2^- ions and two protons) into hypochlorous acid (HOCl). HOCl is the most potent antimicrobial oxidant known to be produced by the neutrophil [324]. MPO is the product [325] of a single gene which is located on the long arm of chromosome 17. It is first translated as an approximately 80kDa protein which then has a 41 amino acid signal peptide removed. Following this it is then subject to N-linked glycosylation to produce an approximately 90kDa inactive proMPO [326, 327]. The insertion of the heme group by three covalent bonds on the peptide backbone converts the enzyme into its active proMPO form [328, 329]. ProMPO is then subjected to the proteolytic cleavage of the N-terminal 125 amino acid pro sequence to produce an approximately 75kDa protein. This is subjected the further proteolytic cleavage resulting in an approximately 64kDa and 14kDa heavy (α) chain and light (β) chain respectively. Native MPO is approximately 150kDa in size and is composed of two heavy-light protomers which are linked together through the heavy chains by a disulfide bond. [330, 331].

Aims

- To select and culture a mouse cell line that produces large amounts of MPO.
- To purify sufficient quantities of pure MPO
- To set up the following mouse models of anti-MPO vasculitis
    - Bone marrow transplant model
    - Splenocyte transfer model
    - Transplacental antibody transfer model
    - Passive antibody transfer model

4.2 Methods

4.2.1 MPO production and purification

4.2.1.1 Cell culture and peroxidase staining.

WEHI-3B, 32Dcl3 and MRPO cell lines were cultured as described in section 2.2. 100µl of a 2 x 10^5 cells/ml solution of each cell line was centrifuged onto a microscope slide by cytospin as described in section 2.3. The cells were fixed using leucognost and stained for peroxidase activity using chloronaphthol as described in section 2.16.4. Large cultures of MPRO cells were grown in the laboratory and the cell pellets were stored at -80°C in buffer A until purification.

4.2.1.2 MPO production

MPO was purified as described in section 2.5. Samples of the MPO were taken at various stages of the purification process and analysed by both gel electrophoresis and western blot as described below. The purity of the MPO was assessed at each stage of the process.

4.2.1.3 SDS-PAGE of mouse MPO

Mouse MPO samples were diluted to a concentration of 100µg/ml in PBS and SDS-PAGE was carried out under reducing conditions as described in section 2.6. Commercially available purified human MPO was also run for comparison.

4.2.1.4 Western blot of mouse MPO

Mouse MPO samples were diluted to concentrations of 2.5 and 1µg/ml in PBS and run on SDS-PAGE under reducing conditions as described in section 2.6. The gel was removed and transferred to the blot as described in section 2.7. The primary antibody was a 1 in 2000 dilution (in 1xTBS tween/5% powdered milk) of goat anti human/mouse MPO antibody (R&D systems, Abingdon, UK). The secondary antibody, a HRP conjugated anti-goat IgG antibody, was diluted 1 in 1000 in 1xTBS tween/5% powdered milk.

4.2.2 Induction of anti-MPO crescentic glomerulonephritis

4.2.2.1 Bone marrow transplant model

Once sufficient quantities of MPO had been purified and its purity determined, MPO-deficient mice were immunised. The MPO was prepared for initial immunisation in complete freund's adjuvant (CFA) and in incomplete freund's adjuvant (iCFA) for subsequent boosts as described in section 2.11.1. For the bone marrow transplant model, mice were immunised and irradiated as per the schedule detailed in section 2.11.2. Bone marrow chimeras were made using wild type C57BL/6 marrow as described in section 2.9. The mice were bled at two week intervals post irradiation in order to measure anti-MPO serum titres by ELISA (as described in section 2.12.1). They were sacrificed eight weeks post bone marrow transplant. Kidneys were processed and analysed for disease as described in sections 2.15 and 2.16.

4.2.2.2 Splenocyte transfer model

For the splenocyte transfer model, MPO-deficient mice were immunised and boosted with MPO as described in section 2.11.4. On day 40, these mice were bled for serum from the saphenous vein in order to measure anti-MPO titres by ELISA. On day 42, the mice were sacrificed and the spleens harvested.

Thy1.1 mice were used as recipients for the spleen cells as this allows for a close examination of the degree of chimerism. Thy1 is glycophosphatidylinositol (GPI) anchored cell surface protein. It is expressed on murine T cells and is often used as a T cell marker. There are two alleles, Thy1.1 and Thy1.2 which differ by one amino acid at position 108. Thy1.2 is expressed by most strains of mice including C57BL/6. By using an antibody that can distinguish between Thy1.1/ThY1.2 it is possible to identify donor T cells in a chimeric mouse.

Eight recipient Thy1.1 mice were sublethally irradiated with 6.5Gy and injected with 5×10^7 splenocytes by I.V injection. On day 77, peripheral blood was collected from each mouse and stained for flow cytometry as described below in section 4.2.4. This was carried out in order to determine the degree of chimerism and to confirm the presence of neutrophils.

The mice were boosted with 10µg MPO in iCFA by I.P injection on day 78. On day 91 the mice were placed in metabolic cages overnight to collect urine and were sacrificed the following day. Kidneys were processed and analysed for disease as described in sections 2.15 and 2.16. Serum was also collected at day 92 and anti-MPO titres were measured by ELISA.

4.2.2.3 Transplacental transfer model

In regard to the transplacental transfer model, adult MPO-deficient female mice were immunised as described in section 2.11.5 and mated with adult wildtype C57BL/6 males on day 42. When the resulting pups reached weaning age they were placed in metabolic cages overnight and sacrificed the following day. Kidneys were processed and analysed for disease as described in sections 2.15 - 2.17.

4.2.2.4 Passive transfer model

In regard to the passive transfer model carried out in this section, a pool of anti-MPO serum was collected from a total of 13 immunised MPO-deficient mice (immunized with MPO RZ=0.62). These mice were immunised, and the serum was collected as described in section 2.11.3. A group of five, adult wild type C57BL/6 mice received a total of 400µl of anti-MPO serum by I.V injection on day 0 and also 200µl of 50µg/ml LPS (Enzo Life Sciences, Exeter, UK) by I.P injection after 1 hour on day 0 and on day 3. On day 6, the mice were placed in metabolic cages overnight in order to collect urine and were sacrificed on day 7.

4.2.3 Histological assessment of disease

The mice were sacrificed at the stated time points and kidney sections were fixed in Bouin's solution and PLP as described in sections 2.15.1 and 2.15.2 respectively. Snap frozen sections were also collected as described in section 2.15.3. Bouin's fixed samples were processed, embedded in paraffin wax, cut into thin sections and stained with periodic acid Schiff stain as described in sections 2.15.1 and 2.16.1 respectively. Glomerular crescents were scored as described in section 2.17.1. Snap frozen kidney samples were cut to 5µm thickness and stained for glomerular IgG deposition as described in section 2.16.3. PLP fixed frozen tissue was cut to 5µm thickness and stained for glomerular macrophage infiltration as described in section 2.16.2 and scored as described in section 2.17.2.

4.2.4 Flow cytometry

100µl of heparin treated blood was treated with 25µl of a 1 in 125 pre-diluted Fc block (Becton Dickinson, Oxford, UK) for 20 minutes at room temperature. A staining solution was prepared using the antibodies and dilutions shown in table 4.1 below. 25µl of the staining solution was added to each appropriate tube and incubated for 20 minutes at room temperature in the dark. Erythrocytes were lysed and the cells were prepared for analysis as is described in section 2.20.1.

Antibody	Dilution in staining solution	Final dilution of antibody in 150µl volume
ThY1.2 FITC clone 53-2.1 (Becton Dickinson)	1 in 333.2	1 in 2000
CD3 PE clone 17A2 (Becton Dickinson)	1 in 33.3	1 in 200
Ly6G – Alexaflour700 Clone 1A8 (Becton Dickinson)	1 in 33.3	1 in 200

Table 4.1 Antibody preparation used for mixed chimera staining

4.2.5 Albuminuria

Albuminuria was quantified using a commercially available ELISA kit (Bethyl laboratories, Montgomery, TX, USA) as described in section 2.12.2.

4.2.6 Statistics

Statistical analysis was performed using Graphpad Prism software (Graphpad software Inc, La Jolla, CA, USA). As the extent of the antibody responses of the immunised mice were expected to follow a normal distribution, data were analysed using unpaired t tests.

4.3 Results

4.3.1 MPRO cells produce MPO

We tested three murine granulocyte cell lines for their ability to produce MPO. These were WEHI-3B cells, the most popular cell line used for this purpose by other groups involved in vasculitis research, 32Dcl3 cells and MPRO cells. The cells were cultured as described in the methods section and were stained for peroxidase activity. The WEHI-3B murine myeloid cell line did not stain positive for the presence of peroxidase as shown in figure 4.1 A. The 32Dcl3 cell line was grown in IL-3 containing medium to a density of 2 x 10^6 cells/ml and then differentiated for 12 days in medium devoid of IL-3, but containing 2ng/ml recombinant murine GCSF. At day 12 the differentiated cells showed the characteristic polymorphonuclear phenotype of mature neutrophils but did not stain for peroxidase activity as shown in figure 4.1 B. The GMCSF dependent MPRO cell line also showed this polymorphonuclear phenotype at an earlier stage and stained for peroxidase activity which was localised within intracellular granules as shown in figure 4.1 C. Based upon these observations it was decided to utilize the MPRO cell line as the source of murine MPO used for the remainder of this thesis.

MPO was purified (as described in section 2.5) by both concanavalin A sepharose and ion exchange chromatography. Concanavalin A sepharose chromatography is routinely used for the purification of glycoproteins. This step of the procedure, as shown in figure 4.2 A resulted in the appearance of three distinct bands on gel electrophoresis. These are the 64kDa α and the 14kDa β chains of MPO. The 75kDa αβ subunit, which is produced as a result of the cleavage of a 125 amino acid sequence from the precursor proMPO, is also visible. At this stage of the purification process the RZ value of the MPO preparation was approximately 0.3. As MPO is a heavily positively charged protein due to the presence of the heme group it was purified further by cation exchange chromatography. The gel profile after this step of the process is similar to that after the concanavalin A step which suggests that it has not removed much impurity from the sample. However, the RZ value after this ion exchange step increased to approximately 0.6. This considerable change in the purity of the sample may be explained by the removal of fragmented heme proteins during the ion exchange step. Commercially available purified human MPO is shown for comparison. Both

the 64kDa and 14kDa chains are clearly visible. These subunits were confirmed to be MPO by western blot using a goat anti-mouse MPO antibody as shown in figure 4.2 B. MPO concentrations of both 2.5 and 1µg/ml at the various stages of the purification process are shown.

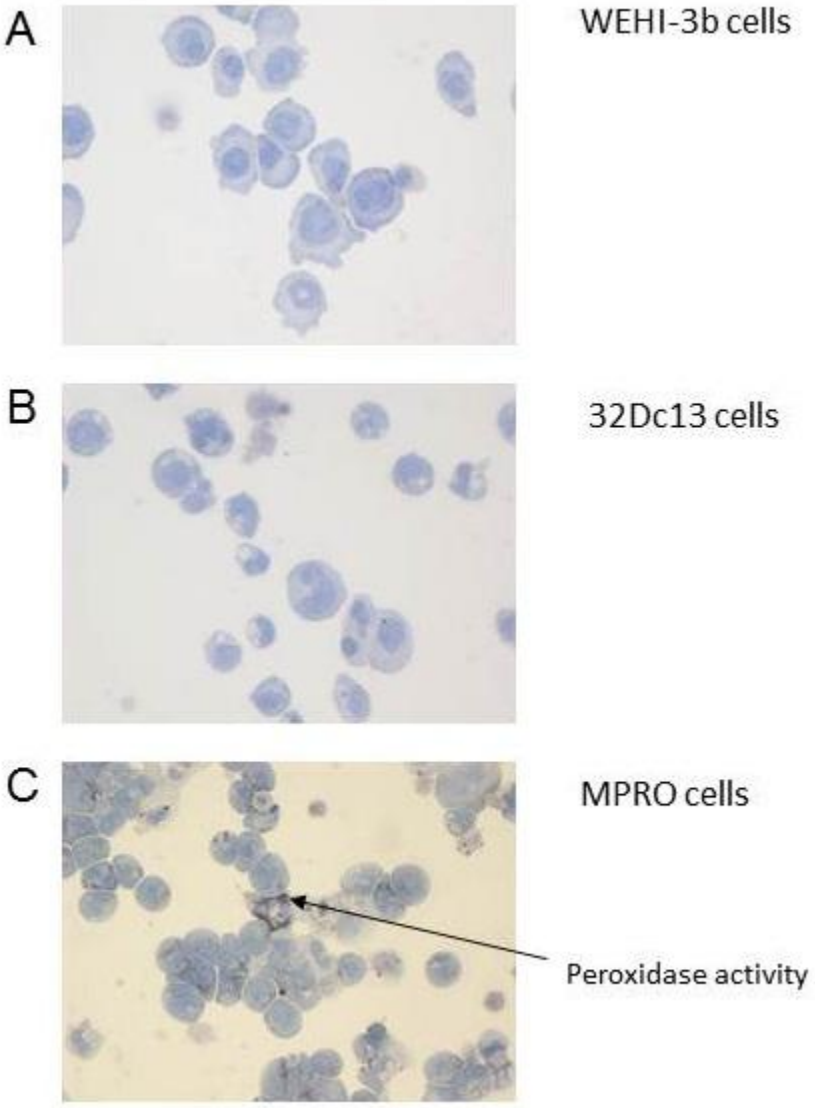


Figure 4.1 MPRO cells express MPO. Mouse myeloid cell lines were stained for peroxidase activity using chloronaphthol, a peroxidase substrate. Both the WEHI-3b (A) and 32Dc13 (B) cell lines did not stain positive for the presence of peroxidase. The MPRO (C) cell line however, did stain positive for peroxidase activity which was localised to the intracellular granules (shown above). All pictures taken at x400 magnification.

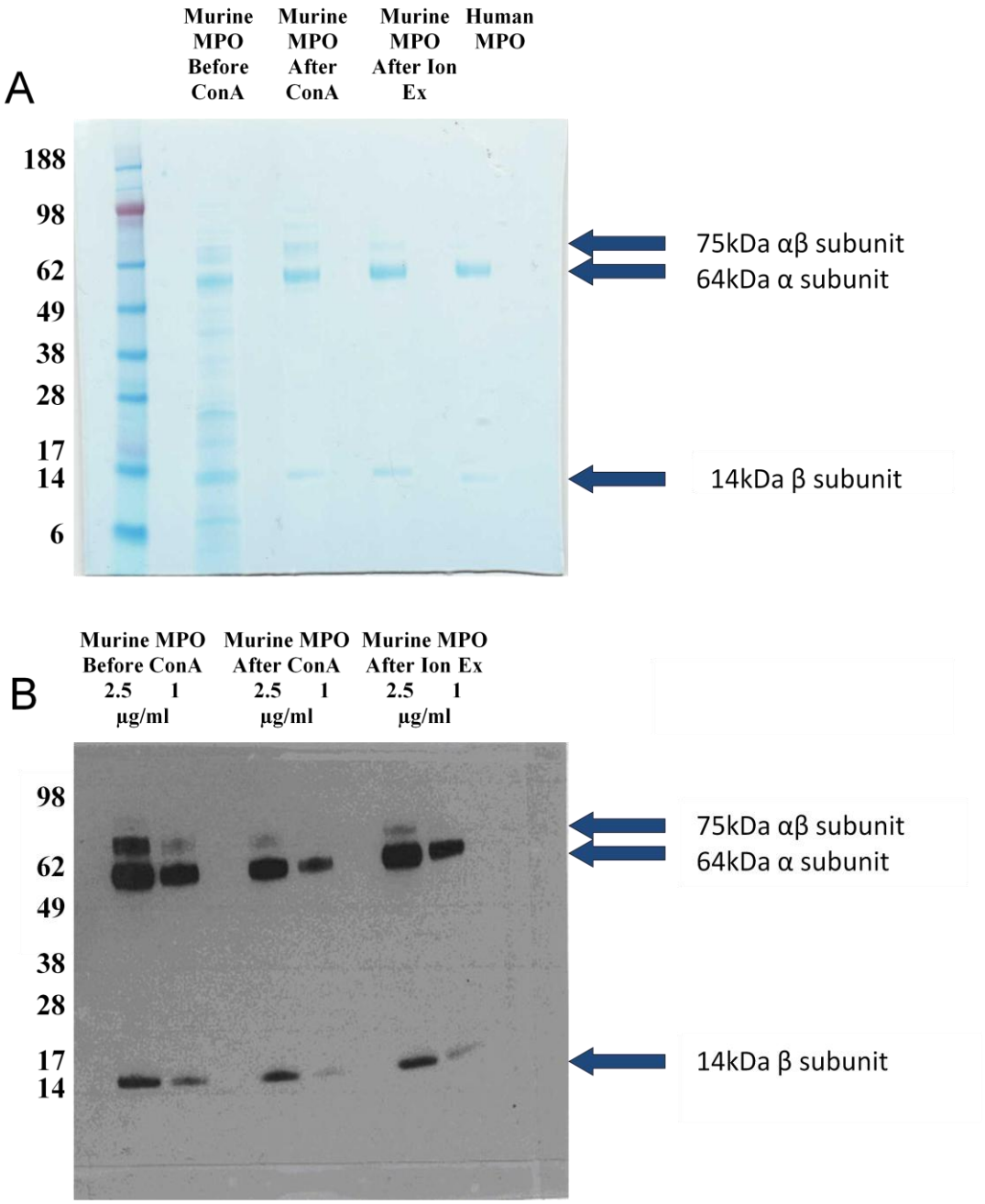


Figure 4.2 MPO can be purified from the MPRO cell line. (A) Purification of murine MPO is shown on a coomassie blue stained gel at various stages of the purification procedure. Run under reducing and denaturing conditions, the 64kDa α chain and the 14kDa β chain are clearly visible after both concanavalin A sepharose and ion exchange chromatogrpahy. The 75kDa αβ subunit is also visible. Commercially available purified human MPO is shown for comparison. (B) Western blot of reduced murine MPO shown at the same stages of the purification process. Concentrations of 2.5 and 1µg/ml MPO were used. Both α and β chains are clearly visible, as is the 75kDa αβ chain.

4.3.2 Attempts to set up various mouse models of ANCA vasculitis

4.3.2.1 Bone marrow transplant model

A group of seven, adult MPO-deficient mice were immunized with 5µg of murine MPO (RZ = 0.3) and boosted as stated in the methods. Anti-MPO titres at day 41 are shown in figure 4.3 A. Control levels were undetectable. At day 42 they were lethally irradiated with 9 Gy and engrafted with 5 x 10^6 wild type BM cells. Anti-MPO titres were analysed every two weeks until sacrifice at week eight. The anti-MPO titres fell dramatically between two and four weeks post engraftment as shown in figure 4.3 B. By week eight the levels were almost undetectable. No evidence of histological disease was found on light microscopy. It was hypothesized that the dose of MPO given and/or the purity of the preparation may have been a factor and that if the antibody levels had been higher at day 42 it may indicate a stronger immune response and thus be more likely to cause disease. We therefore immunized and boosted a group of ten, 6 - 8 week old MPO-deficient mice with 10µg of MPO (RZ = 0.62). Anti-MPO titres at day 41 are shown in figure 4.4A and compared to those of the mice from the previous experiment. Immunization with a higher dose of a more pure preparation of MPO led to a significant increase in anti-MPO titres with means of 61.4 ± 6.8 arbitrary units in the group receiving 10µg MPO (RZ=0.62) compared to 33.2 ± 6.9 in those receiving 5µg MPO (RZ=0.31) (data are mean ± SEM). However, after irradiation and BM cell engraftment, these antibody levels also decreased rapidly between weeks two and four as shown in figure 4.3 B. These mice also failed to develop evidence of histological disease.

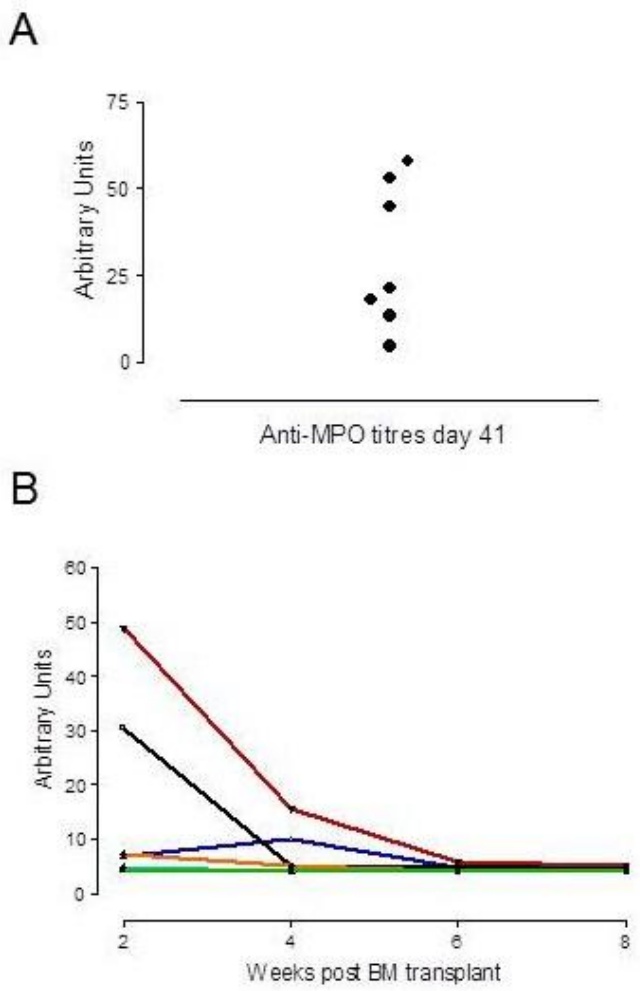


Figure 4.3 Anti-MPO serum titres over an eight week period. (A) Anti-MPO serum titres of MPO-deficient mice immunized with 5µg murine MPO (RZ = 0.31) at day 41, prior to irradiation. Control levels were undetectable. (B) Anti-MPO levels of each mouse measured at 2 week intervals post BM transplant until sacrifice at week 8. The anti-MPO levels of five of the seven mice had declined sharply by 2 weeks post irradiation. There was also a sharp decline in antibody titres in the other two animals between weeks 2 and 4. As a result, there were no signs of disease detectable on light microscopy.

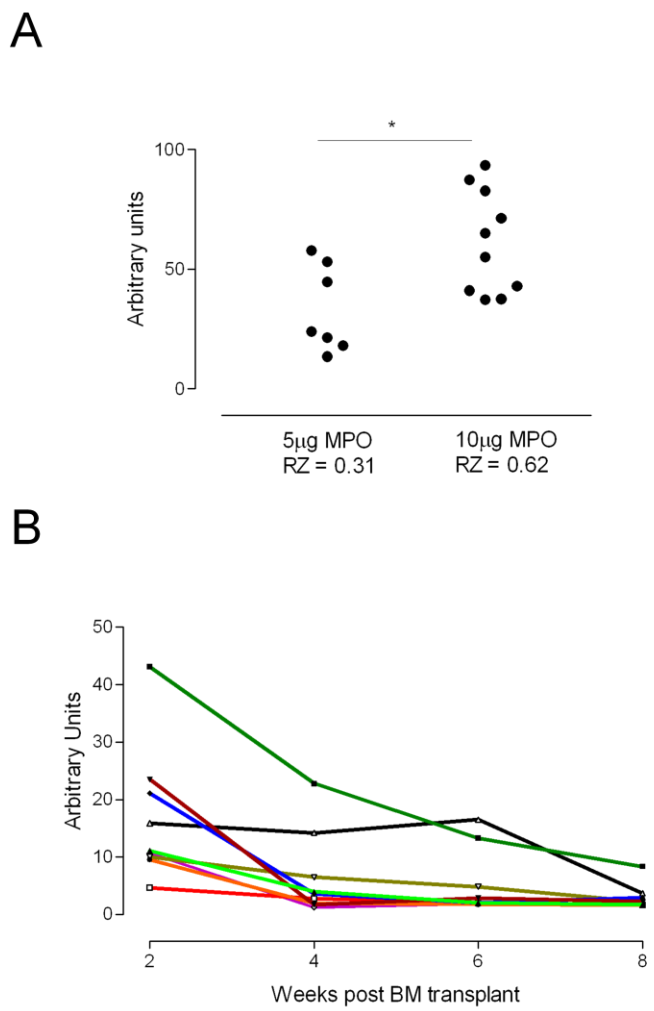


Figure 4.4 Anti-MPO serum titres over an eight week period. (A) MPO-deficient mice immunized with 10µg murine MPO (RZ = 0.62) measured at day 41 compared to those immunised with 5µg of MPO (RZ = 0.31) from the previous experiment. Immunisation with a more pure preparation of MPO caused a significant increase in anti-MPO antibody production ($P<0.05$). (B) Anti-MPO levels of each mouse measured at 2 week intervals post BM transplant until sacrifice at week 8. As in the previous experiment, antibody levels in some mice declined sharply after irradiation. Data were analysed using an unpaired t test.

4.3.2.2 Splenocyte transfer model

The splenocyte transfer model was attempted using splenocytes harvested from eight, adult MPO-deficient mice which had been immunized and boosted with 10µg of MPO (RZ = 0.62). The relative anti-MPO titres at day 42 are shown in figure 4.5 A. Control titres were undetectable. Eight Thy1.1 mice, aged 6 - 8 weeks were sublethally irradiated with 6.5Gy and injected I.V with 5 x 10^7 anti-MPO splenocytes. Five weeks post splenocyte injection the mice were bled and the degree of chimerism was analysed by flow cytometry. The mean percentage of donor (Thy1.2+) CD3+ cells present in the recipient mice was 10.5 ± 1.4 percent of total leukocytes compared to a mean of 19.4 ± 3.6 percent CD3+ Thy1.2 negative cells. A representative dot plot is shown in figure 4.5 B. It was also confirmed that these mice possessed a population of circulating neutrophils (mean 12.9 ± 2.1 percent of total leukocytes). A representative dot plot is shown in figure 4.5 C. The mice were boosted with a further 10µg MPO (RZ = 0.62) by I.P injection on day 78 and sacrificed on day 92. The anti-MPO titres of the eight mice at day 92 is shown relative to those of a known high responder in figure 4.6 A. No evidence of histological disease was seen in these mice and this may be explained by the low anti-MPO levels seen by day 92. To investigate the extent of glomerular immune complex deposition, frozen kidney sections were stained with a FITC-conjugated goat anti-mouse IgG and compared to an MRL/lpr positive control mouse. All eight of the splenocyte recipient mice displayed a lack of glomerular IgG deposition when compared to the positive control. Representative sections are shown in figure 4.6 B.

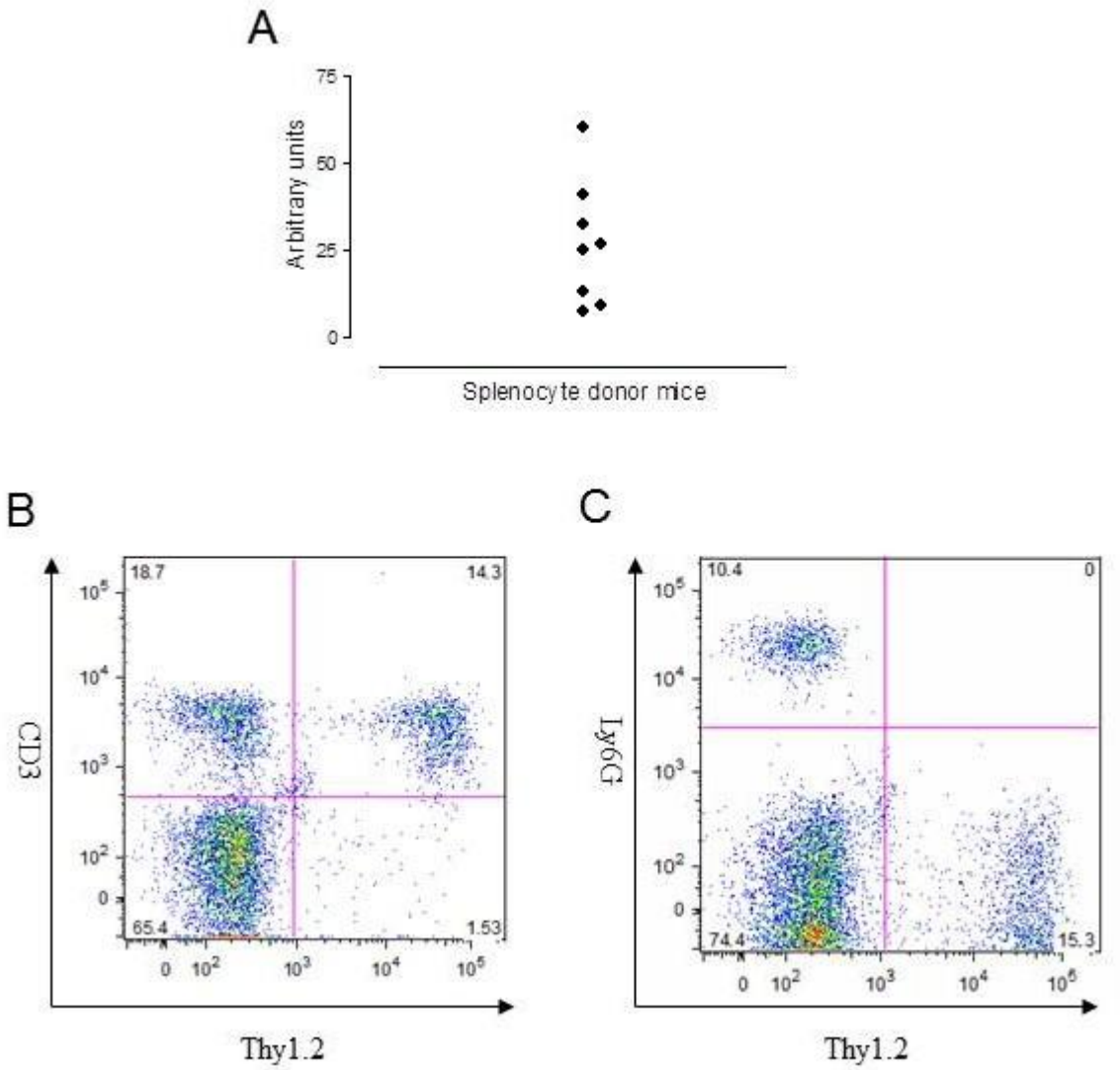


Figure 4.5 Analysis of anti-MPO serum titres and donor splenocyte engraftment**.** (A) Anti-MPO serum titres of spleen cell donor mice on day of sacrifice. Control levels were undetectable. A total of eight sublethally irradiated recipient Thy 1.1 positive mice received 5 x 10^7 spleen cells via tail vein injection on day 42. Chimerism was assessed at day 77 in all eight mice with means of 10.5 ± 1.4 percent (of total leukocytes) Thy1.2+ donor CD3+ cells and 19.4 ± 3.6 percent Thy1.2 negative recipient CD3+ cells. A representative dot plot is shown in (B). Ly6G+ circulating neutrophil counts were assessed in all eight recipient mice with a mean of 12.9 ± 2.1 percent of total leukocytes. A representative dot plot is shown in (C). Data is mean ± SEM.

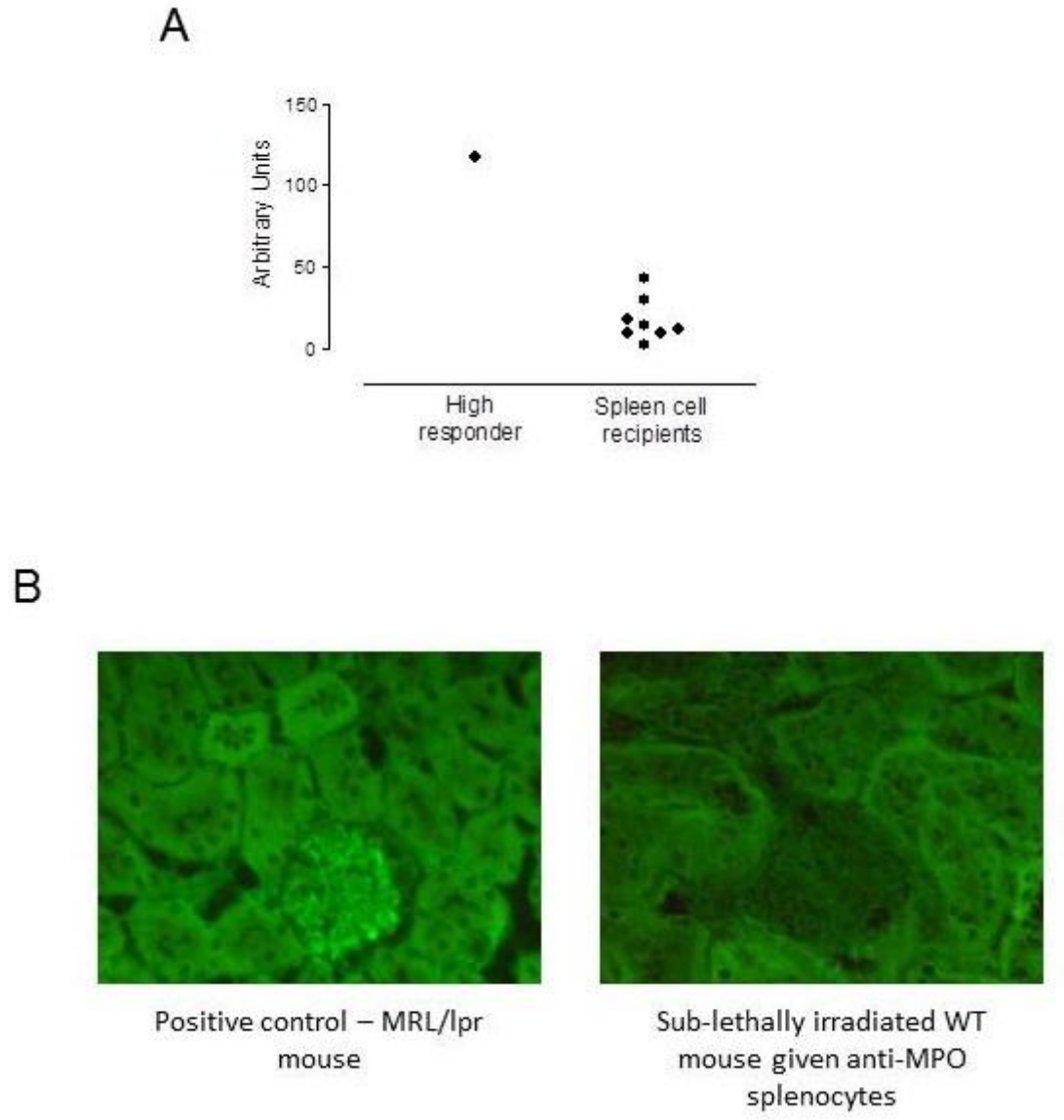


Figure 4.6 Anti-MPO serum titres and glomerular IgG deposition. (A) Anti-MPO serum titres of spleen cell recipient mice at day 92. Titres are compared to those of a known high responding mouse. Control levels are undetectable. (B) Immunofluorescence of mouse glomerulus stained with a FITC conjugated goat anti-mouse IgG (x400 magnification). There is a marked absence of immune complexes in the glomerulus of the sub-lethally irradiated wild-type when compared to that of a positive control MRL/lpr lupus mouse.

4.3.2.3 Transplacental transfer model

Four adult female MPO-deficient mice were immunized with MPO (RZ=0.62) as described in the methods. They were bled from the saphenous vein for the assessment of anti-MPO titres on day 40. On day 42 they were mated with adult C57BL/6 male mice. A total of thirty two pups were born. At weaning age, the pups were placed in metabolic cages overnight for urine collection and sacrificed the following day. Anti-MPO titres of the immunized female MPO deficient mice are shown in figure 4.7 A compared to those of the pups. The antibody titres of one of the four female mice and sixteen of the pups fell below the sensitivity of the assay. Kidney sections were processed and PAS staining was carried out on paraffin embedded tissue. Kidney sections from all of the pups showed no sign of glomerulonephritis and appeared no different from kidney tissue from healthy three week old mice. There was no significant elevation in albuminuria in the pups when compared to urine collected from healthy three week old mice as shown in figure 4.7 B.

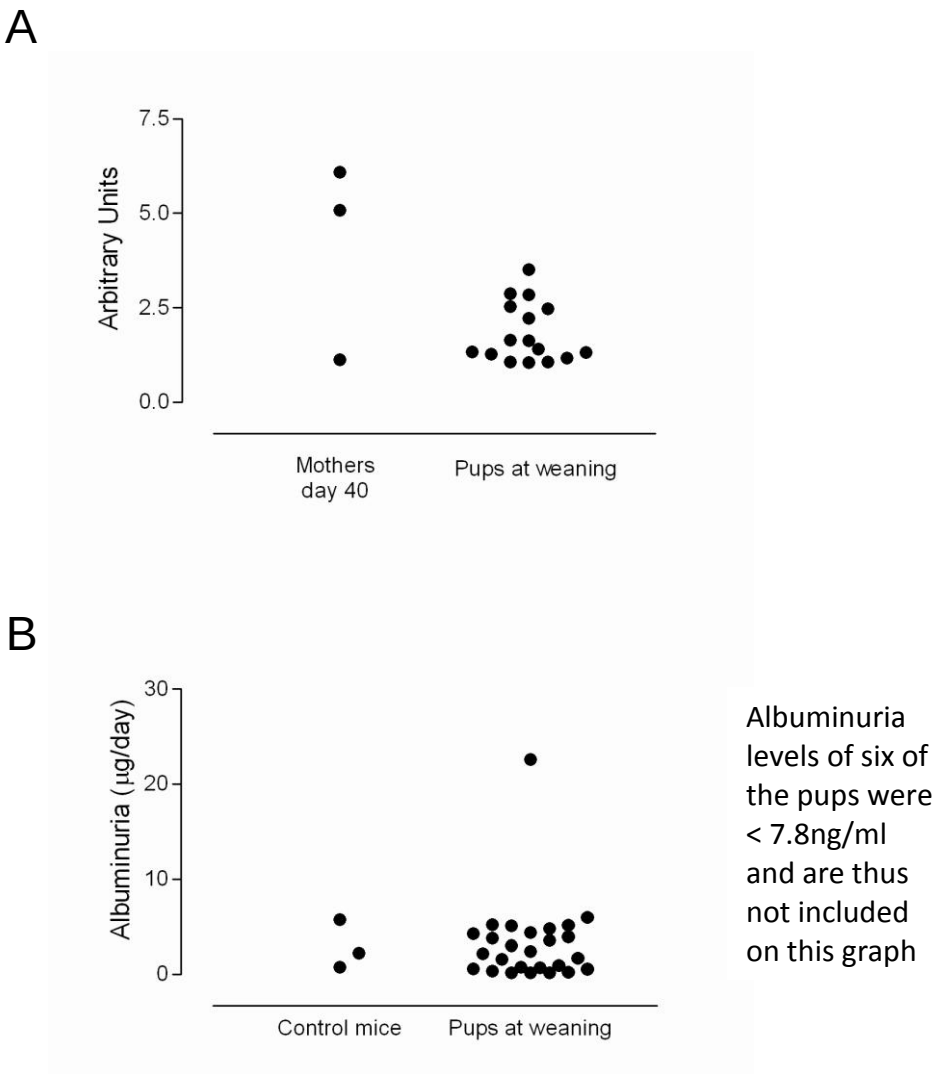


Figure 4.7 Transplacental transfer of anti-MPO IgG did not induce crescentic glomerulonephritis in mice. (A) Anti-MPO titres in immunised female mice at day 40 compared to those of their pups at weaning age. The antibody titres of one of the female mice and of sixteen of the pups are not included in the graph as they fell below the lowest point of the standard curve (1 in 409,600 of a positive serum pool) at a 1 in 12,800 dilution. (B) Albuminuria levels of the pups were of similar levels to those of age matched healthy control mice. The albuminuria levels of six of the pups fell below the sensitivity of the assay (7.8ng/ml). No signs of crescentic glomerulonephritis were seen on PAS stained kidney sections of any of the mice.

4.3.2.4 Passive transfer model

A group of five, adult wild type mice were injected I.V with a total of 400µl of an anti-MPO serum pool on day 0. They also received 10µg of LPS on days 0 and 3 and were sacrificed on day 7. Glomerular crescents were scored by light microscopy on PAS stained sections with a mean of 6.8 ± 1.11% of glomeruli containing crescents as shown in figure 4.8A (data are mean ± SEM). Glomerular infiltration of $CD68^+$ macrophages was also scored with a mean of 0.5 ± 0.06 macrophages per glomerular cross section (gcs) as shown in figure 4.8 B (data are mean ± SEM). By day 7, none of the five mice had developed albuminuria as shown in figure 4.8C. We did not collect baseline urines from these mice, but later experiments showed normal levels to be approximately 20µg/day (see figure 5.3A). Representative light microscopy and immunofluorescence is shown in figures 4.8D-E. A normal glomerulus shown under light microscopy is shown for comparison.

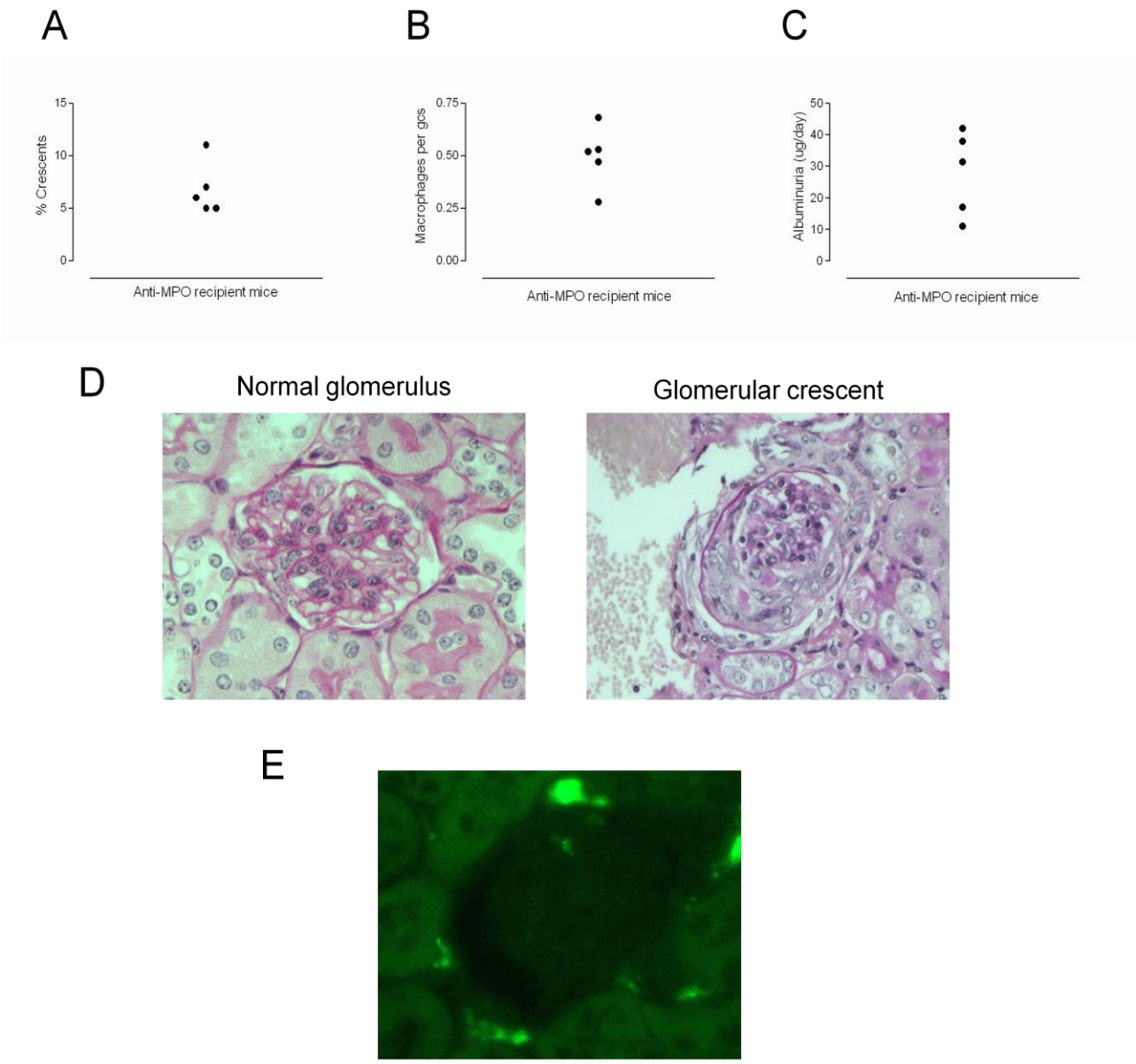


Figure 4.8 Anti-MPO serum induces crescentic glomerulonephritis in mice**.** (A) Glomerular crescent scores at day 7 in wild type mice that received a total of 400µl of an anti-MPO serum pool by I.V injection on day 0 and 10µg of LPS on days 0 and 3. (B) Glomerular macrophage scores at day 7. (C) Urine albumin content (µg/day) at day 7. (D) Respresentative light microscopy of PAS stained kidney sections showing both a normal and glomerulus and a glomerular crescent (x400 magnification). (E) Representative immunofluorescence staining glomerular $CD68^{+}$ macrophages (x400 magnification).

4.4 Discussion

In this section three different murine granulocyte cell lines were tested for their ability to produce MPO. The two most commonly used cell lines for this purpose in relation to vasculitis research, WEHI-3B and 32Dcl3, did not produce significant quantities of MPO in our cultures. This is most likely due to inherent variation in the different batches used by different groups. It is unlikely to be due to the culture conditions as both cell lines grew well and differentiated to maturity as identified by the characteristic poly-lobed nucleus of mature neutrophils. The GMCSF dependent MPRO cell line on the other hand produced significant quantities of MPO from early stages of culture. These cells proved to a reliable source of MPO and multiplied rapidly in culture. Combined with their relative ease to maintain compared to the other cell lines, the MPRO cells were the most suitable for our purposes. These cells have not been previously used in vasculitis research as a source of murine MPO. The purification of large batches of cells (a mimimum of $4x10^{10}$ cells) proved to be the most efficient approach to purifying sufficient quantities of MPO for the immunisation of mice. Its purity was determined by its RZ value and this was approximately 0.3 after the concanavalin A step of the purification process.

The immunisation of a group of mice with 5µg of this preparation for the BM transplant model resulted in relatively low anti-MPO titres, which declined rapidly. This was thought to be due to both the low dose and purity of the MPO. It was decided to purify the MPO further by ion exchange chromatography and to increase the dose for immunisation to 10µg. Ion exchange chromatography increased the RZ value of the MPO preparation to approximately 0.6. Groups of mice were immunised and boosted with 10ug of this preparation and this resulted in a significant increase in anti-MPO titres. However, the antibody titres again declined rapidly between weeks two and four and thus did not induce glomerular disease in these mice. Antibody producing plasma cells are known to be relatively resistant to radiation and were thus expected to remain active after the mice were irradiated. However, it is possible that the high dose of radiation (9Gy) used in these experiments may have destroyed the anti-MPO producing plasma cells. This would explain the decline in anti-MPO levels seen. Perhaps by titrating down the radiation dose, the

antibody levels would remain more constant over the eight week period and be sufficient to induce disease.

The splenocyte transfer model reported by Xiao et al induced glomerular crescents in approximately 85% of glomeruli. This model therefore boasts the most severe glomerular disease of any ANCA vasculitis model currently available. However, disease was induced using Rag2-deficient mice which are devoid of lymphocytes. When they were injected with splenocytes, the cells presumably multiplied to fill the void and compensate for the absence of lymphocytes in the Rag2-deficient recipients. This would account for the immune complexes which were reported in the glomeruli of these mice. We attempted to replicate the severe extent of disease in this model, all the while trying to avoid these glomerular deposits. By utilising partially irradiated wild type recipients we hoped to prevent the homeostatic proliferation of the injected lymphocytes and the resulting immune complexes. In addition, we further boosted the recipient mice with 10µg of MPO five weeks after spleen cell transfer as an additional stimulus. However, despite this approach, there was no sign of glomerular disease in these mice. The anti-MPO titres at day 92 were low when compared to a known high responding mouse from a different experiment (serum taken at day 42). This may help to explain the lack of disease. The antibody titres of the spleen cell donor mice were quite variable suggesting varied immune responses of the mice to the MPO. Perhaps taking spleen cells from mice with stronger antibody responses to the MPO would have successfully induced disease in the recipients. There was however, a lack of glomerular IgG deposition in the recipients as shown by immunofluorescence. This is a substantial advantage to this model as it more closely mirrors the pauci-immune nature of human ANCA vasculitis. With further work, this model has the potential to become extremely useful. Not only does it induce the most severe glomerular disease among the various models, it possesses T cell immunity to MPO, a feature which is absent from some of the other models. Due to the long timescale over which these disease models develop, and their difficulty to set up, it was decided that time and resources could be spent more wisely in developing one of the other available models.

With the report of a new born baby of a woman with MPA developing glomerulonephritis as a result of the transplacental transfer of anti-MPO IgG, the question of whether this could

be possible in mice arose. If the pups of MPO-immunised female mice received enough anti-MPO IgG across the placenta it was hypothesized that glomerulonephritis would develop. In our experience, the antibody titres of the pups remained too low to induce disease. As is the suspected issue with the previous model, if stronger immune responses could be induced in the MPO-deficient mice there is hope that this model could be successful. By using embryo-transfer techniques, the embryos of various genetically modified strains of mice could be implanted into immunised females. This approach would allow for the investigation of any number of potential molecules without the need for complicated breeding of mice. As this is an entirely new model of anti-MPO vasculitis which has not been tested in the past, there was uncertainty as to whether it would induce disease at all. With further refinement it may be possible to induce disease in this way but for the purposes of this thesis a more robust model was required.

The simplest way to induce anti-MPO glomerulonephritis in mice is to directly inject anti-MPO IgG. This will result in a mild glomerulonephritis developing within seven days. We successfully induced disease in a group of wild type mice by injecting anti-MPO serum which had been pooled from a group of immunised MPO-deficient mice. The extent of disease was comparable to that induced by Xiao et al [51]. However, a considerable drawback to this model is the requirement of LPS administration to maximise the extent of glomerular disease. This would considerably complicate the task of trying to determine a role for endogenous TLR4 ligands in disease. Another drawback of this model relates to the number of mice that need to be immunised to produce enough antibody to induce disease. This worked out to be approximately two immunised mice for every one experimental mouse. However, the biggest challenge of this model related to the mild nature of the disease. ANCA vasculitis has been shown to be dependent on neutrophils as their depletion in this model attenuates kidney injury [31]. As only approximately 10% of mouse leukocytes are neutrophils compared with approximately 80% in humans, this is likely to be an important factor in determining the extent of disease. If the numbers of circulating neutrophils could be increased in the mice, it stands to reason that there should be a more severe glomerulonephritis in response to anti-MPO IgG.

Chapter 5

GCSF administration exacerbates a murine model of anti-MPO vasculitis

5.1 Introduction

It was shown in chapter 3 that the circulating levels of GCSF are significantly elevated in the serum of patients with active ANCA vasculitis when compared to those of age matched healthy controls. Elevated levels of GCSF are not unique to vasculitis and have been reported in rheumatoid arthritis patients [191]. Furthermore, it was shown to have a critical role in animal models of inflammatory arthritis [194, 195]. GCSF deficient mice were shown to be resistant to the induction of inflammatory arthritis, and the blockade of endogenous GCSF prior to disease onset reduced its severity [196]. However, these effects could be attributed to reduced neutrophil mobilisation from the bone marrow to the circulation thus resulting in attenuated disease. Aside from its ability to mobilise polymorphonuclear cells, there are studies that show the ability of GCSF to prime isolated human neutrophils for an fMLP induced respiratory burst [186, 187]. Furthermore, as shown in chapter 3, GCSF could prime isolated human neutrophils for an anti-MPO induced respiratory burst. *In vitro* studies using human cells have demonstrated GCSF induced changes in the phenotype of neutrophils in regard to their surface expression of the adhesion molecules Mac-1 and LFA-1 [190]. Subsequent neutrophil binding to the endothelium was shown to be dependent on CD18, the common subunit to both Mac-1 and LFA-1. Despite this evidence using human cells, there remains little data on the effects of GCSF on mouse neutrophils in the context of these inflammatory models. One study showed that the treatment of isolated mouse neutrophils with GCSF caused the shedding of L-selectin and an upregulation of CD11b [332]. These are both indicators of neutrophil activation. The effect of GCSF on neutrophils *in vivo*, however, is less clear.

Aims

- To investigate the effects of GCSF administration on the passive antibody transfer model of ANCA vasculitis.
- To examine the *in vivo* effects of GCSF on neutrophil activation status.

5.2 Methods

5.2.1 Passive transfer model

An anti-MPO serum pool was prepared from 48 MPO-deficient mice which had been immunized with MPO (RZ=0.62) as described in section 2.11.3. A group of ten, adult C57BL/6 wild type mice were split into two groups of five and bled from the saphenous vein on day -5 for assessment of baseline neutrophil numbers. This was done by flow cytometry as described in section 5.2.3 below. On day -5, each mouse was given an S.C injection of either 200µl of PBS as control, or 200µl containing 6µg GCSF (Filgastrin, Neupogen®, Amgen, Cambridge, UK) daily up until sacrifice at day 7. On day -1, the mice were again bled from the saphenous vein for neutrophil assessment. All mice were given a total of 800µl of anti-MPO serum by I.V injection over 2 days (day 0 and day 1). They also received 200µl of 50µg/ml LPS (Enzo Life Sciences, Exeter, UK) by I.P injection after 1 hour on day 0 and on day 3. The mice were placed in metabolic cages overnight on day 6 for urine collection and sacrificed on day 7.

5.2.2 Histological assessment of disease

Histology was assessed as described in section 4.2.3.

5.2.3 Flow cytometry

Flow cytometry was carried out on day -1 in order to assess circulating neutrophil concentrations, as described in section 2.20.1 using the following antibody.

Target	Isotype	Clone	Fluorochrome	Dilution	Supplier
Ly6G	Rat (LEW) IgG2b,k	1A8	Alexafluor 700	1 in 200	Becton Dickinson

Table 5.1 Anti-mouse antibody used for flow cytometry assessment of neutrophils.

5.2.4 Functional data

Albuminura was quantified by ELISA using 1 in 200 dilutions of mouse urine as described in section 2.12.2. Haematuria and serum creatinine analysis was carried out as described in sections 2.14 and 2.13 respectively.

5.2.5 Assessment of murine neutrophil activation markers

A total of 16 adult C57BL/6 mice were divided into four groups with each group containing four mice. The four groups were to be treated as described in table 5.2 below. Mice were bled from the saphenous vein four days after receiving 30µg of pegylated human GCSF (Neulasta®, Amgen, Cambridge, UK) or PBS. The appropriate groups then received either 10µg LPS (Enzo Life Sciences, Exeter, UK) or PBS and were again bled from the saphenous vein after 2 hours. In order to assess neutrophil activation status, flow cytometry was carried out as described in section 2.20.1 using the antibodies and dilutions stated in table 5.3 below.

5.2.6 Statistics

Statistical analysis was performed using Graphpad Prism software (Graphpad software Inc, La Jolla, CA, USA). Experiments containing two independent groups, were analysed using unpaired t tests whereas experiments containing more than two groups were analysed using one way ANOVA. In this case a Tukey's multiple comparison post test was used to assess statistical significance between groups. The specific tests used are stated in the figure legends and data is expressed as mean ± SEM.

Group Number	GCSF	LPS
Group 1	×	×
Group 2	✓	×
Group 3	×	✓
Group 4	✓	✓

Table 5.2 GCSF and LPS treatment status of each group of mice.

Target	Isotype	Clone	Fluorochrome	Dilution	Supplier
CD62L	Rat IgG2a,k	MEL-14	Pacific Blue	1 in 80	Biolegend
CD11b	Rat (LEW) IgG2b,k	MI/70	FITC	1 in 100	Becton Dickinson
CD11c	Armenian Hamster IgG	N418	PE	1 in 62.5	Biolegend
Ly6G	Rat (LEW) IgG2b,k	1A8	Alexafluor 700	1 in 200	Becton Dickinson

Table 5.3 Anti-mouse antibodies used for flow cytometry to assess neutrophil activation status

5.3 Results

5.3.1 GCSF administration exacerbates disease in a murine model of ANCA vasculitis

To investigate the *in vivo* relevance of GCSF in ANCA vasculitis, disease was induced using the passive antibody transfer model in mice that had been given GCSF or PBS as control (as described in the methods). As expected, GCSF administration caused an increase in circulating neutrophil numbers as shown in figures 5.1 A-B. Representative dot plots showing the increase in Ly6G positive cells (neutrophils) after 4 days of GCSF treatment is shown in figure 5.1 C. The extent of glomerular disease was assessed both histologically and functionally at day 7 after disease induction by anti-MPO antibody injection. Disease was significantly greater in mice that received GCSF than those that received PBS control. There were significantly more glomerular crescents in mice that received GCSF with a mean percent of 29.1 ± 4.4 compared to 5.8 ± 1.8 in controls as shown in figure 5.2 A (data are mean ± SEM). There was also an increase in the number of infiltrating $CD68^+$ macropahges with a mean of 3.2 ± 0.5 per glomerular cross section (gcs) in the GCSF group compared to 1.2 ± 0.3 in the controls, as shown in figure 5.2 B. Representative light microscopy and immunofluorescence is shown in figures 5.2 C-D. Functional parameters of disease were assessed in figure 5.3. There was no difference in albuminuria between the two groups as

shown in figure 5.3 A, although the levels in both groups were above normal levels for adult C57BL/6 mice (mean obtained from eight mice is shown by the dotted line). Both haematuria and serum creatinine were significantly raised in the GCSF group as shown in figure 5.3 B-C. Serum creatinine was a mean of 13.6 ± 2.8 µmol/L in GCSF treated mice compared to a mean of 8.3 ± 0.5 µmol/L in controls. This data shows that GCSF administration exacerbates disease in a mouse model of ANCA vasculitis as assessed by both histological and functional parameters.

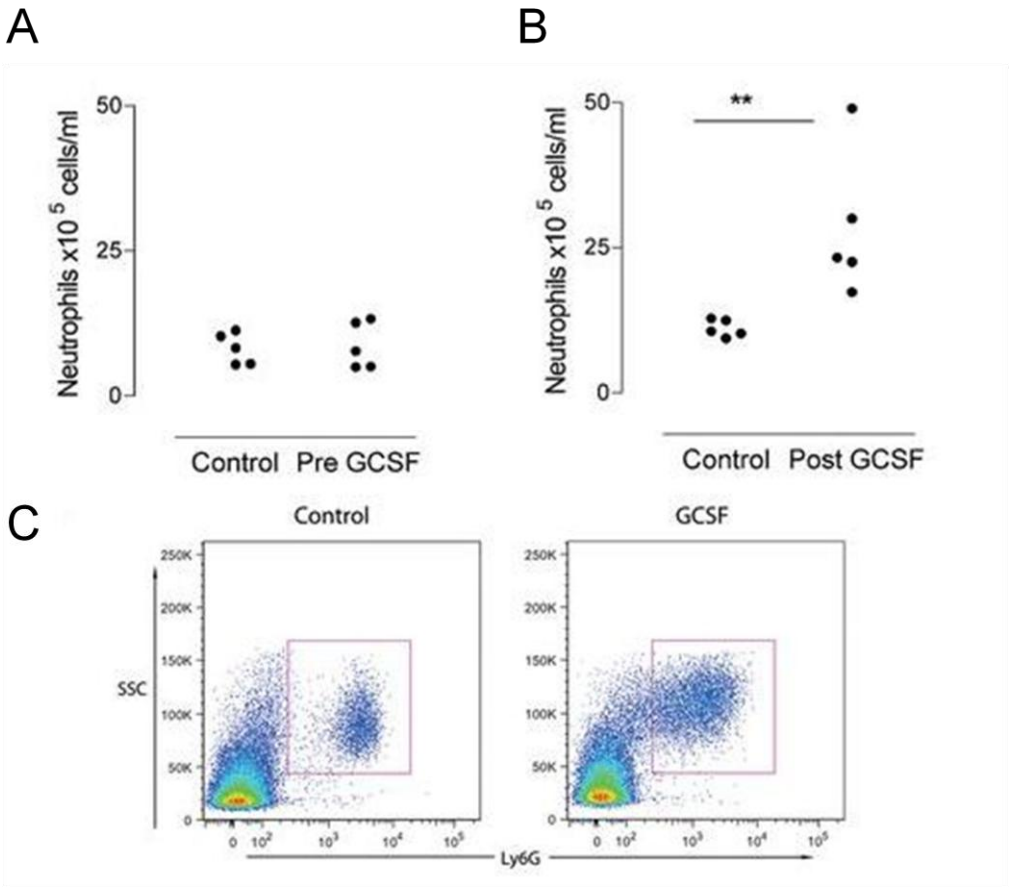


Figure 5.1 GCSF exacerbates disease in a murine model of anti-myeloperoxidase vasculitis (A) Peripheral blood neutrophil counts of both control and GCSF treated mice at day -5 (preGCSF treatment). (B) Peripheral blood neutrophil counts of control and GCSF treated groups at day -1. GCSF treated group has significantly more circulating neutrophills at day -1 ($p<0.05$) (C) Representative dot plots showing the increase in Ly6G positive cells after 4 days of GCSF treatment. Each symbol is data from a seperate mouse. Data was analysed using an unpaired t test.

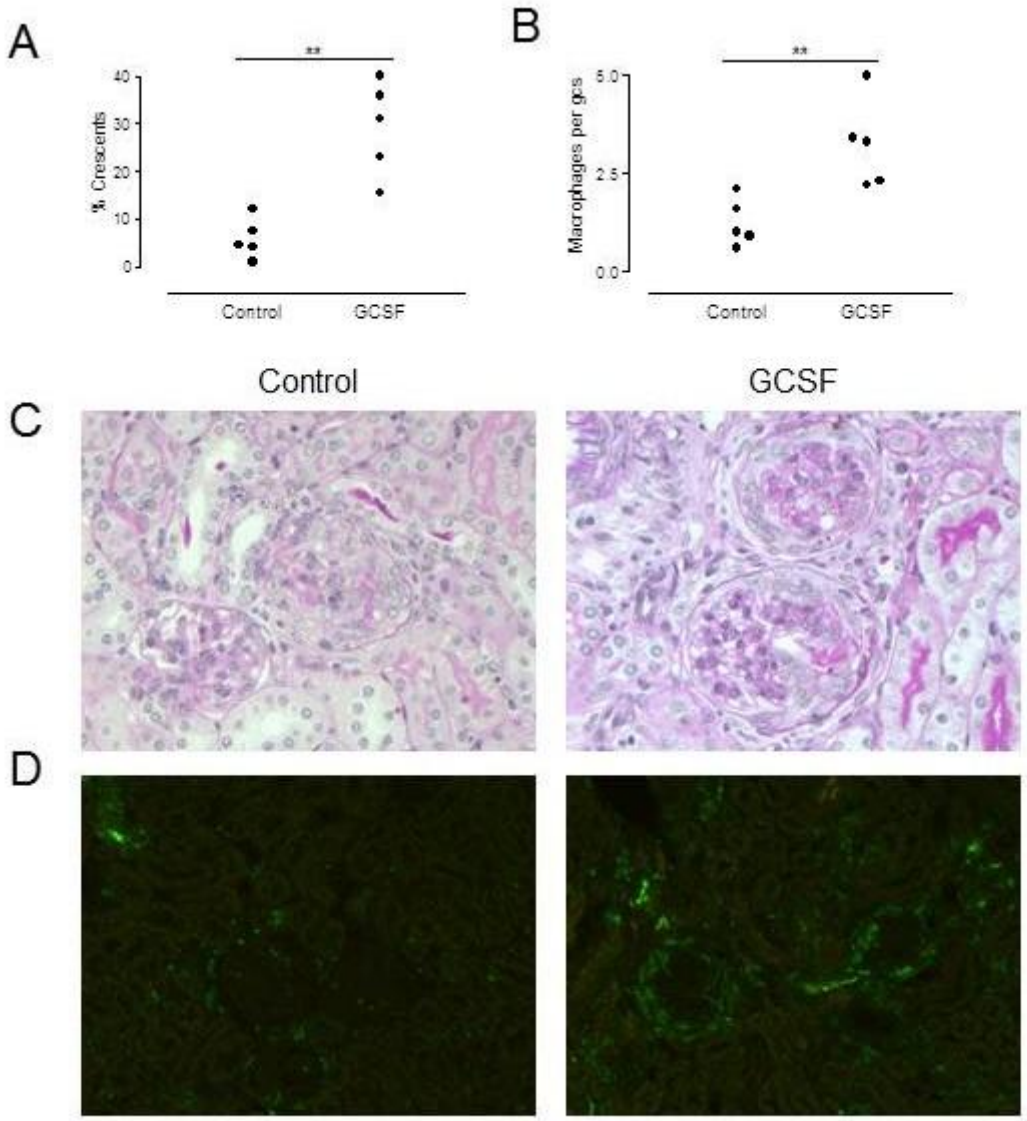


Figure 5.2 GCSF exacerbates disease in a murine model of anti-myeloperoxidase vasculitis. (A) By day 7, GCSF treated mice have significantly more glomerular crescents compared to controls (P<0.01). (B) GCSF treated mice also have significantly more glomerular CD68 positive macrophage infiltration than control mice (P<0.01). (C) Representative light microscopy and (D) representative immunofluorescence staining of CD68 positive macrophages. Each symbol is data from a separate mouse. All above pictures taken at x400 magnification. Data was analysed using unpaired t tests.

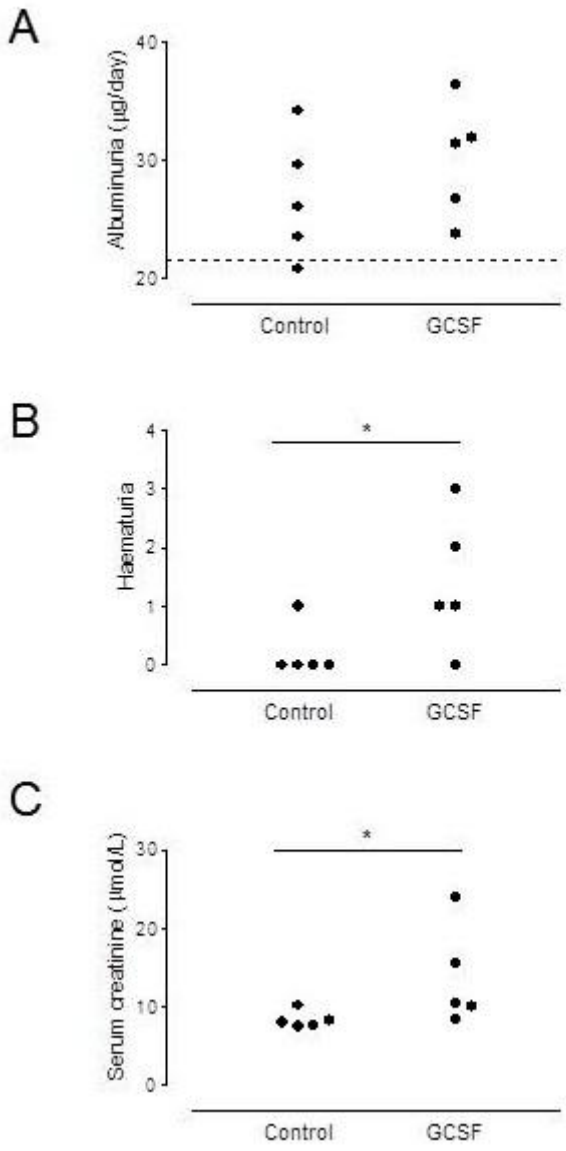


Figure 5.3 Functional data at day 7 of the same experiment. GCSF administration significantly increases haematuria and serum creatinine. (A) There was no difference in albuminuria between the two groups at day 7 (normal levels obtained from mean of eight mice, shown by dashed line). (B) Haematuria was significantly greater in the GCSF treated group than in the control group ($P<0.05$). (C) Serum creatinine was also significantly raised in the GCSF treated group ($P<0.05$). Each symbol is data from a separate mouse. Data was analysed using an unpaired t test.

5.3.2 GCSF primes mouse neutrophils *in vivo*

To further investigate the effect of GCSF in the context of the animal model we examined whether it could prime neutrophils *in vivo*. A total of sixteen, adult C57BL/6 wild type mice were split into four groups. Each group was given either 30µg of pegylated human GCSF or PBS control. After four days, the mice were given either 10ug LPS or PBS control and bled from the saphenous vein for FACs analysis. Neutrophil expression of the adhesion molecules CD62L (L-selectin), CD11b and CD11c was analysed. The purpose of the experiment was to analyse the effect on neutrophil activation of GCSF when given prior to LPS, as in the *in vivo* experiment described above. The experimental groups were the same as in the *in vivo* experiment with the addition of two extra groups. One was given neither GCSF or LPS and the other GCSF in the absence of LPS. As shown in figure 5.4 A, CD11b expression was upregulated in mice given LPS alone when compared to the control group which received neither LPS nor GCSF ($P<0.05$). GCSF alone did not cause an upregulation of CD11b expression above control levels. In mice given both GCSF and LPS, there was a trend towards increased CD11b expression which did not reach significance. As shown in figure 5.4 B, CD11c (LFA-1) expression was upregulated by GCSF alone and in those given GCSF and LPS. There was no CD11c upregulation in response to LPS suggesting that this effect was induced by GCSF. As shown in figure 5.4 C, GCSF and LPS, both independently and in combination caused the shedding of CD62L.

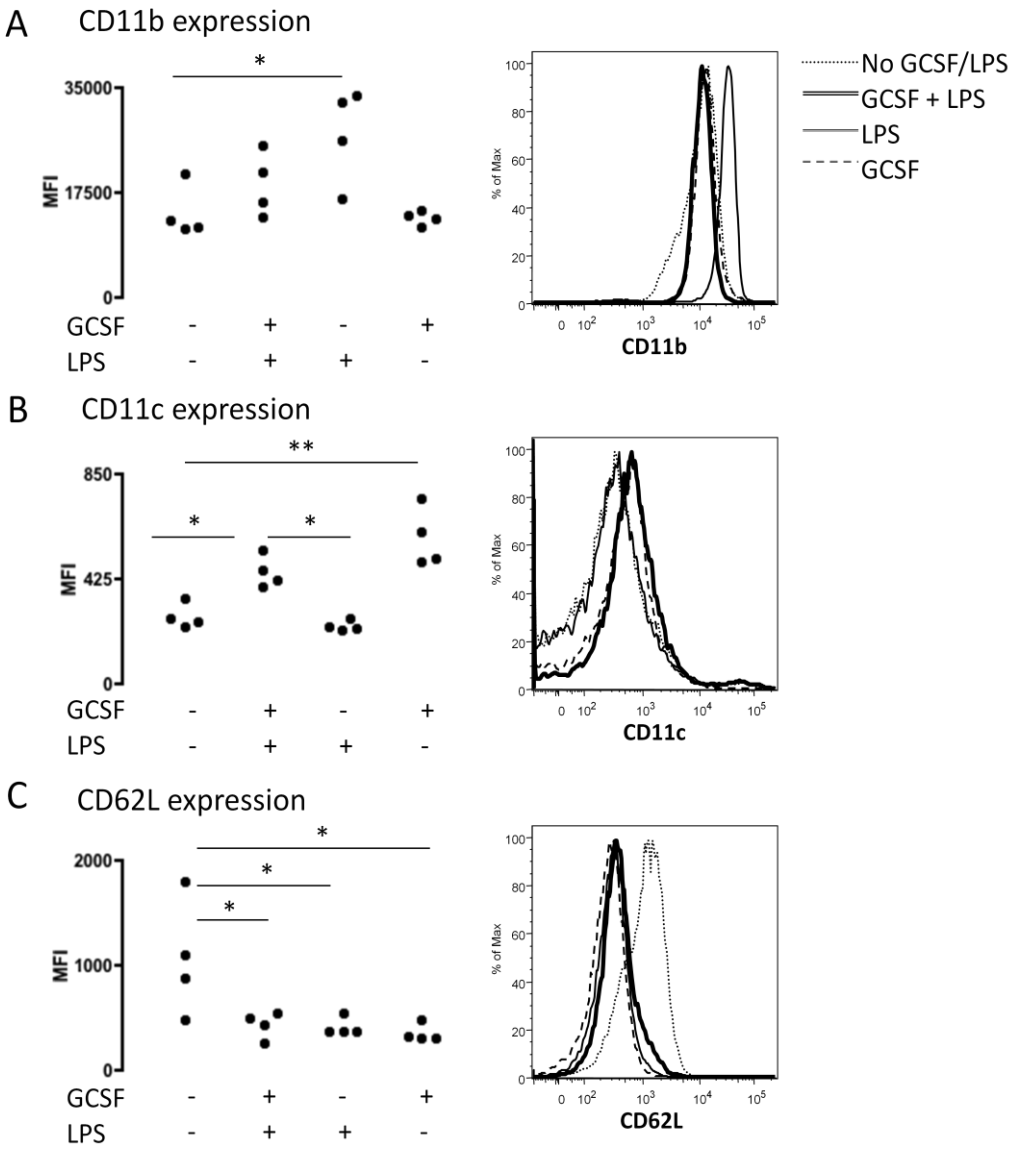


Figure 5.4 *In vivo* activation of murine neutrophils. Mice were treated with 30µg of pegylated human GCSF or PBS as control. On day 4 they were given 10µg LPS or PBS as control and bled from the saphenous vein 2 hours later. Groups are as indicated in the figure and were assessed for (A) CD11b, (B) CD11c and (C) CD62L expression on Ly6G positive neutrophils. Data obtained from individual mice is shown. Data were analysed by one way ANOVA and a Tukey's multiple comparison post test. *p<0.05, **p<0.01, ***p<0.001

5.4 Discussion

To further investigate the relevance of the increased GCSF concentration in ANCA vasculitis patients and its effects on neutrophils, we examined its role in the context of a mouse model of disease. The daily administration of GCSF over the course of twelve days caused a significant exacerbation of disease, both histological and functional, when induced by the passive transfer of anti-MPO antibody. In addition to the extensive histological disease, the presence of a significantly elevated serum creatinine signifies renal failure in these mice. This has not previously been reported in this model. A considerable limitation of the passive model has been the mild nature of the disease induced. In the initial study, wild-type mice that had been injected with anti-MPO IgG developed crescents in a mean of 3.3% of glomeruli [51]. As shown in chapter 4 of this thesis we managed to induce crescents in approximately 7% of glomeruli using this model. However, despite this slight increase, the disease extent remains mild. This would make it difficult to identify any possible differences between experimental groups if this model were to be used. GCSF administration may be an effective method of inducing more severe disease in the mouse model, thus possibly allowing for a reduction in mouse numbers required per group.

The extent of disease exacerbation is likely to be due partly to the increase in circulating neutrophil numbers. However, it is also likely to be due to the proinflammatory action of GCSF on neutrophil function. In order to dissect out the mechanisms by which GCSF exacerbates disease in the mouse model, we examined the expression patterns of neutrophil adhesion molecules in mice that received GCSF with or without LPS. GCSF induced the shedding of L-selectin, an adhesion molecule involved in the earliest stage of neutrophil transmigration, which is consistent with *in vitro* data from another study [332]. L-selectin, which is expressed on the neutrophil surface, binds to P-selectin glycoprotein ligand-1 (PSGL-1), expressed on the endothelium. This initiates rolling of the neutrophil along the endothelium. Upon activation, the neutrophil sheds its L-selectin in favour of another adhesion molecule, CD11b, which initiates firm adhesion to the endothelium. GCSF treatment did not however induce the upregulation of CD11b expression above control levels which is in contrast to the results of *in vitro* work reported in the above study. When treated with LPS alone however, CD11b expression was significantly upregulated, in line

with published *in vitro* data [333]. This study showed that LPS stimulation of isolated mouse neutrophils induced the upregulation of CD11b (but not CD11a or CD11c) through interaction with TLR4 in a manner dependent on JNK and NF-κB signal transduction. Interestingly, in mice that received GCSF alone, CD11c levels were significantly raised above those of control mice and mice receiving LPS alone. This shows that GCSF administration induces CD11c upregulation on the surface of mouse neutrophils *in vivo*. CD11c is a member of the leukocyte function associated (LFA) family of molecules and plays a crucial role in neutrophil adhesion to the endothelium. This may be of significant importance in the murine model as it may play a role in neutrophil migration to the glomerulus. A recent study utilising intravital microscopy showed that in a proinflammatory environment, such as in the presence of LPS, LFA-1 plays a key role in anti-MPO induced recruitment of neutrophils into the glomerulus [334]. Therefore, the upregulation of CD11c by GCSF could be playing an important role in pathogenesis, suggesting an additional mechanism for the observed effect on glomerular disease.

Chapter 6

MASP2 deficiency exacerbates the passive transfer model of anti-MPO vasculitis

6.1 Introduction

The complement system has been linked to many forms of glomerulonephritis in both humans and in animal models of disease. As stated in section 1.7.1.1 recent studies have pointed to a possible role for the complement system in ANCA vasculitis. The alternative, but not the classical or lectin pathways were implicated in the renal injury seen in renal biopsies taken from ANCA vasculitis patients [278]. Another study reported elevated levels of C3a, C5a and C5b-9 in the serum of patients with active disease [279]. A possible genetic component may also be involved as there has been a report of a skewed expression of C3 and C4 gene polymorphisms in ANCA vasculitis patients [335]. Further evidence supporting the involvement of the alternative pathway was uncovered from the mouse models of disease. Mice deficient in factor B were resistant to the induction of disease by anti-MPO antibodies whereas C4-deficient mice were not [287]. Furthermore, the blocking of C5 using a monoclonal antibody given either before or one day after disease induction using anti-MPO antibodies led to a significant reduction in the extent of disease [288]. *In vitro* studies using human blood have shown that TNF-α primed neutrophils which were stimulated with ANCA released a factor which generated C5a in normal serum [46]. The importance of C5a in neutrophil priming was also shown in this study as C5a receptor blockade during priming inhibited ANCA induced respiratory burst *in vitro*. This group went on to show that C5a receptor deficient mice were protected from glomerulonephritis using the BM transplant model of anti-MPO vasculitis. Despite this strong evidence for an important contribution of complement in pathogenesis, the identity of the factor responsible for complement activation has remained elusive. One candidate molecule is properdin. Properdin is a serum protein produced by various cell types including neutrophils, T cells, monocytes and endothelial cells. As described in section 1.6.4, it is the only known positive regulator of complement activation which serves to stabilize the alternative pathway C3 convertase and to inhibit C3b inactivation by complement regulators. It is known to be stored in the secondary granules of neutrophils and is released upon neutrophil activation [237]. Furthermore, various proinflammatory mediators such as LPS and C5a are known to

stimulate the release of properdin from neutrophils [293]. Currently, the most substantial evidence points toward a primary role for the alternative and not the classical or lectin pathways in ANCA-associated disease [278, 287]. However, there is evidence that is suggestive of a fourth pathway of complement activation in which C3 activation in the lectin or classical pathway occurs without the need for C2 or C4 [336]. Activation of the complement system via the lectin pathway is known to require MASP2, which cleaves C4 and C2 to form the C3 convertase. MASP2 is homologous to the classical pathway protease C1s and they are thought to share a common ancestor. The above study showed that the lectin pathway component MBL can bind directly to a bacterial oligosaccharide and cause C3 activation in the absence of C2, C4 and MASP2. There is a requirement however for an intact alternative pathway. It is possible that C3b which is deposited in the presence of MBL is protected from inactivation by complement regulators. This response could then be amplified by the alternative pathway. The identity of the mechanisms responsible for the initial C3 activation however, remains unknown. More recently it was shown that mannan bound MBL can activate the alternative pathway using a mechanism that requires both MASP2 and C4 [337]. Thus there may exist more than one C2 bypass mechanism of complement activation, depending on the ligands to which MBL binds. There also exists a report of a C4 bypass mechanism of complement activation in C4-deficient guinea pig serum [338]. A more recent study investigating the role of MASP2 in a mouse model of myocardial ischemic reperfusion injury identified a MASP2 dependent C4 bypass mechanism involving the lectin pathway [315]. MASP2-deficient mice had significantly less myocardial damage when compared to wild type controls. Furthermore, C4-deficient mice developed disease of a similar severity to wild types suggesting that C3 activation can occur via the lectin pathway independently of C4. This effect was shown to be MASP2 dependent by the use of an inhibitory monoclonal MASP2 antibody. Interestingly the presence of both C2 and MASP1 were required for this MASP2 dependent C4 bypass mechanism to function. This may be explained by the known role of MASP1 in the direct cleavage and activation of MASP2 [339]. This data sheds doubt upon the hypothesis of Xiao et al that the lectin pathway of complement activation is not involved in the disease seen in the antibody transfer model of anti-MPO vasculitis. It is possible that the lectin pathway, acting through this C4 bypass mechanism, made a significant contribution to disease severity in the C4-deficient mice.

Aims

- To investigate the roles of the alternative pathway molecule properdin using the passive antibody transfer model of anti-MPO vasculitis.
- To investigate a possible role for the lectin pathway in disease using the passive antibody transfer model in MASP2-deficient mice.

6.2 Methods

6.2.1 Passive antibody transfer model

An anti-MPO serum pool was generated from a group of MPO-deficient mice that had been immunised with 10µg MPO (RZ=0.44) as described in section 2.11.3. IgG was purified from this serum by ammonium sulphate precipitation and protein G chromatography as described in section 2.10. Purified antibody was used instead of anti-MPO containing serum as used in chapter 5, because this serum would also contain complement products such as properdin. In an experiment investigating the role of properdin in disease using a properdin-deficient mouse, this antibody purification step was necessary to remove contaminating complement. On days -8 and -4, all experimental mice (properdin-deficient and wild type, n=8 per group) were given 30µg of pegylated human GCSF (Neulasta®, Amgen, Cambridge, UK) by S.C injection. This GCSF remains in the circulation for longer than the GCSF used in chapter 5 and was administered every four days. This was used primarily to reduce stress on the mice. On day -1, the mice were bled from the saphenous vein for FACs analysis of circulating neutrophil concentrations and serum creatinine measurement as described in sections 2.20 and 2.13 respectively. Baseline urines were also collected. On day 0, each mouse was given 2mg of the purified IgG via I.V injection followed by 10µg of LPS (Enzo Life Sciences, Exeter, UK) on day 0 and day 3. They were also given additional doses of 30µg GCSF on days 0 and 4. Urine was collected prior to sacrifice on day 7. In the experiment investigating the role of MASP2 in disease, the MASP2-deficient mice and wild type controls were treated as above.

6.2.2 Histological assessment of disease

Histology was assessed as described in section 4.2.3.

6.2.3 Flow cytometry

Assessment of circulating neutrophil numbers at day -1 was performed as described in section 5.2.3.

6.2.4 Functional data

For the experiment investigating the role of properdin in disease, 24 hour urines were collected using metabolic cages and total albumin content was determined as described in section 2.12.2.

In regard to the experiment investigating the role of MASP2, spot urines were collected on day 7, immediately prior to sacrifice of the mice. This was done due to the unavailability of the metabolic cages in the facility housing these animals. Albumin creatinine ratios of the urines at days -1 and day 7 were calculated to assess albuminuria as described in section 2.13.

6.2.5 Statistics

Statistical analysis was performed using Graphpad Prism software (Graphpad software Inc, La Jolla, CA, USA). Data was analysed using unpaired t tests and expressed as mean ± SEM. The data presented in figure 6.8 B-C were logarithmically transformed prior to analysis in order to equalize the variances.

6.3 Results

6.3.1 Properdin-deficiency does not protect mice from anti-MPO induced glomerulonpehritis

In order to investigate the role of properdin in a mouse model of anti-MPO vasculitis, disease was induced in both wild type and properdin deficient mice and its extent was compared between the groups after 7 days. A preliminary experiment was carried out in order to determine the optimal number of doses of pegylated GCSF (Neulasta®, Amgen, Cambridge, UK) to give prior to disease induction. As the disease is known to be neutrophil dependent, we hoped to maximise the circulating neutrophil counts prior to anti-MPO administration. A group of four adult C57BL/6 mice were bled from the sapenous vein on day 0 (prior to GCSF treatment) and then given 30µg of pegylated GCSF on days 0 and 4. The mice were again bled from the sapenous vein on days 4 and 8 to assess circulating neutrophil counts by FACs. As shown in figure 6.1, neutrophils levels had increased from a mean of 0.3 ± 0.07 at day 0 to 17.5 ± 1 x 10^6 cells/ml by day 4. This had further increased to 22.2 ± 1.7 x 10^6 cells/ml by day 8 after an additional dose of GCSF on day 4. From this data, it was determined that two doses of 30µg GCSF was optimal before disease induction on day 0.

Both groups of mice (properdin-deficient and WT controls) received 30µg of pegylated human GCSF at days -8 and -4 and were bled from the saphenous vein for the assessment of neutrophil numbers at day -1. There was no difference in circulating neutrophil concentrations at this time point between the wild types and properdin knockouts as shown in figure 6.2A (mean concentrations of 1.42 ± 0.1 and 1.31 ± 0.05 x 10^7 respectively). When collecting baseline urines, one of the properdin-deficient mice died prior to assessment of circulating neutrophil numbers. Representative dot plots showing Ly6G positive neutrophil levels are shown in figure 6.2B. The percentage of glomerular crescents between the groups was similar as shown in figure 6.3A, with means of 27.5 ± 6.9 and 27.8 ± 8.2 for the wild type and properdin-deficient mice respectively. There was also no difference in the extent of glomerular macrophage infiltration as shown in figure 6.3B, with means of 5.6 ± 0.4 and 4.9 ± 0.6 macrophages per glomerular cross section in control and properdin-deficient mice respectively. Representative light microscopy and immunofluorescence of glomerular CD68

macrophages are shown in figures 6.3 C-D. There was no significant difference in albuminuria between the groups with means of 31.7 ± 1.5 and 32.5 ± 3.1µg/day in control and properdin-deficient mice respectively, as shown in figure 6.4A. There was no significant difference in haematuria between the groups at day 7 as shown in figure 6.4B. Mean serum creatinine concentrations were 18 ± 2.7 and 15.6 ± 2.7µmol/L in control and properdin-deficient mice respectively.

6.3.2 MASP2-deficiency exacerbates a murine model of anti-MPO glomerulonephritis

The experimental design with regard to the experiment investigating the role of MASP2 was identical to that described above. However, instead of collecting 24 hour urines, spot urines were collected on the day of sacrifice as the metabolic cages were unavailable in the facility housing these mice. By day -1, after two doses of 30µg pegyulated GCSF mean circulating neutrophil counts were 1.3 ± 0.09 and 1.1 ± 0.06 $x10^7$ cells/ml in control and MASP2-deficient mice respectively, as shown in figure 6.5A. Representative dot plots showing Ly6G positive neutrophils at day -1 are shown in figure 6.5B. At day 7 the MASP2 deficient group had significantly more glomerular crescents than controls with means of 30.9 ± 4 and 17.1 ± 2.8 percent respectively, as shown in figure 6.6A ($p<0.05$). There was no significant difference in glomerular macrophages numbers between the groups with means of 1.76 ± 0.18 and 2 ± 0.15 macrophages per gcs in controls and MASP2-deficient mice respectively, as shown in figure 6.6B. In order to verify this observation, a second method of macrophage scoring was employed which involved quantifying the percentage area of each glomerulus that stained positive for macrophages. A mean percentage was then calculated for each mouse. Using this method, there was still no detectable difference between the groups as shown in figure 6.6B. Representative light microscopy and immunofluorescence staining of CD68 positive macrophages are shown in figures 6.7 A-B. There was also a significantly higher amount of haematuria in the MASP2-deficient group compared to the control at day 7, as shown in figure 6.8A ($p<0.05$). Serum creatinine concentrations were significantly elevated in the MASP2-deficient mice at day 7 with means of 22.3 ± 4.2µmol/L compared to 11.9 ± 0.9 in controls, as shown in figure 6.8B ($p<0.01$). However, they also had significantly higher levels at day -1 ($p<0.05$) which is most likely due to the MASP2-deficient mice being, on average, slightly larger (mean 21.4 ±1.1 vs 16.4±3.8 grams). While every effort is made to weight match the mice in each of the groups, slight variations in weight do occur. Albumin

creatinine ratios at day 7 were significantly elevated in the MASP2-deficient mice compared to controls with means of 222.4 ± 48.39 and 114.2 ± 23.31 mg/g respectively ($p<0.05$), as shown in figure 6.8C.

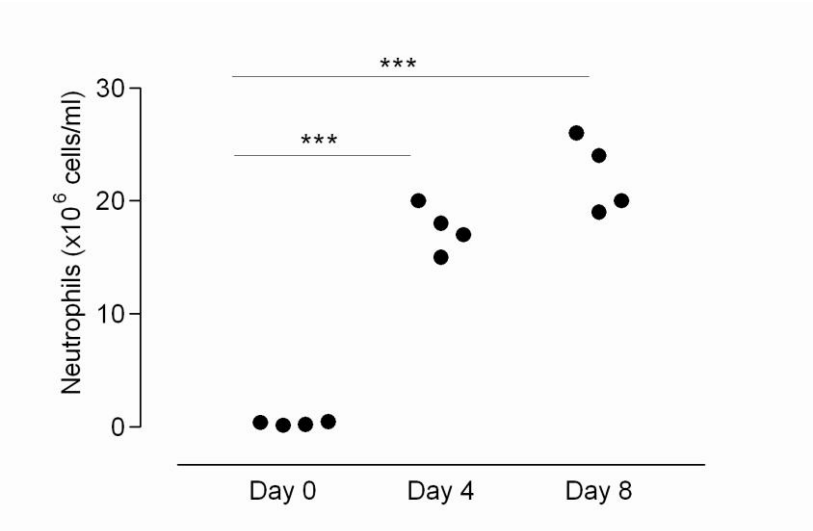


Figure 6.1 Circulating Ly6G-positive neutrophil numbers after one (day 4) or two (day 8) doses of pegylated GCSF. Each symbol represents a separate mouse. Data was analysed using a paired t test. ***p<0.001

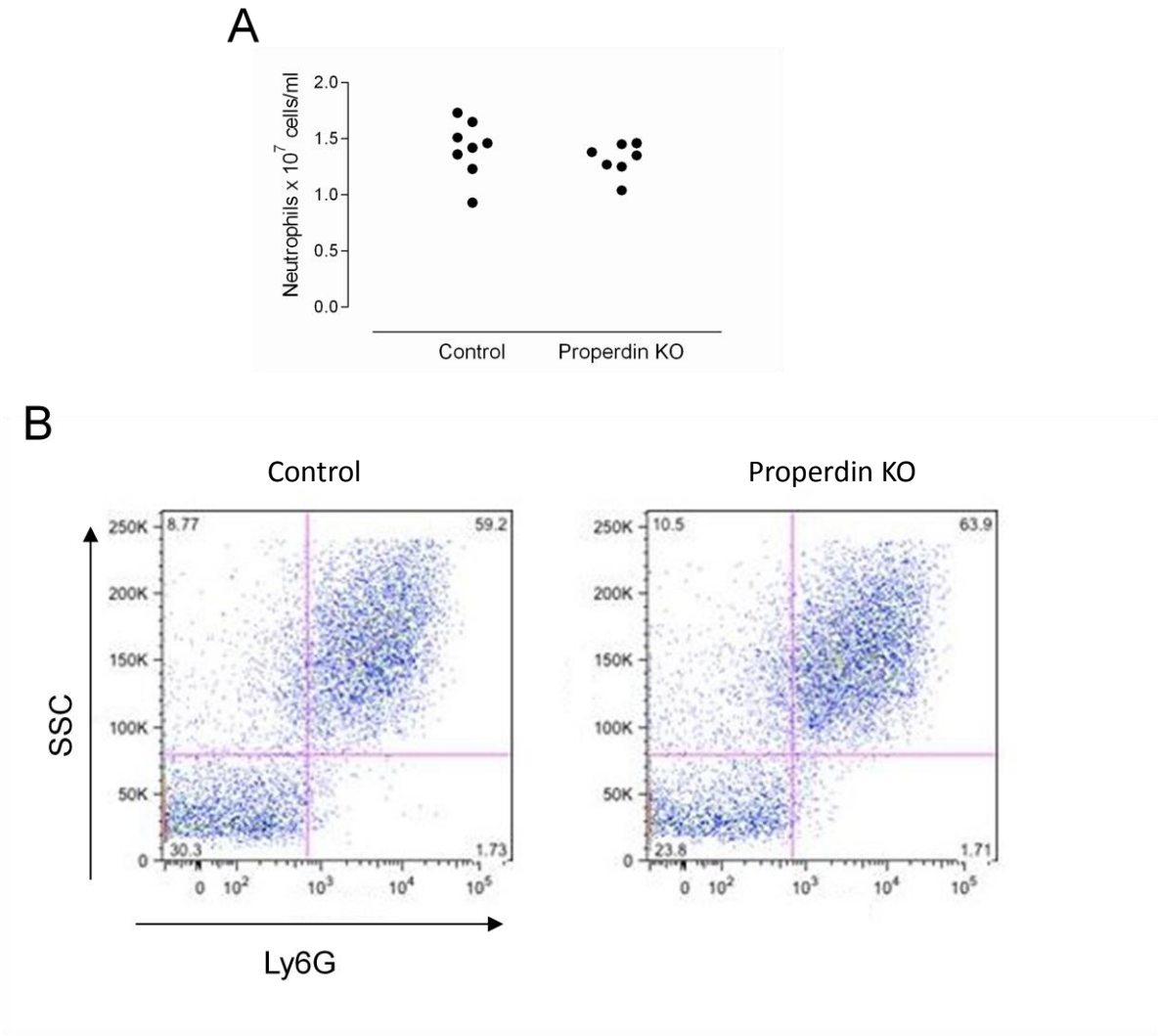


Figure 6.2 (A) Peripheral blood neutrophil counts of control and properdin-deficient mice at day -1 (after two doses of 30µg pegylated human GCSF). There is no significant difference between the groups. Each symbol represents a separate mouse. (B) Representative dot plots showing Ly6G positive neutrophils expressed as a percentage of total leukocyte number in both control and properdin-deficient groups.

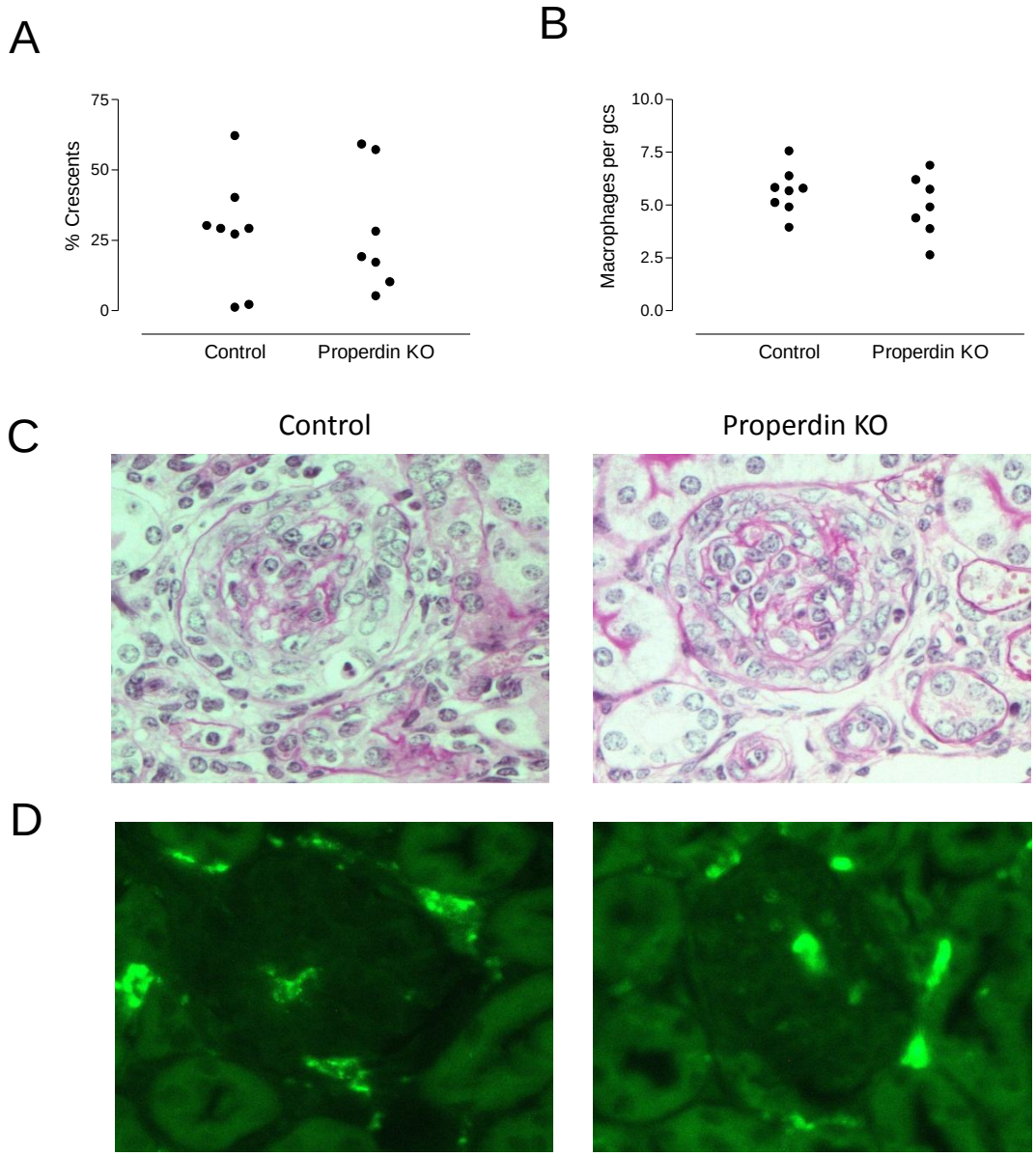


Figure 6.3 (A) Glomerular crescent counts in control and properdin-deficient mice at day 7 after disease induction. (B) Glomerular macrophage numbers in the same mice at day 7. Each symbol represents a separate mouse. There is no significant difference between groups with regard to glomerular crescent counts or macrophage infiltration. (C) Representative light microscopy and (D) immunofluorescence staining of CD68 positive macrophages at day 7 (x400 magnification).

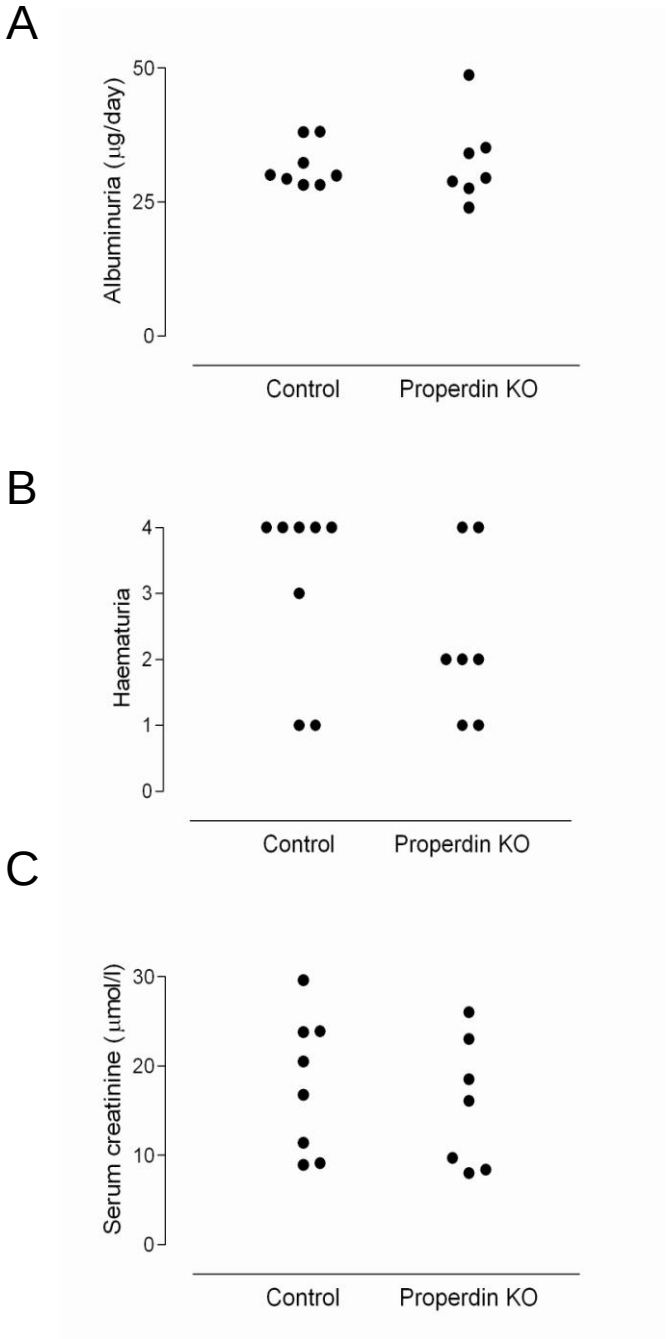


Figure 6.4 Properdin-deficiency does not influence anti-MPO induced glomerulonephritis. Functional data at day 7 of the same experiment. (A) There was no difference in albuminuria levels between both groups. There were no significant differences in (B) haematuria and (C) serum creatinine between the groups.

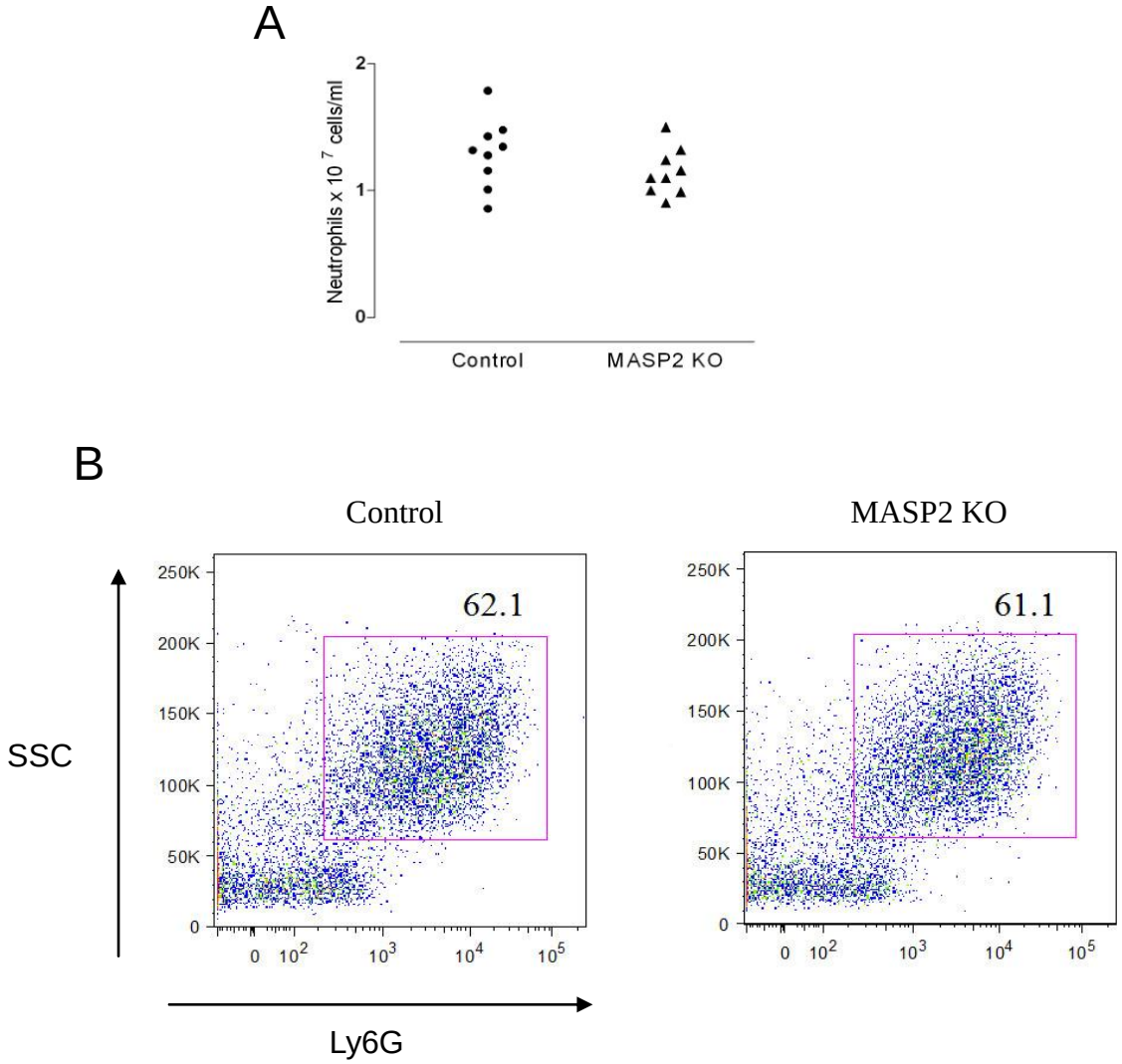
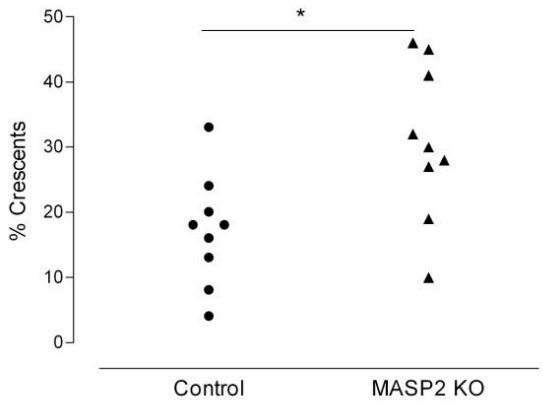


Figure 6.5 (A) Peripheral blood neutrophil counts of control and MASP2-deficient mice at day -1 (after two doses of 30µg pegylated human GCSF). There was no significant difference between the groups. Each symbol represents a separate mouse. (B) Representative dot plots showing Ly6G positive neutrophils expressed as a percentage of total leukocyte numbers in both control and MASP2-deficient groups.

A



B

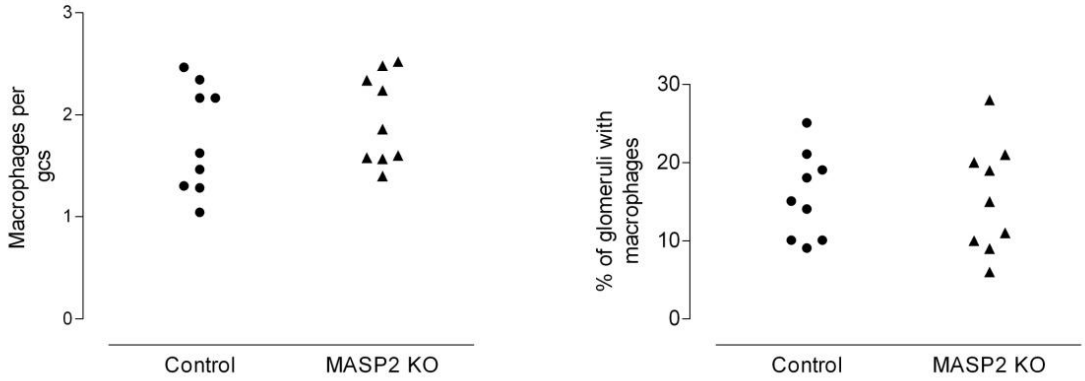


Figure 6.6 MASP2 deficiency exacerbates a murine model of anti-MPO glomerulonephritis. (A) MASP2-deficient mice had significantly more glomerular crescents than the control group 7 days after disease induction ($p<0.05$). (B) There was however no significant difference in glomerular macrophage infiltration between the groups as determined using two separate methods of quantification. Data was analysed using an unpaired t test.

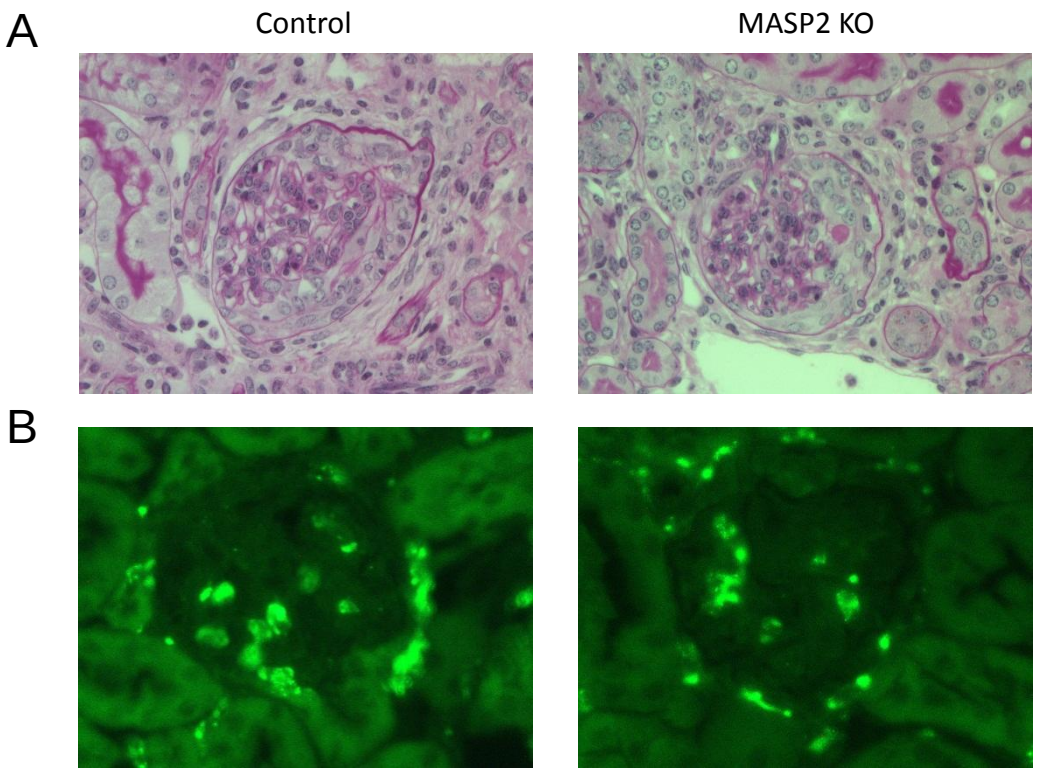


Figure 6.7 MASP2 deficiency exacerbates crescentic glomerulonephritis in a mouse model of anti-MPO vasculitis. (A) Representative light microscopy showing glomerular crescents and (B) immunofluorescence showing glomerular $CD68^{+}$ macrophages in both groups of mice. All pictures taken at x400 magnification.

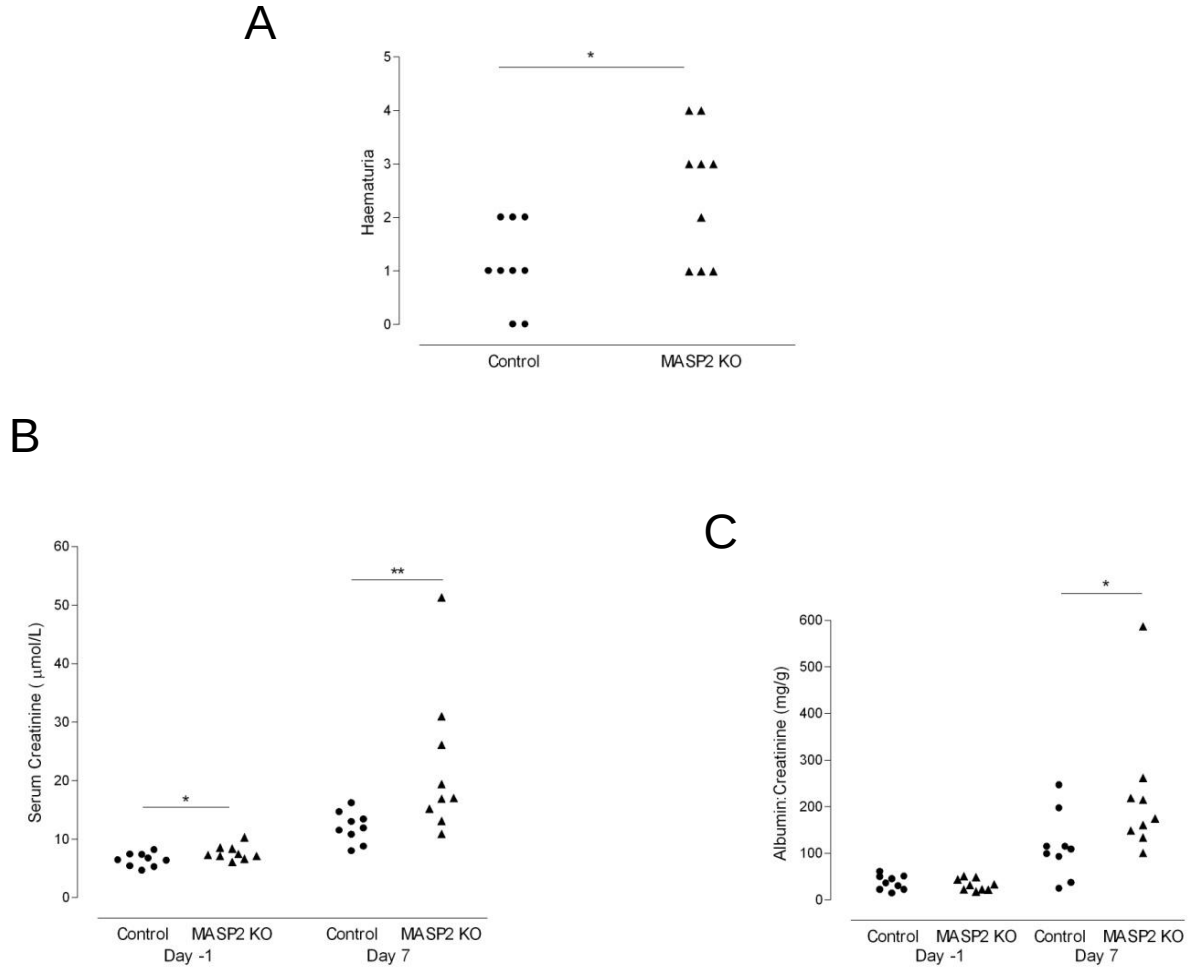


Figure 6.8 MASP2 deficiency causes a significant increase in haematuria, serum creatinine and albumin creatinine ratio. (A) MASP2-deficient mice have significantly more haematuria than controls 7 days after disease induction ($p<0.05$). (B) There was a significant difference in serum creatinine between the groups at both day -1 ($p<0.05$) and day 7 ($p<0.01$). (C) There was also a significant elevated albumin creatinine ratio in the MASP2-deficient mice compared to controls at day 7 ($p<0.05$). Data was analysed using an unpaired t test.

6.4 Discussion

To investigate the potential roles of properdin and MASP2 in a mouse model of ANCA vasculitis, mice with genetic deficiencies in these molecules were utilized. Using the passive antibody transfer model, properdin-deficient mice developed renal disease of equal severity to the control group, thus eliminating a potential role for properdin in initiating or amplifying the alternative pathway in the context of this model. There still remains however, various other candidate molecules which are known to be released by activated neutrophils and are capable of activating C3 and C5 in normal serum. These range from oxygen radicals, myeloperoxidase products, and proteases [289, 290, 340]. It is possible that any of these factors, acting either independently or in combination could cause the initial activation of C3 which is then quickly amplified by the alternative pathway. C5a is generated in this process and has been shown to be required for the induction of disease [288]. By blocking C5 with a C5 inhibiting antibody, mice were protected from disease induced by anti-MPO IgG. C5a is also known to have a priming effect on neutrophils which acts through the C5a receptor. This process has been shown to be of particular significance in the bone marrow transplant model of anti-MPO vasculitis as MPO-deficient mice which had been immunized with MPO and later engrafted with C5a receptor deficient bone marrow had significantly milder disease than controls. This process represents a feedback loop in which neutrophil activation induces the release of activated complement products which then act as a priming agent for neutrophil activation. Another way in which the alternative pathway may be initiated is by the release, from neutrophils, of the proteases kallikrein and elastase. These molecules share structural similarities to factor D, the molecule responsible for factor B cleavage [341]. Kallikrein has been shown to cleave factor B thus leading to activation of the alternative pathway C3 convertase [342]. A recent report demonstrated the importance of MASP2 in disease severity using a mouse model of ischemia reperfusion injury [315]. MASP2 is known to be essential for the activation of the lectin pathway and its deficiency resulted in a marked reduction in disease severity. Therefore, a similar effect was anticipated in regard to the mouse model of anti-MPO vasculitis. Interestingly, MASP2-deficiency led to a significant exacerbation of renal disease when compared to controls in the context of the passive transfer model. MASP2-deficient mice had significantly more glomerular crescents, haematuria, serum creatinine and albumin creatinine ratios than

controls at day 7. This data suggests that in the context of this model, MASP2 has a somewhat protective effect. The exact mechanisms responsible for this effect are not clear. The possibility of whether this effect on disease severity is mediated through the alternative pathway remains to be determined and is a priority for future study. By comparing disease severity in factor B knockout mice to that of MASP2/factor B knockouts one could determine if the exacerbatory effect of MASP2-deficiency acts through the alternative pathway. If more extensive disease was seen in MASP2/factor B-deficient mice than in the factor B knockouts it would suggest that MASP2-deficiency is exacerbating disease via a mechanism independent of complement activation. Investigating whether MASP2/C3 knockouts develop disease to a similar extent to C3 knockouts would shed light on this question.

The primary role of the MASP proteins is believed to be the activation of complement components C4 and C2 in a manner homologous to the classical pathway proteases C1r and C1s. However, the exact function of each of the MASP proteins remains unclear. MASP1 is thought to activate MASP2 which in turn acts as the primary activator of C4 and C2 in the lectin pathway. There is as yet, no known function or target of MASP3. In recent years, additional functions of the MASP proteins have begun to emerge. One of these appears to be a link to the coagulation system. For example MASP1 has been shown to activate pro-thrombin and factor XIII and can lead to fibrin formation in a thrombin-dependent manner [343]. In addition, activated MASP2 is known to cleave pro-thrombin into thrombin thus leading to the formation of a fibrin clot [344]. The absence of MAPS2 may lead to a possible defect in the coagulation cascade at sides of infection or immune mediated injury which may contribute to tissue injury.

Chapter 7

Discussion

7.1 Summary of results and future work

7.1.1 Chapter 3 – GCSF concentration in acute ANCA-vasculitis and its role in neutrophil priming

This chapter showed that ANCA vasculitis patients presenting with acute disease have significantly higher serum concentrations of GCSF than those of age matched healthy controls. The clinical features and extent of treatment of each of the acute patients was carefully documented as this would be expected to have an impact on our study. Serum GCSF concentrations are generally low in a healthy population (<50pg/ml) and thus we found that a commercially available GCSF detection ELISA lacked the sensitivity required to accurately measure GCSF. We therefore opted for an ultra-sensitive electro-chemiluminescent assay. To investigate the implications of this elevation in GCSF concentration we sought to determine if it could prime isolated human neutrophils for respiratory burst in response to stimulation with a monoclonal anti-MPO or anti-PR3 antibody. GCSF was shown to have a priming effect for anti-MPO stimulation but not for anti-PR3. This priming effect was not as prominent as that of TNFα and was seen in some but not all of the blood donors used. The reason for this difference remains unclear. It may be due to differences in the priming mechanisms of GCSF and TNFα. Unlike TNFα, GCSF priming was shown to be unable to induce an upregulation of MPO and PR3 expression on the surface of isolated neutrophils [188, 345].

7.1.2 Chapter 4 – Setting up of various murine models of anti-MPO vasculitis

Chapter 4 focused on the setting up of several mouse models of anti-MPO vasculitis which had not been previously set up in the lab. As all of these models required the immunisation of MPO-deficient mice with mouse MPO, we first had to identify a suitable myeloid cell line that produced it. We first tested the commonly used cell lines (WEHI-3b and 32Dcl3) used by other groups interested in vasculitis research but found them inadequate for our needs. The MPRO cell line however, produced MPO from early stages of maturity and large batches of cells were cultured. MPO was then purified from these cells by concanavalin-A sepharose

and cation exchange chromatography. The purity of heme proteins is assessed by their RZ value where RZ>0.84 is considered to be of high purity.

Our attempts to develop the bone marrow transplant model of anti-MPO vasculitis were unsuccessful. This was most likely due to the sharp decline in serum anti-MPO levels after irradiation. Although antibody producing plasma cells are known to be relatively radiation resistant it remains a distinct possibility that they were destroyed during irradiation. Although the dose of radiation used (9Gy) was the same as that used by the Schreiber group, slight variations between irradiators means that the dose may have been slightly greater, resulting in the observed effects. Perhaps by titrating down the dose of radiation the anti-MPO levels would remain elevated in the serum and be sufficient to induce glomerulonephritis. The purity and quantity of MPO used for immunisations may also be an important factor for disease induction as mice immunised with 10µg of an MPO preparation of RZ=0.62 purity had significantly higher levels than those immunised with 5µg of an RZ=0.31 preparation.

The setting up of a modified version of the splenocyte transfer model was met with mixed success. This model was initially set up in Rag2-deficient mice and induced severe disease with approximately 80% of glomeruli being crescentic. However, the model was hindered by the presence of immune deposits in the glomeruli, complicating its use as a model of pauci-immune crescentic glomerulonephritis. This is most likely caused by the homeostatic proliferation of the injected lymphocytes in the Rag2-deficient recipients. By using sublethally irradiated wild type recipients there was a marked absence of glomerular deposits. However, unfortunately these mice failed to develop glomerular disease and by the end of the experiment the anti-MPO titres were markedly lower than those of a known high responder. The reasons for this are unclear. Perhaps the anti-MPO splenocytes were being regulated in the wild type recipients thus inhibiting anti-MPO production. Alternatively, had the antibody responses been greater in the splenocyte donor mice, it may have resulted in higher anti-MPO titres in the recipients, and thus led to glomerular disease. Despite its drawbacks, the splenocyte model induced the most severe disease seen in any model of anti-MPO vasculitis and thus it remains an appealing model. If severe disease could

be achieved in this way without glomerular immune depositis this would be a very useful model.

This chapter also described a potentially new model of anti-MPO vasculitis based on the transplacental transfer of anti-MPO IgG from the immunised MPO-deficient mother to neonates during gestation. By immunising female MPO-deficient mice with MPO and then mating them with wild type males, it was hoped that the anti-MPO IgG that the neonates received from the mother would bind to their MPO sufficient neutrophils and cause glomerular disease. This approach however, failed to induce glomerulonephritis in these mice at weaning age. In theory, this model would be extremely useful if successful as embryos of various types of genetically altered mice could be implanted into immunised females. In this way, the roles of many candidate molecules could be investigated.

The passive transfer model was successfully set up in this chapter with crescentic glomerulonephritis and macrophage infiltration observed in wild type mice that had been injected with an anti-MPO containing serum pool. Although disease is generally mild in this model its simplicity combined with its reliability and ability to cause disease in a short time period made this the preferred disease model used in this thesis.

7.1.3 Chapter 5 – GCSF administration exacerbates a murine model of anti-MPO vasculitis

This chapter detailed the effect of GCSF administration on the passive antibody transfer model. It showed that GCSF administration caused a significant exacerbation of disease with increases in glomerular crescent scores, macrophage infiltration, haematuria and serum creatinine. *In vivo* experimentation showed that GCSF administration caused a significant upregulation of surface CD11c expression and shedding of L-selectin on mouse neutrophils. While LPS administration caused L-selectin shedding and upregulation of CD11b it did not induce CD11c upregulation. In light of a recent study implicating LFA1 in anti-MPO neutrophil recruitment to the glomeruli [334] it is likely that CD11c upregulation in response to GCSF is having an important role in disease. As to whether the elevlated levels of GCSF seen in patients with acute ANCA vasculitis (discussed in chapter 3) is having an effect is unclear. A key experiment is to investigate the role of endogenously produced GCSF in

disease using the passive antibody transfer model. This could be done using a commercially available GCSF blocking antibody and is a priority for future study.

7.1.4 Chapter 6 – MASP2 deficiency exacerbates the passive transfer model of anti-MPO vasculitis

This chapter utilized the passive transfer model with the use of GCSF to investigate the roles of properdin and MASP2 in the pathogenesis of anti-MPO vasculitis. A pegylated form of GCSF was used for both experiments in this chapter. This GCSF stays in the circulation for longer than that used in chapter 5 and required injection every four days, as compared to daily. It was used primarily in order to reduce stress on the animals. This GCSF did however cause much larger increases in circulating neutrophil numbers. Despite this, the extent of disease seen in wild types was in general no more severe than that seen in the experiment detailed in chapter 5.

In vitro experiments reported that TNFα primed neutrophils stimulated with ANCA release a factor into the supernatant that possesses the ability to cause complement activation in normal serum as measured by the presence of C3a and C5a [46] This factor has yet to be identified. Activated neutrophils are known to secrete components of the complement system such as C3, C6, C7 and factors that are unique to the alternative pathway, namely factor B and properdin [291, 293, 346]. Properdin has been implicated as a candidate molecule involved in the initial steps of complement activation, through stabilisation of the alternative pathway C3 convertase. As properdin is known to be contained within and released from activated neutrophils [293] it was hypothesized that it may play an important role in the activation of complement in ANCA vasculitis by stabilizing the alternative pathway C3 convertase. However, in the context of the passive antibody transfer model of anti-MPO vasculitis, properdin-deficiency did not lead to any reduction in disease severity assessed both histologically and functionally. This suggests that properdin does not play a significant role in ANCA vasculitis. However, many other neutrophil derived candidate molecules exist including oxygen radicals and proteases [289, 340, 347, 348] and we plan to investigate this further.

Previous reports implicated the alternative but not the classical or lectin pathways in the passive transfer mouse model of anti-MPO vasculitis, as factor B-deficient but not C4-deficient mice were protected [287]. However, with the recent discovery of a MASP2 dependent C4 bypass mechanism of complement activation via the lectin pathway, the possibility of lectin pathway involvement has re-emerged [315]. In this chapter, disease was induced in MASP2-deficient mice with the hypothesis that its severity would be reduced compared to controls, thus implicating a pathologic role for the lectin pathway. However, the opposite proved to be the case. MASP2-deficient mice developed significantly more severe disease with increased glomerular crescent scores combined with elevated serum creatinine and albumin creatinine ratios. The mechanism behind this effect is currently unclear, but it appears that MASP2 possesses some protective effect. Further investigation is required to determine whether this effect is mediated through the complement system or more specifically, through the alternative pathway. We plan to investigate this by comparing disease severity (using the passive antibody transfer model) in MASP2/factor B-deficient mice to that of factor B-deficient mice. If the extent of disease was more severe in the MASP2/factor B-deficient group it would suggest that the effect is due to a mechanism other than the alternative pathway. To confirm that this possible effect was complement related we would compare disease severity in MASP2/C3-deficient and C3-deficient mice.

Summary of future work

- To block endogenous GCSF in the passive antibody transfer model
- To investigate complement activation by neutrophils in response to ANCA *in vitro*.
- To further investigate the mechanism behind the increase in disease severity in MASP2-deficient mice. We will compare disease severity (using the passive antibody transfer model) in MASP2/factor B-deficient and factor B-deficient mice.

7.2 Limitations

7.2.1 *In vivo* models

Much of what is known about the pathogenesis of ANCA-associated disease has been derived from the mouse models. These models have proven to be extremely useful and have been used to demonstrate the pathogenicity of anti-MPO antibodies and the

important contribution of neutrophils. Furthermore, the data relating to the contribution of complement have come from studies in mice. However, these models do come with drawbacks which lead to significant limitations in their use. One substantial limitation to the animal studies is the absence of a model based on immunity to PR3. Several attempts to develop a mouse model have been unsuccessful. The reasons for this remain unclear but may relate to differences in the structure and expression patterns of human and rodent PR3. Attempts to develop a humanised mouse model, in which NOD-scid IL2rγ deficient mice which have been reconstituted with a human immune system are injected with purified human ANCA, have been made. Although these mice have been shown to possess functional human neutrophils [349] the induction of crescentic glomerulonephritis has not been reported. One study has however reported mild disease in this model [147].

The passive antibody transfer model which was utilized throughout this thesis has proven to be the most reliable model of anti-MPO vasculitis. However, the mild nature of the glomerulonephritis, and the requirement of LPS administration to increase disease severity, place significant limitiations on the model. Disease severity was increased significantly by GCSF administration as shown in chapter 5. This was speculated to be due to a combination of both an increase in circulating neutrophil numbers and a GCSF priming effect on neutrophils. Combined with the observation in chapter 3 of a significantly increased GCSF concentration in patients with acute ANCA vasculitis it is hypothesized that endogenous GCSF may play an exacerbatory role. We plan to investigate this possibility by blocking endogenous GCSF in the passive transfer model. However, the mild nature of disease in the absence of GCSF makes this difficult. There is therefore a need to develop the model further so that a severe glomerulonephritis could be induced by anti-MPO IgG without the need for exogenous GCSF or LPS. This would require the preparation of a batch of anti-MPO IgG with the ability to induce more severe disease. To accomplish this it may require the immunisation of mice with greater quantities of pure preparations of MPO. This in itself poses difficulties of a technical nature as preparing larger quantities of MPO is both labour intensive and financially expensive. The development of a monoclonal antibody capable of inducing disease would be a major advantage in the models but is currently not yet available.

The bone marrow transplant model has an advantage over the passive antibody transfer model in that disease can be induced without the need for LPS administration. This model however, in our hands has proven to be difficult to set up possibly relating to the variable antibody responses seen in the immunised mice. The splenocyte transfer model, although reporting crescents in approximately 80% of glomeruli is hindered by immune complex deposition. In our hands this model also failed to induce disease, although this may be due to the splenocytes being injected into sublethally irradiated wild type mice instead of Rag2-deficient mice.

Several models described above, have been developed based on immunity to MPO but all are induced models and rely on the production of MPO antibodies in response to immunisation of MPO-deficient mice. Therefore, these are not, by definition, autoimmune models. The spontaneous crescentic glomerulonephritis/Kinjoh (SCG/Kinjoh) strain is known to possess anti-MPO antibodies and develop spontaneous glomerulonephritis. They however, also possess other autoantibodies and glomerular immune complexes and are thus not ideally suited for ANCA vasculitis research. The only currently available model which includes both the humoral and cellular immunity to MPO was achieved by the immunisation of wild type mice with human MPO. These mice develop antibodies against human MPO which cross reacts with mouse MPO. This cross reactivity alone is insufficient to induce disease however, and the additional administration of glomerular binding antibody is required to attract neutrophils to the glomeruli. This model is unique in that it has the advantage of an autoimmune response to MPO. It has proven to be a useful model and has provided data relating to the role of the autoimmune T cell response in inducting glomerulonephritis in the absence of anti-MPO antibodies. However, the requirement for a heterologous glomerular binding antibody to attract neutrophils into the glomerulus is a significant drawback since immune complexes are not usually present in the glomeruli of patients with ANCA vasculitis.

7.2.2 *In vitro* assays

The neutrophil respiratory burst assays shown in chapter 3 were carried out using isolated, primed human neutrophils stimulated with a monoclonal anti-MPO or anti-PR3 antibody. Monoclonal antibodies were used for stimulation as experiments using purified polyclonal

human ANCA gave variable results (data not shown). Not all ANCA purified from patient plasma had the ability to induce respiratory burst. The reasons for this are unclear but may relate to the presence of non specific IgG. As ANCA induced respiratory burst has been shown to be dependent on Fc receptor engagement it is possible that non specific IgG is blocking these Fc receptors [72-74]. Perhaps by affinity purifying patient antibody a more reproducible respiratory burst assay could be set up. Particular caution would have to be taken to avoid endotoxin contamination of the antibody preparation however.

7.3 Conclusions

ANCA-associated vsculitis is a complicated autoimmune inflammatory disease involving both the humoral and cellular arms of the immune system. Infection is thought to exacerbate disease through the production of the proinflammatory cytokine TNFα which primes neutrophils for respiratory burst. In this thesis it was shown that another cytokine, GCSF, is elevated in patients with active ANCA vasculitis and that it could prime isolated human neutrophils for anti-MPO induced respiratory burst. The subsequent work of this thesis focused on the setting up of, and assessment of GCSF administration in, a mouse model of anti-MPO vasculitis. It also focused on the effects of properdin and MASP2 deficiencies in the mouse model and the findings include the following:

- GCSF administration exacerbates a mouse model of anti-MPO vasculitis
- Properdin-deficiency does not influence disease severity in the mouse model
- MASP2-deficiency led to a more severe disease severity

The murine model of anti-MPO vasculitis presents a number of technical challenges, is difficult to establish and usually results in mild disease. Few reports have shown disease severe enough to cause kidney injury with a raised serum creatinine, as in these studies. Therefore the administration of GCSF may be a useful strategy to induce more severe and robust disease in the model. There have been case reports of GCSF administration exacerbating disease in patients with ANCA vasculitis [197]. Although such reports cannot prove causation, results presented here give experimental and mechanistic data to support the suggestion that GCSF administration can exacerbate disease in patients. It is a

suggestion that exogenous GCSF should be administered with caution in patients with ANCA vasculitis and in addition endogenous GCSF may be a potential therapeutic target.

The mechanisms of complement activation in ANCA vasculitis remain to be solved. However it is closely related to neutrophil activation. The mechanism by which neutrophils activate the complement system is still unknown but results presented here suggest that properdin is not involved. Furthermore MASP2 may have some protective role in ANCA vasculitis, through a mechanism which is currently unknown, and future work is planned to investigate this further.

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