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The Role of Complement in Pre-Clinical Models of ANCA-associated Vasculitis

Dr Jonathan Dick
BA. MBBS

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Department of Medicine,
Southern Clinical School
Monash University

Abstract

The anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV) are diseases in which autoimmunity to the neutrophil granule proteins myeloperoxidase (MPO) or proteinase-3 (Pr3) results in multi-organ damage including rapidly progressive glomerulonephritis. Murine models have demonstrated a critical role for complement component C5a in promoting neutrophil activation by anti-neutrophil cytoplasmic antibodies. This discovery has led to clinical trials of an oral C5a receptor 1 (C5aR1) antagonist. In this thesis, work using murine models is presented which demonstrates that in addition to its effect on neutrophils, signalling through the C5aR1 on antigen presenting cells promotes anti-MPO cellular immunity and humoral immunity. This C5aR1 mediated potentiation of nephritogenic Th1 response and reduction of anti-inflammatory T regulatory cells results in exacerbation of anti-myeloperoxidase glomerulonephritis. This suggests that therapeutic targeting of this receptor may have beneficial immunomodulatory effects in addition to its known effects on neutrophil behaviour.

The C3 split product C3a mediates its effects through the C3a receptor (C3aR). The C3aR has been reported to have diverse effects in murine models of inflammation and immunity. In this thesis, the role for the C3aR in the generation of both anti-MPO autoimmunity and glomerular injury was examined using two complimentary models of anti-myeloperoxidase glomerulonephritis. Whilst the C3aR did not modulate glomerular injury in either model, signalling through the C3aR did promote macrophage infiltration to the glomerulus and moderate ANCA production. This change in humoral immunity was not due to any measurable changes in B cell development in the spleen or bone marrow, nor did the C3aR affect T cell mediated immunity. These data suggests that the C3aR has minor pro and anti-inflammatory effects in this disease and overall does not support therapeutic targeting of C3aR.

The alternative pathway of complement plays an important role in several human diseases. There are data to suggest that the alternative pathway generates the complement mediated priming required for neutrophil activation by ANCA. To examine the possibility for additional roles of the alternate pathway in this disease, the generation of anti-MPO autoimmunity and subsequent T cell mediated anti-MPO glomerulonephritis was investigated in complement factor B deficient mice. Absence of the alternative pathway altered neither autoimmunity nor glomerular injury in this model, suggesting that any role for the alternative pathway in this disease is confined to its effect on neutrophils.

The classical pathway of complement is not thought to play a pathogenic role in AAV. However, evidence against its role stems from a murine model in which classical pathway activity is very weak. The potential for classical pathway activation by ANCA to initiate the positive feedback loop of complement and neutrophil activation was therefore investigated. *In vitro*, sera from the majority of patients with anti-MPO AAV fixed C1q on single antigen beads and was therefore capable of activating the classical pathway. In addition, purified IgG from patients with AAV induced C3 deposition on human neutrophils suggesting that ANCA targeting of neutrophils results in surface C3 activation. To determine whether the initial pathway of complement activation was via the classical pathway, an anti-C1s monoclonal antibody was used to specifically inhibit this pathway. Classical pathway inhibition resulted in profound decrease in neutrophil surface C3 in neutrophils treated with either ANCA IgG or control IgG, suggesting that in this experimental system there is a significant classical pathway activation. Further modification is therefore required for this assay to specifically examine the role of ANCA activated classical pathway in neutrophil activation.

In summary this thesis builds on the current knowledge of the role of complement in AAV by suggesting novel roles for the complement system acting through the C5aR1, the C3aR and the classical pathway in this disease.

Thesis including published works General Declaration

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

This thesis includes 2 original papers submitted for publication. The core theme of the thesis is the role of the complement system in pre-clinical models of ANCA associated vasculitis. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Medicine, Monash University under the supervision of Professor Stephen Holdsworth and Professor Richard Kitching.

Consistent with Monash University guidelines the chapters in paper format are presented with inclusive pagination and referencing to give a uniform appearance to the thesis. The presentation of papers as thesis chapters does result in some minor, unavoidable repetition. Efforts have been made to keep this to the minimum necessary.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.
In the case of the submitted papers my contribution to the work involved the following:

Chapter	Publication title	Publication status*	Nature and extent (%) of student's contribution	Co-Authors' names and contribution (%)
2	The C5a Receptor 1 promotes autoimmunity, neutrophil dysfunction and injury in experimental anti-myeloperoxidase glomerulonephritis	Submitted-manuscript currently under revision (Kidney International)	Designed and performed all experiments with exception of intravital microscopy. Analysed the data and wrote the manuscript. (66%)	**P. Gan-** Assistance with designing and performing experiments (4%) **D. Odobasic** Assistance with designing and performing experiments (3%) **M. Alikhan** Data analysis and interpretation (1%), **S. Loosen** Performing intravital microscopy experiments (4%) **P. Hall:** Preparation of mice for intravital microscopy experiments (3%), **C Westhorpe-** analysed data(2%) **A. LI** performed intravital microscopy experiments (1%) **J. Ooi-** Contributed reagents (1%) **T.Woodruff** Contributed reagents (1%) **C. Mackay:** Contributed reagents (1%) **A.R. Kitching-** Designed

				experiments and wrote the manuscript (4%) **M. Hickey:** Designed experiments, analysed data and wrote the manuscript (4%) **S. Holdsworth:** Designed experiments and wrote the manuscript (5%)
3	The C3aR promotes macrophage infiltration and regulates ANCA production but does not affect glomerular injury in experimental anti-myeloperoxidase glomerulonephritis.	Submitted (PLOS one)	Performed all the experiments, analysed the data, wrote the manuscript. (85%)	**P. Gan**. Assistance with designing and performing experiments (5%). **A.R. Kitching**. Designed experiments and wrote the manuscript (5%) **S. Holdsworth.** Designed experiments and wrote the manuscript. (5%)

Student signature: [redacted] **Date: 20/07/17**

Dr Jonathan Dick

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

Main Supervisor signature: [redacted] **Date: 20/07/17**

Professor Stephen Holdsworth

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Chapter 1: Introduction

The ANCA associated Vasculitides

The anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAVs) are necrotising vasculitides primarily affecting small vessels. They include the diseases clinically described as microscopic polyangitis (MPA), Granulomatosis with polyangitis (GPA; previously Wegner's Granulomatosis) and Eosinophilic granulomatosis with polyangitis (EGPA; previously Churg-Strauss syndrome)[1]. They are often associated with the presence of autoantibodies against the neutrophil granule proteins myeloperoxidase (MPO) or proteinase 3 (Pr3).

Epidemiology and Clinical Presentation

The AAVs have an annual incidence of approximately 1:100,000 population, the disease is associated with aging, with peak incidences in the ages 50s-70s. There is both ethnic and regional variation in disease incidence. In the Northern Hemisphere GPA is more common in northern latitudes and MPA in southern latitudes[2]. In Asia, anti-MPO disease is by far the predominant cause of AAV[3, 4]. The AAVs cause significant mortality with 1 and 5 year survival of 88% and 76% respectively in a European cohort which equates to a mortality ratio of 2.6 compared to the general population[5]. Survivors are left with a heavy burden of morbidity related to both the disease and treatment with approximately 90% having irreversible damage in at least one organ[6].

The AAVs have traditionally been seen as an overlapping spectrum of a similar disease process marked by small vessel vasculitis. Multiple organs can be involved including the kidneys, lungs, skin, GI tract, ENT system and nervous system. Two of the most feared manifestations are rapidly progressive pauci-immune crescentic glomerulonephritis in the kidney and pulmonary haemorrhage in the lung. Additional features prominent in particular clinical disease

groups are the necrotising upper airway granulomas characteristic of GPA and the asthma and eosinophilia of EGPA. Whilst due to their rarity the AAVs have often been studied together in clinical trials, it is now clear the diseases classified by their autoantibody specificity often have a different clinical presentation, different genetic associations,[7] and divergent disease courses[8]. Disease pathogenesis, natural history, organ system involvement and optimal treatment regimens are therefore likely to vary between the diseases when classified by autoantibody specificity.

Current treatment of AAV

Current treatment for AAV involves immunosuppression, usually combined with adjunct medications to ameliorate some of the side effects of treatment. Untreated, AAV is almost universally fatal. In what remains the seminal advance in treatment of AAV, reports from the National Institute of Health of durable remission in the majority of patients treated with cyclophosphamide and prednisolone demonstrated the efficacy of immunosuppression[9] and treatment regimens based on this remain the most commonly used worldwide today. However, cumulative cyclophosphamide dose is associated with significant toxicity including infertility, myelosupression and increased malignancy risk. As rare diseases, multinational collaborative efforts have been required to conduct the randomised clinical trials that provide evidence based therapeutic strategies for AAV. These have resulted in important advances by defining safer, more effective treatment strategies. The key results of these trials are summarised in table 1.

Table 1

Trials of Induction Therapies		
Trial name	**Design**	**Key Outcomes**
NORAM[10]	Oral cyclophosphamide 2mg/day vs. oral methotrexate 20-25mg/week for early AAV without severe renal impairment/ critical organ manifestations.	Methotrexate is an alternative to cyclophosphamide for early disease although its use is associated with greater risk of relapse in long-term follow-up and more steroid + cyclophosphamide use after the trial[11].
MEPEX[12]	Standard therapy + plasma exchange or I.V. methylprednisolone in patients with severe renal involvement (creatinine >500 μmol/L)	Plasma exchange associated with improved renal survival up to 1 year, this difference was not sustained in longer-term follow-up[13].
CYCLOPS[8]	Comparison of pulsed I.V. cyclophosphamide 15mg/kg 2-3 weekly vs. daily oral cyclophosphamide 2mg/kg as induction regimens.	I.V. cyclophosphamide associated with 50% less cumulative dose, but increased risk of relapse, no difference in overall survival.
RAVE[14]	Rituximab (4x375mg/m^2) vs. daily oral cyclophosphamide (2mg/kg) induction in severe AAV	Rituximab non-inferior to cyclophosphamide and superior in patients with relapsing disease.
RITUXIVAS[15]	Rituximab (4x375mg/$m^{2)}$ + 2 doses I.V. cyclophosphamide 15mg/kg vs. standard I.V. cyclophosphamide 15mg/kg/2 weekly for 3-6 months for induction of remission in patients with a new diagnosis of AAV.	Rituximab was not superior to cyclophosphamide for induction, adverse events were similar between groups and there was no separation between groups in outcomes after 2 years follow-up[16]

Trials of Maintenance Therapies		
Co-Trimoxazole for prevention of relapse in Wegner's[17]	Co-trimoxazole vs. placebo for patients with Wegner's/GPA in remission after induction therapy.	Co-trimoxazole reduced risk of relapse and respiratory infection during study period.
CYCAZAREM[18]	Comparison of azathioprine 2mg/kg/day vs. cyclophosphamide 1.5mg/kg/day maintenance immunosuppression after cyclophosphamide induction.	Rate of relapse in azathioprine arm not significantly increased, therefore valid alternative to reduce cyclophosphamide exposure.
WGET[19]	Patients with GPA in remission after cyclophosphamide or methotrexate/ prednisolone induction randomised to received etanercept/ placebo in addition to azathioprine or methotrexate maintenance therapy.	Addition of TNF blockade to standard therapy was not beneficial in maintenance of GPA remission.
German Network of Rheumatic Diseases study[20]	Maintenance therapy prednisolone + either methotrexate 7.5-20mg/week or leflunomide 30mg/day in patients with GPA after cyclophosphamide induction.	Maintenance therapy with leflunomide associated with less relapse but more adverse effects.
WEGENT[21]	Patients in remission after cyclophosphamide/ prednisolone induction randomised to maintenance tapering prednisolone + either azathioprine 2mg/kg or methotrexate (up to 25mg/week)	Two agents similar in terms of efficacy and adverse effects.
MAINRITSAN[22]	Patients in remission after cyclophosphamide/prednisolone induction randomised to maintenance immunosuppression with either 22 months of azathioprine (tapered) or 18 months of rituximab (5x500mg). Steroids tapered at clinician's discretion.	Sustained remission at 28 months was more common with rituximab than azathioprine.

A huge collaborative effort has resulted in a very significant body of trial-based evidence, however, whilst this has led to improvement treatment strategies this progress has been incremental and treatment regimens and outcomes have not dramatically altered over the last 30 years. The most recent modification to treatment has been the introduction of rituximab, and whilst this may offer a modest benefit compared to cyclophosphamide in relapsing patients, adverse effect rates are similar to that of conventional cyclophosphamide treatment. Treatment related adverse effects are high, with almost 60% of deaths in the first year resulting from

therapy related adverse events [23]. There remains a significant need for efficacious and safer therapies. Current therapies are generally modifications of treatment strategies used for other rheumatologic and haematological conditions. Understanding of the pathogenesis of AAV and potential disease modifying targets specific to this disease could lead to the definition of novel therapeutic strategies that provide meaningful advances in patient care.

Pathogenesis of ANCA associated vasculitis

The autoantigens: MPO and PR3

Myeloperoxidase (MPO) is a 150-kDa peroxidase present in the azurophil granules of human neutrophils and comprising approximately 5% of the dry weight of neutrophils, it is also present in lower amounts in monocytes[24]. In the presence of hydrogen peroxide, it catalyses the formation of multiple reactive agents including hypochlorous acid. Myeloperoxidase can be released from neutrophils by degranulation, the production of neutrophil extracellular traps (NETs), apoptosis and necrosis[25]. Myeloperoxidase plays roles in host defence including killing of intracellular bacteria and clearance of fungal infections. Myeloperoxidase deficiency is relatively common with an incidence of around 1:2000. It has a mild phenotype as most cases are asymptomatic although it is associated with a minor increase in risk of candida fungemia[26]. In addition to its role in the innate immune system, myeloperoxidase also influences the generation of adaptive immunity by supressing DC mediated T cell priming[27].

Proteinase 3 (Pr3) is a neutral serine protease located in the azurophil granules and secretory vesicles. Its expression is restricted to neutrophils and monocytes. Pr3 is expressed on the membrane of resting neutrophils in a bimodal distribution[28]. Surface expression of Pr3 is increased in response to neutrophil priming e.g. by TNF or IL-8[29]. Proposed physiological

functions of Pr3 include killing of phagocytosed pathogens[30], degradation of extracellular matrix protein and cleavage of C1 inhibitor, TNF and IL-8[31]. It is likely that properties of Pr3 impact on AAV disease susceptibility as polymorphisms in the Pr3 protein have a genetic association with anti-Pr3 disease[7].

Other autoantigens in AAV

A small proportion of patients have features of AAV but negative serological testing for anti-MPO and anti-Pr3 antibodies. Some of these may have autoantibodies against other antigens such as neutrophil elastase, bactericidal/permeability-increasing protein, capthepsin G[4], a MPO epitope ($MPO_{447-459}$) which is masked by caeruloplasmin in serum[32] or human lysosome membrane protein 2 (LAMP-2)[33].

The loss of self-immune tolerance

Under normal conditions, humans are able to distinguish between self and non-self antigens, a phenomenon termed immune tolerance. Immune tolerance occurs centrally in the thymus where T cells that form high affinity T cell receptor (TCR)-major histocompatibility complex class II (MHC-II) interactions are deleted. Peripheral autoreactive T cells are suppressed by natural and induced peripheral T regulatory cells. To develop autoimmune disease, the restraining influence of these suppressive cells must be overwhelmed by a population of autoreactive T effector cells. MPO immune tolerance is likely to be maintained by both central and peripheral mechanisms, and in mice, interference with either leads to an increase in anti-MPO autoimmunity and exacerbation of autoimmune anti-MPO glomerulonephritis[34].

The steps leading to loss of tolerance to antigens in autoimmune disease are not well understood. In AAV although some environmental triggers such as silica exposure and propylthiouracil have been suggested, it is probable that these are not contributing factors in the majority of cases[35].

Infection has been proposed as a potential trigger for AAV. There is an association between nasal carriage of *Staphylococcus aureus* and disease relapse in AAV [36]. To date, this association has only been shown for relapse (possibly due to immune stimulation via pathogen associated molecular patterns) rather than true disease susceptibility. Molecular mimicry has been proposed as the mechanism by which infection might induce autoimmunity to the ANCA antigens. Molecular mimicry is the hypotheses that similarities between self-proteins and foreign (e.g. bacterial) proteins results in generation of immunity against the foreign protein accompanied by autoreactivity against the self-protein. Two possible instances of this in AAV have been proposed. One is that homology between the complementary Pr3 (cPr3) peptide and bacterial proteins leading to the subsequent formation of pathogenic anti-idiotypic Pr3-ANCAs. In one study, 7/34 patients with Pr3-AAV were reported to have anti-cPr3 antibodies[37]. However, this finding was not reproduced in a separate cohort of patients with AAV (n=57) in whom anti-cPR3 reactivity was lower in patients with Pr3-AAV than both healthy controls and patients with MPO-AAV[38].

A second potential instance of molecular mimicry is of the FimH protein found in gram-negative bacteria which leads to an immune response with cross-reactivity to human lysosome associated membrane protein-2 (LAMP-2). In the described cohort of patients, a significant proportion had infection with a FimH expressing bacteria that preceeded glomerulonephritis. Evidence of pathogenicity was demonstrated by the development of necrotising pauci-immune GN in rats injected with anti-LAMP-2 antibody [39]. This hypothesis also requires further verification as conflicting findings regarding the prevalence of anti-LAMP-2 antibodies in patients with AAV have subsequently been published[40], and the presence of antecedent urinary tract infections remains to be validated.

An additional mechanism by which infection could trigger AAV is by the presence of the

autoantigens on neutrophil extracellular traps (NETs). These are networks of chromatin fibres decorated with anti-microbial proteins that are extruded from neutrophils in response to inflammatory stimuli. NETosis plays an important role in host defence against bacteria and other pathogens. Neutrophils stimulated with ANCA form NETs which contain MPO and Pr3. Analysis of renal biopsies from patients with active AAV showed that patients with neutrophil infiltration had evidence of robust NET formation which co-localised with the antibacterial peptide LL37[41]. LL37 has been shown to convert inert self-DNA into TLR9 ligands thereby activating plasmacytoid dendritic cells and breaking self-tolerance[42]. NETs therefore co-locate the autoantigens with immune stimulation, potentially resulting in autoimmunity. Support for this hypothesis is provided by an animal model, in which co-culture of NETotic neutrophils and dendritic cells (DCs) resulted in DC uptake of MPO and Pr3, and anti-MPO autoimmunity when these DCs were transferred into mice[43].

B cells produce ANCA

As a disease that is autoantibody induced, B cells are a requirement for the pathogenesis of AAV. A humoral immune response to self-antigen results in the production of ANCA against MPO or Pr3. The importance of B cells in the disease has been supported by efficacy of B-cell targeted therapy with rituximab, as in long term follow up of the RITUXIVAS trial cohort, clinical relapse was only observed in patients with return of peripheral B cells[16].

In addition to their role as antibody producing cells, B cells play other immune roles including antigen presenting, stimulatory and regulatory functions. Clinical studies have reported both reduced and unaltered numbers of IL-10 producing regulatory B cells (Bregs) in active disease[44-46]. The use of CD5 as a marker for Bregs has been investigated as a biomarker in patients treated for AAV and has been found to be useful in anticipating relapse in some, but not all studies[47, 48].

The success of rituximab therapy has stimulated the investigation of other B cell therapeutic strategies such as the use of the anti-B cell activating factor (BAFF) monoclonal antibody belimumab in GPA (NCT01663623). Results from this trial have not yet been released.

ANCA are pathogenic and activate neutrophils

After the association of ANCA with necrotising segmental glomerulonephritis was first described in 1982[49], a key step forward in elucidating the pathogenesis of the disease was the demonstration by Falk et al that ANCA could cause isolated human neutrophils primed with cytochalasin-B and tumour necrosis factor (TNF) to de-granulate, and produce reactive oxygen species[50].

It is presumed that the fragment antigen-binding (Fab) portion of ANCAs bind to their cognate autoantigens on the surface of neutrophils. There is strong evidence for neutrophil surface expression of Pr3. Surface staining for Pr3 appears in washed cells but is significantly attenuated in the presence of autologous plasma[51] suggesting that serum factor(s) such as alpha-1-anti-trypsin inhibit Pr3 binding[52]. Alpha1-anti-trypsin (α1-AT) is a serine protease inhibitor whose enzymatic targets include Pr3. The importance of α1-AT-PR3 interaction is emphasised by the finding that a single nucleotide polymorphism in α1-AT was associated with disease susceptibility in a genome wide association study of patients with AAV[7].

MPO is not highly expressed on the membrane of resting neutrophils[53] but has been demonstrated on stimulated and apoptotic neutrophils, although the magnitude of expression appears to be much less than that of Pr3[51, 54, 55]. MPO externalisation from intracellular granules during neutrophil "priming" is therefore required for antigen surface expression. Experimentally, in *in vitro* studies, this has been most commonly achieved with TNF[50], though other priming agents such as C5a and IL-18 have also been reported [56, 57].

Both Fab and fragment crystallisable (Fc) regions of ANCA are required for pathogenicity. The

Fab fragment determines autoantigen specificity and Fab binding alone to its target antigen has been reported to cause neutrophil activation in some studies, [50, 58] with intracellular signal transduction involving GTPase $p21^{ras}$ and phosphatidylinositol-3 kinase (PI3K)[59]. However, not all studies support a role for the Fab fragment directly causing neutrophil activation[53, 60] and, as neither MPO nor Pr3 have transmembrane domains, any signal transduction is likely to involve other molecules. Membrane bound Pr3 is associated with a CD177/Mac-1 complex which may contribute to anti-Pr3 induced neutrophil activation[61]

The Fc portion of antibody mediates effector function via two mechanisms. The Fc portion of IgG interacts with cellular Fc receptors (FcR). Neutrophils, the main target cell of ANCA express FcγRI, FcγRIIa, FcγRIIc, FcγRIIb, FcγRIIIb as well as the receptor for IgA FcRαI and the human neonatal Fc receptor (FcRn). Of these, the activatory FcγRIIa and FcγRIIIb are thought to be the most important in AAV pathogenesis[62], although some data exist for most of these receptors playing a potential role in this disease under experimental conditions (see Table 2).

Table 2

Receptor	**Expression on human neutrophils**	**Properties**	**Evidence for a role in AAV**
FcγRI (CD64)	Inducible on neutrophil activation[63]	Activating	Patients with ANCA have unregulated FcγRI[64]. Blockade of FcγRI attenuates ANCA induced superoxide release[65].
FcγRIIa (CD32A)	Yes[66]	Activating	Inhibition of FcγRIIa reduces neutrophil activation by ANCA[53, 67]
FcγRIIb (CD32B)	mRNA expressed[68] but surface membrane expression may be low[66]	Inhibitory	FcγRIIb$^{-/-}$ mice have enhanced anti-MPO immunity and glomerulonephritis[69]
FcγRIIc (CD32C)	Yes [70]	Activating	No data.
FcγRIIIb (CD16B)	Yes[71]	Activating	ANCAs engage FcγRIIIb[72], blocking FcγRIIIb attenuates ANCA induced superoxide release[65]
FcRαI (CD89)	Yes [73]	Activating[74]	IgA ANCA found in 30% of patients with GPA and can cause degranulation in neutrophils from patients with susceptible FcRαI genotype. Presence of IgA ANCA is associated with less severe renal disease[75].
FcRn	Expression mostly intracellular, co-localises with MPO containing azurophilic granules[76]	Important for IgG transport may also enhance phagocytosis of IgG opsonised pathogens[76]	No direct evidence for a pathogenic role in AAV though as a major determinant of IgG half life has been proposed as a potential therapeutic target in antibody mediated autoimmune disease[77]

The Fc portion of IgG can also activate the classical pathway of complement by binding C1q, the importance of this in AAV pathogenesis has not been extensively investigated.

The downstream effects of neutrophil stimulation by ANCA include signalling through the phosphatidylinositol 3 kinase (PI3K) and spleen tyrosine kinase[65, 78]. This results in a cascade of pathological changes including: an increase in actin polymerization[79], upregulation of cytokines IL1-β[80] and IL-8[81], reactive oxygen species production, degranulation and NETosis[41]. ANCA activation of neutrophils also modifies their interaction with the endothelium, this results in a change from rolling to adhesion that is β-2 integrin (CD11b/CD18) dependent[82, 83]. In murine glomerular capillaries anti-MPO IgG induces neutrophil retention that is leucocyte function associated antigen-1 (LFA-1) and α-4 integrin dependent[84]. The net result of these changes is that the neutrophils, a key part of host innate immune response are aberrantly activated to cause endothelial damage in small vessels[85].

Determinants of ANCA pathogenicity

Not all ANCA antibodies appear to be equally pathogenic. Clinical studies of the association between crude ANCA titre and disease activity have had varied results, but for the most part do not support a strong association between these variables[86-89]. Characteristics both of ANCA themselves, and other factors, are therefore likely to influence disease activity in AAV.

Factors intrinsic to ANCA that may determine pathogenicity include epitope specificity. Roth et al demonstrated that one linear epitope ($MPO_{447-459}$) was exclusive to active disease and antibodies against this epitope induced glomerulonephritis in mice[32]. Other antibody characteristics that may influence pathogenicity include Fc glycosylation which alters binding to both Fc receptors and C1q[90, 91] and inversely correlates with disease activity in anti-Pr3 disease[92]. Antibody subclass is an important determinant of its effector functions. *In vitro,* ANCA preparations with higher levels of IgG3 induce more neutrophil reactive oxygen species

(ROS) production, and more potently change behavior in an endothelial flow chamber than other subclasses[93, 94]. However, correlation between IgG3 ANCA and clinical disease activity has not been consistently demonstrated[95, 96].

In addition to properties of ANCA themselves, other factors that may modify disease severity or risk of relapse include autoantigen methylation and expression[97], the coexistence of other autoantibodies such as anti-plasminogen or anti-moesin[98-100], T cell mediated immunity[101, 102] and colonisation with *Staphylococcus aureus*[36].

Monocytes/Macrophages: Targets for ANCA and effectors of glomerular injury.

There are two potential mechanisms by which monocytes/macrophages may participate in glomerular injury. Firstly, they may themselves be targets of ANCAs resulting in monocyte activation and tissue injury. Secondly, they may be indirectly recruited to the glomerulus either by activated neutrophils[103] or by antigen specific T cells[104]. Monocyte/macrophages are prominent infiltrating leucocytes in kidney biopsies from humans with AAV[105] and $CD163^{+}$ activated macrophages are found in the glomerular lesions of early necrotising glomerulonephritis[106]. In active disease, markers of circulating monocyte activation such as CD64, CD11b, neopterin and urinary soluble CD163 are increased[107, 108].

Monocytes express the ANCA autoantigens MPO and Pr3. *In vitro*, ANCA can activate human monocytes causing release of ROS, TNF, IL-1β and monocyte chemotactic protein-1 (MCP-1) [109-111]. The expression of MPO and Pr3 is highest on $CD14^{++}CD16^{+}$ intermediate monocytes, a subset that is increased in patients with AAV and produces the most IL-1β in response to ANCA stimulation[112]. It is therefore possible that monocytes/macrophages represent an additional target cell for ANCA and the resulting dysfunction contributes to glomerular injury.

Macrophages are also a key effector of T cell driven type IV hypersensitivity reactions. Transfer of T cell clones specific for a myeloperoxidase peptide planted in the glomerulus

induces necrotising glomerulonephritis accompanied by macrophage infiltration in mice[104]. These macrophages are likely to be a significant contributor to glomerular injury as in a murine model of T cell dependent crescentic glomerulonephritis, macrophages play an important role in the progression of established disease[113].

CD4$^+$ T cells, key participants in AAV

There are several strands of evidence implicating T cells in the pathogenesis of AAV. T cells provide help for antibody response against protein antigens. As ANCA are class switched IgG antibodies against proteins they are almost certainly T-dependent. The association of HLA-DQ and HLA-DP polymorphisms with anti-Pr3 and anti-MPO disease respectively, suggests that the mode of antigen presentation to T cells is an important disease susceptibility trait[7]. There is also an association between markers of T cell activation such as soluble IL-2R and disease activity[114]. Several groups have sought to describe the presence of autoreactive T cells in AAV. *In vitro,* T cell responses specific to the ANCA autoantigens have been described in patients with active disease although these have also been described in a significant proportion of healthy controls[115-118].

Additional support for a role for T cells in human disease comes from the study of human biopsies in which the presence of activated T cells, macrophages, tissue factor and fibrin suggest T-cell directed injury[105]. Whilst the ANCA autoantigens are usually found in neutrophils, degranulation results in their glomerular deposition leading to significant extracellular glomerular MPO in patients with active AAV[119]. Data from mouse models suggest that a T cell mediated response specific to glomerular myeloperoxidase deposited by neutrophils results in a T cell mediated glomerulonephritis[104, 120].

Naïve T cells differentiate into effector subsets that are characterised by the profile of cytokines they secrete. Both IFN-γ secreting Th1 and IL-17A secreting Th17 cells can be injurious in

glomerulonephritis[121]. Increases in both IFN-γ secreting [102] and IL-17A secreting T cells have been described in patients with active AAV[122]. IL-17A acts as a powerful chemo-attractant for neutrophils and monocyte/macrophages as well as stimulating release of pro-inflammatory cytokines such as TNF from macrophages[123]. IL-17A may also play a role in the humoral immune response by promoting formation of germinal centres[124]. Supporting evidence for the pathogenic involvement of Th17 cells comes from work in mice in which deficiency of IL-17A results in almost total protection from autoimmune anti-MPO GN[125]

T regulatory cells (Tregs) are important for preventing autoimmunity and resolving inflammation. Investigation of Tregs in patients with AAV has demonstrated Tregs with an overrepresentation of a FOXP3 splice variant associated with reduced suppressive ability[101]. Whilst it has been challenging to consistently identify Treg abnormalities in human autoimmune disease[126], evidence from animal models suggest that that the T regulatory compartment is involved both in the loss of tolerance to MPO and in limiting subsequent glomerular injury[34, 127].

$CD8^+$ T cells exacerbate disease and influence prognosis

$CD8^+$ or cytotoxic T cells recognise antigen presented on MHC class I and are important in host response to viral infection and malignancy. $CD8^+$ T cells have been implicated in autoimmune diseases such as rheumatoid arthritis and type I diabetes[128]. Data from humans, and experimental work in animals suggests that $CD8^+$ T cells may also participate in AAV. Immunostaining of kidney biopsies from patients with MPO-ANCA AAV suggests that $CD8^+$ T cells are at least as common as $CD4^+$ T cells[119]. Additionally, a $CD8^+$ T cell transcription profile suggestive of an expanded $CD8^+$ memory T cell population is associated with poor prognosis in patients with AAV[129]. Data from animal studies support a directly injurious role of

$CD8^+$ T cells in autoimmune anti-MPO glomerulonephritis, which is attenuated by their depletion, and enhanced by the transfer of MPO specific CD8 T^+ cell clones[130]

Mast cells in AAV: Potential for both injurious and immunomodulatory roles

Mast cells are innate immune cells classically involved in the allergic immune response. Degranulated mast cells are found in the biopsies of patients with AAV, particularly in the interstitium, where their frequency correlates with severe injury[131]. Activated mast cells are able to produce pro-inflammatory cytokines such as histamine, proteases, TNF-α, and IL-17A[132]. It is therefore possible that these activated mast cells are directly contributing to tissue damage. Mast cells also modulate T effector and regulatory response via the secretion of IL-10[133]. In murine models IL-10 production by mast cells attenuates both MPO autoimmunity and anti-MPO glomerulonephritis[131, 134].

Animal models of AAV

A significant proportion of our understanding of the pathogenesis of AAV is derived from rodent models of the disease. Whilst these models are reductionist, they have resulted in significant insight into disease mechanisms and informed potential therapeutic strategies.

Although several models of anti-MPO disease have been reported, success in defining an animal model of anti-Pr3 disease has been limited. This may be because murine neutrophil expression of Pr3 is modest[135] or because CD177, which is thought to determine Pr3 surface expression and hence neutrophil activation by ANCA does not associate with Pr3 in mice[136].

The animal model that provided key evidence confirming ANCA pathogenicity was reported by Xiao et al.[137] who immunised $Mpo^{-/-}$ mice with murine MPO resulting in the production of high

titre anti-MPO antibodies. When this anti-MPO IgG was transferred into wild type (WT) C57BL/6 or Rag2$^{-/-}$ mice it induced a pauci-immune crescentic glomerulonephritis. They also transferred splenocytes from immunised Mpo$^{-/-}$ animals into Rag2$^{-/-}$ mice producing a glomerulonephritis. The glomerular injury induced by splenocyte transfer was however associated with immune complex deposition, suggesting that it may share few parallels with the pauci-immune disease observed in humans. As the anti-MPO IgG transfer model often results in relatively mild glomerular changes, augmentation of this model with bacterial lipopolysaccharide (LPS) or granulocyte-colony stimulating factor (GCSF) has been used to exacerbate injury [138, 139].

An alternative murine model was reported by Schreiber et al [140] who immunised Mpo$^{-/-}$ mice with MPO, these mice were subsequently irradiated and transplanted with WT bone marrow (BM). The mice developed necrotising crescentic glomerulonephritis, with mice receiving higher radiation doses, and therefore greater engraftment of WT BM developing more severe disease. This disease model presumably induces disease through the residual effect of anti-MPO IgG or differentiated plasma cells produced prior to irradiation on neutrophils derived from WT BM. The passive transfer of anti-MPO IgG and the BM transplant model have provided important insights into the role of ANCA and neutrophils as key mechanisms in the disease, however, both models have a number of shortcomings. Neither model requires a loss of self-tolerance as both in both cases *Mpo*$^{-/-}$ mice produce a response against an alloimmune antigen. In addition, antigen specific adaptive immune cells are not present in the disease induced by transfer of anti-MPO IgG, and do not appear to actively participate in the injury phase of the bone marrow transplant model.

In contrast, auto-immunity but not autoantibodies are involved in the model of experimental anti-myeloperoxidase glomerulonephritis reported by Ruth et al.[120] In this model,

glomerulonephritis is triggered in MPO immunised C57BL/6 mice by the administration of a sub-nephritogenic dose of sheep anti-GBM IgG. The role of the anti-GBM IgG is to recruit neutrophils to the glomerulus where they degranulate, depositing myeloperoxidase where it acts as a glomerular planted antigen. In this model, mice immunised with MPO develop crescentic glomerulonephritis with significantly worse severity than those immunised with bovine albumin or OVA as control antigens. This model is T cell but not B cell dependent (as it occurs in μ-chain deficient mice) and has provided important insights into the role of $CD4^+$ T-cells as effectors of glomerular injury and permitted investigation of potential immunomodulatory strategies. This model has several disadvantages. The requirement for Freund's complete adjuvant (FCA) to induce robust anti-MPO autoimmunity means that the mode and polarisation of the loss of tolerance and resulting autoimmunity is likely to differ from that seen in human disease. The use of heterologous anti-GBM to recruit neutrophils provides both a small contribution to renal injury and limits duration of the disease model to around 4 days before an autologous anti-sheep globulin response. In addition, whilst MPO autoantibodies are produced, they do not themselves activate neutrophils or contribute to glomerulonephritis in this model.

A rat model of autoimmunity producing crescentic pauci-immune glomerulonephritis, pulmonary haemorrhage and anti MPO antibodies has been described in Wistar-Kyoto (WKY) rats immunised with human MPO (which has significant homology to the rat protein). Other strains of rat immunised with human MPO did not develop evidence of vasculitis suggesting a significant role for genetic background in the pathogenesis of the disease[141]. A disadvantage of this model is the lesser availability of knockout strains and experimental reagents available for rats compared to mice.

The Complement System

The complement system is system of circulating and membrane bound proteins, receptors and regulators. It was named by Bourdet in 1895 as it complements the lysis of bacteria by immunoglobulin. Complement plays many roles in host defence including amplification of danger signals, opsonisation, pathogen lysis, chemo-attraction to areas of inflammation, modification of adaptive immune responses and immune complex clearance. Complement proteins are predominantly synthesised in the liver, however recent work has shown that they are also synthesised in a large number of other cells tissues including leukocytes and renal tissue. An approximation of the renal contribution to the total C3 pool was derived from study of patients who received renal transplants from donor kidneys with mismatched C3 allotypes. At steady state with functioning grafts approximately 5% of the C3 pool was allograft derived suggesting that the kidney is a minor site of C3 synthesis[142].

The complement cascade

There are three main complement activation pathways- the classical, lectin and alternative pathway, all of which converge in generating C3 convertases: C4bC2b in the case of the classical and lectin and C3bBb in the case of the alternative pathway. The classical pathway is predominantly activated by the Fc region of immunoglobulin. It is important for antibody effector functions and clearance of immune complexes. The lectin pathway is activated by carbohydrates found on the surface of many pathogens. The alternative pathway is constitutively active with spontaneous hydrolysis of C3 resulting in low level "tickover". The hydrolysed C3 associates with factor B to form a precursor to the C3 convertase, which once cleaved by factor D is active (C3bBb) and can generate further C3b and initiate a positive feedback loop. Factors that promote alternative pathway activation include the leukocyte secreted protein properdin and pathogen surfaces devoid of complement regulatory proteins.

The alternative pathway also forms a positive amplification loop for activation signals from the classical and lectin pathways.

The complement cascade produces bioactive split products. These mediate inflammation by either signalling through cellular receptors or by direct cell damage. The C3 convertase produces C3a and C3b, which, in addition to forming an essential component of the convertase enzymes, also can covalently bind to pathogens, cellular debris and immune complexes. C3b, along with its breakdown products (iC3b and C3d) interacts with receptors on phagocytes, erythrocytes and lymphoid cells. This process is important for the elimination of pathogens, clearance of immune complexes and induction of adaptive immune response.

The C5 convertase produces splits C5 to C5a and C5b. C5a has pro-inflammatory effects on leucocytes signalling through its main cellular receptor, C5aR1. C5b is the initial component of the terminal membrane attack complex (MAC, C5b-9) which forms transmembrane channels to directly lyse cells.

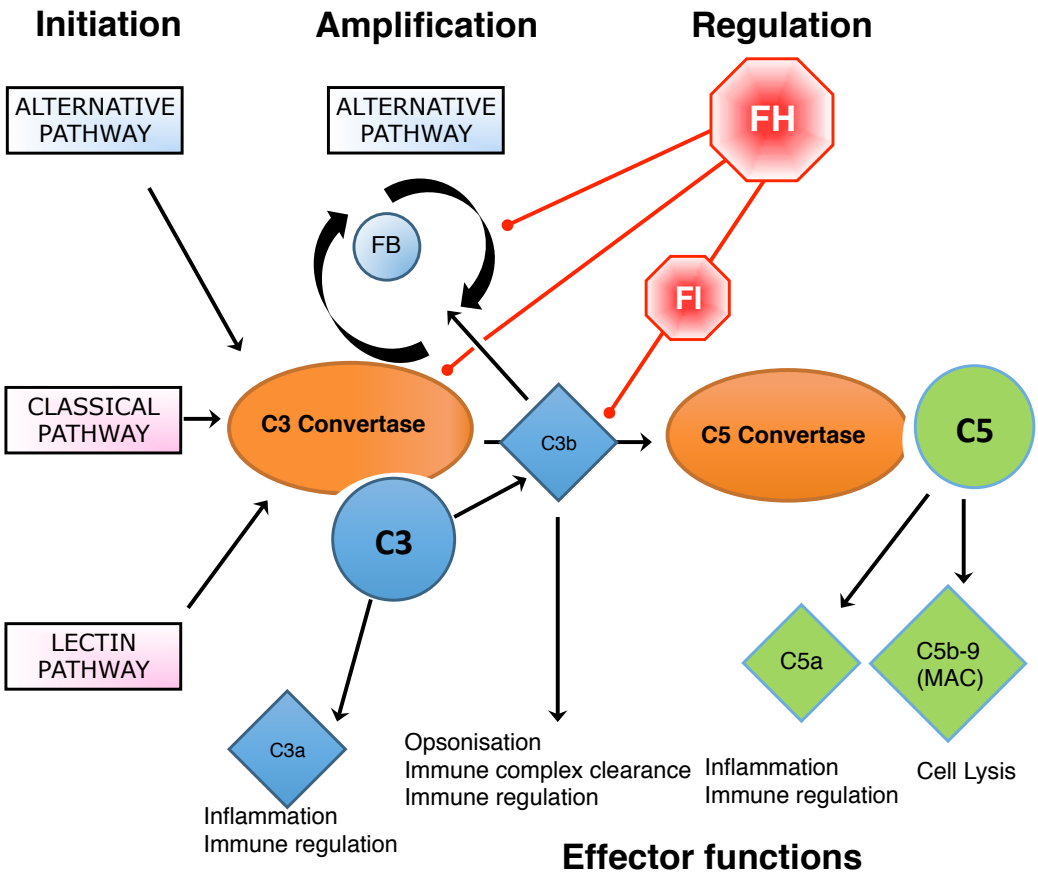

Figure 1.1: *Diagram of key features of the complement system.*

Complement regulation

Regulation of the complement system is essential to limit damage to the host. It is achieved by fluid-phase and membrane-bound proteins which degrade active components of the pathways. Regulation of the alternative pathway positive feedback loop is particularly important, as unrestrained activity results in runaway C3 activation. This mechanism amplifies signals from all three pathways and is active in both the fluid phase and cell surfaces.

The key regulator of the alternative pathway is factor H. Factor H both obstructs factor B binding to C3b to form the C3 convertase and inactivates existing C3 convertases. It also acts as a co-factor for factor I mediated degradation of C3b, an essential step to prevent formation of further C3 convertases[143].

Other complement regulatory proteins include C4 binding protein, CD56 (Membrane co-factor protein), CD55 (Delay accelerating factor- which accelerates the dissociation of the two partners comprising the C3 convertases), CD35 (complement receptor 1) and CD59.

The complement System in renal disease

Defective complement function drives the pathogenesis of a number of human diseases including paroxysmal nocturnal haemaglobinuria, atypical haemolytic uremic syndrome, hereditary angioedema, and C3 glomerulopathy. In addition, complement participates as an effector of injury in other immune mediated glomerulonephritides.

Factor H mutations

Mutations in factor H can result in the clinical disorders of atypical haemolytic uremic syndrome (aHUS) or C3 glomerulopathy (C3G). In aHUS, most factor H mutations impair C terminal protein function, which is important for binding of factor H to C3b and to negatively charged glycosaminoglycans on cell surfaces[144]. The result is uncontrolled C3 activation on cell surfaces, whereas regulation of the alternative pathway in the circulation is often preserved. Accumulation of C3b molecules on endothelial cell surfaces leads to surface C5 convertase formation and surface assembly of the MAC. Clinically this process manifests as acute and/or chronic relapsing microvascular endothelial injury and thrombosis (thrombotic microangiopathy).

In C3G, defective alternative pathway regulation appears to occur both in the fluid phase and on surfaces, in particular the glomerular basement membrane (GBM)[145]. Factor H mutations that impair protein secretion into the circulation or inhibit N-terminal AP regulation result in chronic C3 activation and accumulation of C3 breakdown products along the GBM. The renal pathology is characterized by glomerular deposition of ‘dense deposits’ containing C3 breakdown products (iC3b and C3d) with consequent damage and inflammation. The functional

effect of the factor H mutation thus appears to be critical in determining whether an individual develops C3G or aHUS. One possibility is that the cell surface density of C3b, which determines the relative formation of C3 and C5 convertases[146] and their respective split products may account for the different clinical presentations of C3G and aHUS.

A number of other genetic and acquired complement abnormalities have also been implicated in the complement mediated renal diseases. The combination of rare and common genetic variations in complement proteins and regulators determines the net susceptibility to complement mediated inflammation, and is commonly referred to as "complotype"[147]. It is likely that the susceptibility to complement mediated diseases is a product both of complotype and genetic factors which determine endothelial and immune response to injury.

Atypical haemolytic uraemic syndrome

Atypical haemolytic uraemic syndrome (aHUS) is a thrombotic microangiopathy (TMA) syndrome. TMA is a disease process of microvascular endothelial injury and thrombosis which typically manifests as microangiopathic haemolytic anaemia, thrombocytopenia, end-organ ischaemia and infarction[148]. In aHUS, TMA is due primarily to uncontrolled activation of the complement alternative pathway. Renal failure is usually prominent, although neurological, cardiac and gastro-intestinal sequelae can be severe. In around 60% of cases, a genetic factor affecting AP regulation is identified[149]. The most frequently identified abnormalities in two large European cohorts are complement factor H mutations, polymorphisms or autoantibodies. Mutations in complement factor I, C3, the complement factor H related proteins and membrane cofactor protein (MCP) comprise a minority of cases. Compound heterozygosity and co-existence of mutations and factor H autoantibodies also occur[149].

Prior to the introduction of eculizumab, plasma therapy formed the mainstay of treatment for aHUS and approximately 50% of adults reached ESRD within a year of diagnosis. Eculizumab

is a humanised monoclonal antibody that binds to C5, preventing the formation of C5a and C5b. The efficacy of eculizumab treatment for aHUS in adults with and without demonstrable complement mutations or autoantibodies was illustrated by two phase II prospective cohort studies [150]. In both trials approximately 90% of patients had normalisation of haematological parameters following treatment with eculizumab.

C3 Glomerulopathy

C3 glomerulopathy (C3G) refers to a number of glomerular disorders characterized by predominant C3 staining and the presence of electron-dense deposits within the glomerulus[151]. The light microscopic appearance is varied but includes mesangioproliferative, membranoproliferative and endocapillary proliferative patterns. Electron microscopy enables the ribbon-like, very dark intramembranous deposits of dense deposit disease (DDD) to be seen, whereas the deposits of C3 glomerulonephritis (C3GN) are less dense and are often found subendothelially and in the mesangium[152].

Both acquired and genetic factors that produce uncontrolled C3 activation via the AP have been identified in patients with C3G. Acquired factors include autoantibodies that stabilise the C3-convertase (C3 nephritic factors) or that inhibit factor H. Genetic factors include mutations in factor H or factor H-related proteins [153-155].

Clinically, C3G has a very wide range of renal manifestations including isolated microscopic haematuria, synpharyngitic haematuria, nephrotic syndrome and rapidly progressive glomerulonephritis. DDD is more common in children than adults, is usually associated with a C3 nephritic factor and is occasionally associated with partial lypodystrophy and ocular drusen. There is also a well described, but as yet unexplained association of C3GN with monoclonal gammopathy [156]. Progression of CKD is extremely varied but ESKD occurs in approximately 40% of patients after 10 years.

Paroxysmal Nocturnal haemoglobinuria

Paroxysmal nocturnal haemaglobinuria (PNH) is a complement mediated disorder associated with intravascular haemolysis, thrombosis and bone marrow failure. The underlying defect is an acquired genetic mutation that results in disruption to the synthesis of glycosylphosphatidylinositol, a molecule that anchors proteins to the cell surface. When this mutation occurs in haematopoietic stem cells, it results in an abnormal clone of erythrocytes which lack expression of two complement regulatory proteins CD55 and CD59 and are therefore susceptible to haemolysis[157]. Eculizumab is an effective therapy for PNH, which, in clinical trials results in over 80% reductions in haemolysis, significant reductions in transfusion dependence and thrombotic events and the stabilisation or improvement of CKD in the majority of patients[158].

C4 Glomerulopathy, a new disease entity?

Recently, evidence has emerged of an additional form of complement mediated glomerular injury termed C4 glomerulopathy. A small number of cases of proliferative glomerulonephritis have been reported without significant C1q, C3 or immunoglobulin staining but with C4d staining and sometimes accompanying electron dense deposits of C4d [159-161]. The absence of C1q or C3 suggest that neither the classical nor alternative pathways are activated. To date, a distinct disease mechanism has not been proposed.

IgA nephropathy

IgA nephropathy (IgAN) results from the formation of circulating immune complexes of abnormally glycosylated IgA1 and glycan specific IgG or IgA autoantibodies. These complexes deposit in the mesangium, where IgA is thought to activate complement via the lectin and alternative pathways[162]. Two lines of evidence implicate complement in the subsequent

glomerular injury. First, studies have demonstrated that complement activation in the form of mesangial C3 or C4d is an independent risk factor for ESRD [163, 164]. Second, compelling evidence that complement is more than just a marker of immune complex deposition comes from genome-wide association studies. These have identified an association of protection from disease with a deletion in CFHR3 and 1[165, 166] that is associated with higher factor H levels in both patients with IgA nephropathy and healthy controls. The allele seems to reduce disease severity as it associates with reduced tubulointerstitial injury[167]. The implication of this finding is that the robustness of alternate pathway regulation determines IgAN susceptibility and/or severity and suggests that complement is a potential therapeutic target. In support of this, case reports of eculizumab use in rapidly progressive IgAN have suggested some clinical benefit [168, 169]. A phase II trial of a C5aR antagonist (NCT0238431) will help to inform the validity of this approach.

Lupus Nephritis:

Complement is a potent risk factor for development of SLE, and may also play an injurious role both as an antigen targeted by autoantibodies and as a mediator of inflammation in lupus nephritis. Deficiencies in classical pathway components including C1q, C1s, C2 and C4 are the most potent risk factors for the disease. In homozygous C1q deficiency, disease penetrance is in excess of 90% with approximately half developing nephritis[170]. This susceptibility is thought to result from the physiological role of the classical pathway in the processing and clearance of immune complexes, abnormalities of which lead to defective processing of apoptotic cells and the production of autoantibodies.

Anti-C1q autoantibodies are present in around a quarter of patients with SLE and are associated with lupus nephritis[171]. They are thought to cause additional complement activation at the site of immune complex deposition and increase glomerular injury[172]. Anti-C3b autoantibodies also

occur in SLE and appear to be highly specific for lupus nephritis[173]. Although successful use of eculizumab has been reported in cases of lupus nephritis resistant to conventional immunosuppression[174, 175], an earlier, unpublished trial of eculizumab in lupus nephritis failed to show significant effect[176].

Membranous nephropathy:

The discovery of autoantibodies to glomerular antigens in many cases of primary membranous nephropathy (MN) have increased understanding of the pathogenesis of this disease [177, 178]. Immunostaining of MN biopsies characteristically show complement activation with C3, C4d, and the MAC. C1q is typically not seen[179]. The rat model of Heymann nephritis has similarities to human disease including sub-epithelial immune complex deposition and complement activation. This model has demonstrated the importance the MAC in injuring podocytes by causing sub-lethal cytoskeletal changes, loss of podocyte integrity and proteinuria[180, 181]. Elevated urinary levels of the MAC have been reported in patients with MN[182].

A key remaining question is the mode of complement activation in primary MN, as the dominant immunoglobulin IgG4 does not activate the classical pathway. Possible explanations include activation of the classical pathway by the IgG1 seen in early MN, activation of the MBL pathway, [183], or activation of the alternative complement pathway as patients with combined MBL deficiency and MN have been described to have both Ig4 and C3 deposition [184]. Complement inhibition has not been shown to be effective in MN: an unpublished multi-centre trial of eculizumab in 130 patients found no difference in reduction of proteinuria, the primary endpoint.

Complement in ANCA associated vasculitis

Relative to other forms of crescentic glomerulonephritis, complement is not an obvious participant in AAV as the histological findings are classically "pauci-immune". In addition, the disease is not traditionally associated with abnormal levels of the most commonly measured circulating complement components: C3 and C4. The unexpected discovery for a role for the complement system in AAV is an example of bench to bedside research in which observations in animal models drove the generation of supportive data from clinical cohorts and has led to the introduction of new therapeutic in clinical trials.

The role of complement in AAV: Animal models

The first compelling data supporting a role for complement in AAV came from the observation that *C5*$^{-/-}$, *factor B*$^{-/-}$ but not *C4*$^{-/-}$ mice were protected from glomerulonephritis induced by transfer of anti-MPO IgG[185]. This data suggest that the alternative pathway and C5, but not the classical pathway of complement are critical to the pathogenesis in this disease model. Whilst the absence of protection of *C4*$^{-/-}$ mice was interpreted to mean that the lectin pathway did not participate in disease, subsequently, a C4 bypass mechanism for the lectin pathway in mice has been described[186]. The role for the lectin pathway was therefore re-investigated using *MASP-2*$^{-/-}$ mice (which have no functioning lectin pathway), which developed more severe glomerulonephritis. This may be due to interactions between the complement and coagulation pathways as *MASP-2*$^{-/-}$ had increased serum prothrombin activation[187].

Subsequent work using the model of anti-MPO IgG transfer investigated the effect of C5 inhibition using the mAb BB5.1. This antibody binds to C5, preventing its cleavage to C5a and C5b, a mechanism of action analogous to eculizumab. Pre-treatment with anti-C5 mAb reduced both early glomerular neutrophil influx, and abrogated glomerulonephritis[188]. The C5 dependence of this model was subsequently demonstrated to be mediated by the cellular receptor for C5a, the C5a receptor 1 (C5aR1), on BM derived cells. In the bone marrow

transplant model of anti-MPO glomerulonephritis $Mpo^{-/-}$ mice immunised with MPO and subsequently engrafted with *C5aR1*$^{-/-}$ BM developed minimal histological abnormalities compared to those receiving WT BM who developed glomerular necrosis and crescents[56]. Supportive of C5a being the key injurious mediator, C6 deficient C3H/Hej mice are not protected from anti-MPO IgG induced disease, suggesting that the membrane attack complex is not critically involved in this model[189].

The potential of C5aR1 as pharmacological target was confirmed by the use of transgenic mice with a knocked-out murine C5aR1 and a knocked-in human C5aR1 to demonstrate that the C5aR1 inhibitor (CCX168) attenuated glomerulonephritis induced by anti-MPO IgG. In contrast, mice deficient a second receptor for C5a, the C5aR2 developed more severe glomerulonephritis.

Additional detail on the source of the complement required for anti-MPO IgG induced glomerulonephritis was provided by demonstration using bone marrow chimeras, that circulating C5 derived from hepatocytes rather than C5 produced by leucocytes mediated disease. Interestingly, the same paper reported that properdin deficiency did not alter the severity of disease[187]. Properdin is the only known endogenous positive regulator of complement, it is secreted from neutrophil secondary granules and increases the half-life of the AP C3 convertase. Properdin deficiency may not effect all AP activation equally with a greater effect on LPS induced activation than on activation by the classical pathway[190]. The lack of effect of properdin deficiency in experimental anti-MPO glomerulonephritis suggests that AP activation in the glomerulus by neutrophil secreted properdin is not a significant contributor to injury.

In a series of experiments it has therefore been shown that the C5a, acting on the C5aR1 is a requirement for induction of glomerulonephritis in the murine disease induced by anti-MPO IgG. The amplification loop of the AP appears to be required for C5a generation although isolated deficiency of C4 or properdin does not seem influence disease severity, leaving the mechanism of initial AP activation undetermined.

Complement in AAV: humans

The body of work implicating complement in murine models of AAV has been supported by observational data in humans.

Circulating complement measurements: evidence of complement activation

Analysis of complement components in samples from patients with AAV demonstrates significant evidence of complement activation. Patients with active anti-MPO disease were found to have elevated levels of C3a, C5a, C5-9 and Bb compared to remission[191]. Work by the same group found that urinary C5a, C3a , factor B, MBL and C1q were all elevated in patients with active disease compared to remission[192]. Whilst the findings of elevated levels of components of the MBL and classical pathway are surprising considering the lack of proposed role in disease pathogenesis, this may be related to their function in clearing apoptotic or necrotic cells.

Recent studies have also suggested the association of low serum C3 levels at diagnosis with adverse prognosis. A study of 40 patients with AAV reported that a third of patients had low serum C3 at diagnosis; these patients had worse renal and overall survival. In this study, a quarter of patients also had some histological evidence of thrombotic microangiopathy on biopsy, a finding associated with more severe renal disease[193]. The association of low, or low-normal C3 with adverse outcome has subsequently been confirmed in two separate cohorts,

although these studies did not comment on the prevalence of changes consistent with TMA[194, 195]. The reason for this association has not been defined. Possibilities include that patients with severe AAV have significant neutrophil activation and endothelial damage which results in complement activation and consumption. A more intriguing possibility is that low C3 may be a marker of patients with a susceptible complotype i.e. are more prone to complement activation, and that this leads to more severe AAV. Whilst features of TMA have been observed in a small proportion of patients with AAV in other studies[196], this has not been accompanied by assessment of complement parameters.

Histopathological findings: complement activation occurs in the glomerulus

Staining for complement components in renal biopsies has formed part of the diagnostic framework for nephropathologists for several decades. Whilst the histological findings in AAV are classically pauci-immune several groups have reported evidence of complement deposition in patients with AAV. Studies have documented evidence of C1q, factor B, properdin C3c, C3d, and the MAC in patients with AAV using immunofluorescence and immunohistochemistry. The deposition of the alternative pathway product Bb also correlated with renal damage[191, 197-199]. In one study, the majority of biopsies also stained positive for C4d by immunofluorescence on frozen sections in a mesangiocapillary pattern[200]. This finding is difficult to interpret considering the previously reported positive C4d staining by immunofluorescence in a mesangial pattern in healthy controls[201, 202].

There is therefore compelling evidence of complement activation in human serum, urine samples and renal biopsies. Whilst there appears to be activation of all the pathways, the relationship of alternative pathway activation to disease activity is strongest. These clinical data support a role for complement in AAV though it does not elucidate whether it plays an important role in the disease pathogenesis or whether it is a product of disease induced activated neutrophils and endothelial damage.

Genetic studies

A study of 105 patients with AAV with 200 healthy controls using a candidate gene approach to investigate the association of AAV with polymorphisms in 4 candidate genes, one of which was C3[203]. They found an association of the C3 fast (C3F) allotype (p=0.041) in patients with anti-PR3 disease compared to controls. This allotype has previously been implicated in other diseases in which complement plays a pathogenic role including age related macular degeneration[204]. However, no association was found between C3 allotype and risk of AAV in a subsequent study with a larger cohort[205], nor in the much larger cohort studied in a genome wide association study[7]. There therefore currently appears to be little evidence linking genetic polymorphisms in complement susceptibility to AAV, although this does not exclude an important role in disease pathogenesis.

***In vitro* studies with human neutrophils: C5a is required for priming**

Early reports of the complement dependence of murine models of AAV were accompanied by evidence that complement primed neutrophils for activation by ANCA. These included the observation that neutrophils, when activated by ANCA, released factors that activated complement in autologous serum leading to generation of C3 cleavage products. This "activated serum" was shown to increase neutrophil Pr3 surface expression[185].

This active serum factor was demonstrated to be C5a as blocking the C5aR1 inhibited ROS and neutrophils could be primed for ANCA activation by recombinant C5a. The property of C5a to prime neutrophils for ANCA activation has been proposed to be due to up-regulation of MPO and Pr3 expression on the neutrophil surface. Whilst two groups have showed this for Pr3[56, 206] it remains to be demonstrated that this also applies to surface expression of MPO.

Clinical Studies of C5aR1 inhibition:

Preclinical data suggesting the importance of signaling through the C5aR1 in the pathogenesis of AAV has lead to clinical studies of C5aR1 inhibition in humans. To date, the oral small molecule C5aR1 inhibitor CCX168 (Avacopan) has been studied in two phase II clinical trials. The CLEAR (NCT01363388) and CLASSIC (NCT02222155) studies.

At the time of writing, results from the CLEAR study have been published[207]. This was a randomised, placebo controlled trial in which 67 patients with either new or relapsing AAV receiving rituximab or cyclophosphamide induction were allocated to one of three arms: 60mg of prednisolone, avacopan + 20mg of prednisolone, or a steroid free arm with avacopan alone. The primary endpoint was a 50% reduction during the 12 week study period in the Birmingham Vasculitis Activity Score (BVAS), secondary endpoints included albuminuria and quality of life scores. Avacopan alone was non-inferior to conventional care in terms of BVAS response and was associated with a greater reduction in albuminuria and higher quality of life scores. As high-dose glucocorticoids are likely to be a significant contributor to treatment related morbidity the possibility of steroid avoidance in treatment of AAV might represent a major advance. The phase III ADVOCATE trial (NCT02994927) is currently recruiting and should provide definitive evidence as to the validity of this approach.

The anaphylatoxin receptors

Complement activation products mediate most of their effects through cellular receptors. These include the complement receptors 1, 2 and 3 as well as the receptors for C3a and C5a. The work in these thesis concentrates on the cellular receptors for C3a and C5a.

C5aR1

Cleavage of C5 by a C5 convertase results in two bioactive split products. C5a and C5b. To moderate its inflammatory action, C5a is metabolised by rapid cleavage by carboxypeptidases to the less active C5a-desArg [208]. There are two known receptors for C5a: the C5aR1 and the

C5aR2. The C5aR1 (CD88) is a G-protein coupled receptor, ligation results in cellular calcium influx, mitogen-activated protein kinase, phosphoinositide-3-kinase-γ and phospholipase C β2 signaling[209]. In contrast, the C5aR2 (C5L2) is expressed at a much lower level than the C5aR1, and is unable to couple G-proteins. Its role is controversial, it may act as a decoy receptor reducing the availability of C5a[210], although under some conditions it can be inflammatory[211].

The C5aR1 is expressed on neutrophils, macrophages, monocytes, dendritic cells, eosinophils, mast cells and platelets [212-216]. Expression on T cells is an area of controversy. Whilst expression has been reported in murine T cells by flow cytometry and mRNA analysis by some groups[217, 218], two separate C5aR1 reporter mice have reported no expression on either naïve or stimulated T cells. Some of the confusion may be related to the observation that the commonly used anti-C5aR1 mAb clone 20/70 binds weakly to a neo-epitope on apoptotic T cells giving the misleading impression of C5aR1 expression[219, 220]. The C5aR1 is also expressed in a variety of non-immune cells including in the kidney, where it is expressed in the tubules but not the glomerulus [221, 222].

The C5a acting at the C5aR1 has potent inflammatory action on a variety of cells. It causes histamine release from mast cells[223], is a chemo-attractant for neutrophils, macrophages and mast cells, and triggers oxidative burst in neutrophils and macrophages[209, 224]. C5a also induces transcription of TNF and IL-1[225]. On the vascular endothelium, C5a increases secretion of pro-inflammatory cytokines, enhances tissue factor expression and up-regulates p-selectin mediated neutrophil adhesion [226-228].

The complement system has multiple interactions with other parts of both innate and adaptive immunity. An example of this is the two way signalling between the C5aR1 and Fcγ receptors (FcγRs)[229]. C5aR1 signaling promotes inflammation by causing up-regulation of inflammatory FcγRs and down-regulating the anti-inflammatory FcγRIIb, [230, 231]. Conversely, binding of

FcγRIIb by murine IgG1 results in intracellular signaling that blocks C5aR1 mediated effector functions both *in vitro* and *in vivo*[232]. There is also cross-talk between C5aR1 and the toll-like receptors, the coagulation cascade, and the NRLP3 inflammasome[233].

C5aR1 in models of inflammation.

Animal models suggest that the action of C5a is profoundly pro-inflammatory. Genetic knockout or blockade of C5aR1 has been found to be protective in multiple models of inflammation including sepsis[234], LPS induced shock[235], cisplatin induced acute kidney injury[236], renal ischemia reperfusion injury[237] and monosodium urate crystal induced inflammation[238]. C5aR1 has also been shown to be required in multiple models of IgG induced inflammation including K/BxN arthritis[239], anti-collagen antibody induced arthritis[240], collagen induced arthritis[241], immune complex induced lung inflammation[230] and bullous pemphigoid[242]

C5aR1 modulates T cell immunity

Traditionally, the complement system was viewed as being a first line immune defence, which, aside from complementing the killing response to IgG fixed pathogens largely operated independently from the adaptive immune system. However, recently, there has been a growing understanding of the ability of complement to modulate the adaptive immune response.

Dendritic cell expressed C5aR1 promotes induction of T cell responses

Dendritic cells (DCs) are innate antigen presenting cells (APCs) resident in both lymphoid organs and peripheral tissue. Upon activation, they present antigen in MHC class II peptide complexes to T cells. They are crucial in both initiating and polarising the antigen specific effector response, most commonly to Th1, Th2, Th17 or Treg phenotypes. DCs both express the C5aR1 and can synthesis complement components[243].

DCs from *C5aR1*$^{-/-}$ mice have reduced expression of co-stimulatory molecules, secrete more IL-10 and less IL-12p70. They have a reduced capacity to stimulate allospecific T cells and induce Th1 differentiation but enhanced ability to induce Foxp3^{+} Tregs [244-246]. *C5aR1*$^{-/-}$ bone marrow derived DCs (BMDCs) have also been reported to have impaired antigen uptake capacity, reduced CD11b expression and an expanded population of myeloid derived suppressor cells[247]. Further evidence for the immune stimulatory capacity of C5a is the enhancement of both Th1 polarised cellular immunity and antibody response when a C5a agonist is used as a vaccine adjuvant[245]. The effect of APC expressed C5aR1 on Th17 immunity appears to be variable with both promotion and attenuation of Th17 responses reported[246-248].

Whilst the majority of the data derives from animal studies, in human monocyte derived DCs, C5a enhances co-stimulatory molecule expression and allostimulatory capacity suggesting that the findings in mice are translatable to humans[249].

Evidence for immunomodulation by T cell intrinsic C5aR1: mice

There is a body of literature suggesting that T cell intrinsic C5aR1 influences T cell polarisation in mice. Reported T cell intrinsic effects of C5aR1 include the inhibition of T cell apoptosis[218], the attenuation of alloresponses in graft versus host disease[250], induction of Th1 and Th17 response in experimental autoimmune encephalomyelitis[251] and inhibition of induction of Foxp3^{+} Tregs[252] How this data can be reconciled with the lack of T cell expression of C5aR1 in the C5aR1-GFP reporter mouse strains remains to be resolved.

T cell expressed C5arR1 humans induces Th1 response

In humans, surface and intracellular C5aR1 has been reported to influence T cell immunity. Surface C5aR1 modulates generation of human iTregs with inhibition causing increased FOXP3^{+} Treg generation in cells cultured with DCs, anti-CD3 and Il-2[253]. The importance of

intracellular complement activation and signalling through the C5aR1 was shown by Arbore et al. who demonstrated that human T cells express the C5aR1 intracellularly and that ligation of intracellular C5aR1 results in ROS production, activation of the NRLP3 inflammasome and autocrine IL-1β signalling inducing Th1 polarisation[254].

C5aR1 in models of immune mediated renal disease.

The role for the C5aR1 in generating adaptive effector responses is supported by work using animal models relevant to renal disease. The MRL/lpr mouse, when backcrossed onto the *C5aR1*$^{-/-}$ strain develops reduced renal injury, Th1 response and autoantibody titres[255]. Absence of C5aR1 is also protective in a model of renal allotransplantation with prolonged graft survival and reduced infiltration of IFN- γ^+ cells[256].

C3aR

C3a is 77 amino acid polypeptide split product of C3 produced, along with C3b by the action of the C3 convertases. C3a is rapidly inactivated by carboxypeptidases that cleave off the C-terminal arginine resulting in C3a-desArg. C3a-desArg was traditionally viewed to have minimal biological activity[257] although recent evidence suggests it may have paracrine and endocrine functions such as modulation of lipid metabolism[258].

The cellular receptor for C3a was identified and cloned in 1996[259]. It is a G-protein coupled receptor with 7 trans-membrane domains and high homology to the human C5aR1. Activation of the receptor leads to robust calcium mobilisation from intracellular stores [260, 261]. C3a, in combination with CPG is also a ligand for the receptor for advanced glycation end products (RAGE)[262]. The physiological consequences of this interaction are uncertain.

Expression

Expression of the C3aR in humans and mice has been investigated using a variety of techniques, including flow cytometry, mRNA analysis and functional studies. Results from these studies have at times been conflicting. Expression has been consistently reported on monocytes, neutrophils, eosinophils, basophils, mast cells, dermal, monocyte derived and plasmacytoid dendritic cells[214, 243, 261, 263-265]. Reports on expression of the C3aR on human and murine B and T cells have been less consistent with both expression[217, 266-269] and absence of expression reported[214, 263]. An investigation of renal expression of the C3aR by mRNA, flow cytometry, immunohistochemistry and in-situ hybridisation reported expression on glomerular epithelial and renal tubular cells but not glomerular endothelial or mesangial cells [270]

Ligation of the C3aR has been reported to cause histamine release from mast cells[261], enhance IL1-β and TNF secretion from monocytes[271, 272], promote NETosis in neutrophils[273] and act as a chemotaxin in the macrophage cell line J774a[274].

C3aR enhances antigen presenting cell activation

BMDCS from *C3aR*$^{-/-}$ mice express lower levels of costimulatory molecules such as MHC class II, and secrete fewer inflammatory cytokines accompanied by increased anti-inflammatory IL-10. *In vitro* and *in vivo* these DCs induce reduced allo-specific T cell proliferation and IFN-ϒ production [275, 276]. In human monocyte derived DCs, C3a induces upregulation of MHC-II, CD40 and CD86 as well as enhancing secretion of TNF IL-1β and IL-6[249, 271]. Ligation of the C3aR has also been reported to induce IL-23 production from murine BMDCs with subsequent Th17 and Th2 polarisation of T cells [277, 278].

C3aR modulates T cell immunity

C3 deficient humans mount attenuated Th1 responses[279]. This is partially mediated by surface C3aR and CD46 (which binds C3b), along with intracellular C3a generation signalling through the C3aR which promotes mammalian target of rapamycin (mTOR) signalling and T cell survival[268, 269, 280]. There is no murine homologue for CD46 however there is some evidence that C3aR alone is able to influence the generation of T cell responses by effects on both antigen presenting cells (APCs) and T cells.

Two groups have reported a modest increase in Tregs in *C3aR*$^{-/-}$ mice[252, 281]. However the majority of work in this field has used *C3aR*$^{-/-}$*C5aR1*$^{-/-}$ double knockout mice, in which deficiency of both anaphylatoxin receptors has an additive effect on Treg generation. Inhibition or knockout of both receptors has been reported to increase generation of induced Tregs and enhance suppressive function of natural Tregs, accompanied by diminished Th1 responses in both mice and humans [217, 252, 253, 282]. The mechanism of this effect has been reported to be both T cell intrinsic and DC mediated. The relative contributions of C3aR and C5aR to the phenotype of the double knockout remains to be fully defined.

C3aR in humoral immunity

C3 split products are potent inductors of humoral immunity. In 1975 it was shown that depletion of complement by cobra venom factor inhibited the generation of antibody response to human IgG by mice.[283] Subsequent work showed that the C3b breakdown product C3dg functions as a natural adjuvant, lowering the threshold of B Cell activation by engagement of the complement receptor 2 (CD21) [284].

There are limited data on the role of C3a/C3aR in humoral immune responses. Purified C3a has been reported to suppress IgG production from cultured B cells[266], *C3aR*$^{-/-}$ mice have also been reported to have exaggerated IgG1 production due to an increased Th2 response[285].

The role of the C3aR in murine models of inflammation and autoimmunity

Published studies on C3aR in murine models of inflammation have reported both pro and anti-inflammatory actions depending on the target organ and context of the stimulus. C3aR was reported to be protective in LPS induced shock with higher mortality observed in *C3aR*$^{-/-}$ mice accompanied by elevated levels of Il-1β[286]. C3aR was also protective in a model of intestinal ischemia-reperfusion, in which *C3aR*$^{-/-}$ mice developed exacerbated intestinal injury. This was associated with a marked neutrophilia in *C3aR*$^{-/-}$ mice, which also occurred after GMCSF infusion suggesting that C3aR may be a negative regulator of neutrophil bone marrow mobilisation [287].

In contrast, C3aR has been shown to be pro-inflammatory in other models of inflammation including passive anti-collagen antibody induced arthritis[240], dextran sulphate induced colitis [288] and experimental autoimmune encephalomyelitis [289],

C3aR in models of renal disease

Both protective and injurious roles for the C3aR have also been reported in models of renal disease. In renal ischemia-reperfusion injury *C3aR*$^{-/-}$ mice developed reduced renal injury and attenuated infiltrate of neutrophils and macrophages [237]. C3aR was also reported to exacerbate injury in a mouse model of Adriamycin induced proteinuric nephropathy in which *C3aR*$^{-/-}$ mice had reduced albuminuria and reduced injury of the glomeruli and tubulointerstitial compartment. This was accompanied by reduced interstitial type I collagen, macrophage infiltration and myofibroblast staining. These data, along with the demonstration that C3a induced epithelial to mesenchymal transition in proximal tubular epithelial cells suggests that C3aR signalling may promote chronic fibrotic renal injury[290].

To determine the role of C3aR in complement receptor mediated immune cell infiltration and renal injury Bao et al. conducted a series of experiments in which *Crry*$^{-/-}$*C3*$^{-/-}$ kidneys were transplanted into complement sufficient syngeneic recipients with knockout of the C3aR and/or the C5aR1[291]. CR1-related protein/protein y (Crry) is a surface bound complement regulator that has similar properties to human decay accelerating factor and membrane co-factor protein. Cryy deficiency is embryonic lethal but can be rescued by co-existing C3 deficiency. Transplanting kidneys from *Cryy*$^{-/-}$*C3*$^{-/-}$ mice into complement sufficient mice results in defective complement regulation in the kidney with subsequent inflammation and renal failure[292]. This model is of note as it allows dissociation of the role of immune cell expressed C3aR from renal expressed receptor and is independent of immune complexes. Recipient mice with C3aR deficiency developed reduced tubulointerstitial injury, along with attenuated influx of macrophages and T cells. These data suggest that C3aR signalling drives macrophages to sites of renal complement activation.

In contrast, C3aR was reported to be anti-inflammatory in the MRL/lpr model of lupus nephritis as *C3aR*$^{-/-}$MRL/lpr mice developed significantly higher autoantibody titers (predominantly IgG2a), and accelerated onset, although not severity of renal injury[293]. In this model the C3aR inhibitor SB290157 gives conflicting results to *C3aR*$^{-/-}$ mouse as C3aR inhibition was associated with reduced albuminuria, leukocyte influx and serum urea nitrogen[294]. The divergence between reported outcomes in knockout and inhibitor treated mice may result from the fact that SB290157 is now recognized to have partial C3aR agonist activity[295]

Neutrophil-Complement interaction

Previous studies have suggested a key role for complement-neutrophil interactions in AAV. Complement and neutrophils have multiple and complex two-way interactions. Complement fragments activate neutrophils via surface complement receptors. Activated neutrophils expel their granule contents, which themselves have complement activating properties. Circulating and membrane bound complement regulatory proteins preserve homeostasis by limiting complement activation. The close relationship between complement and neutrophils is illustrated by the association of complement factor H polymorphisms linked to more stringent complement regulation with lower serum myeloperoxidase levels (a marker of neutrophil activation) in a genome wide association study[47].

Complement activates neutrophils

Neutrophils express multiple complement receptors, these are tabulated below along with the effect of receptor ligation

Receptor	Endogenous complement ligand	Functional effect on neutrophil (H=human, M=Mouse)
C5aR1 (CD88)	C5a	Promotes neutrophil chemotaxis and degranulation (H). Upregulates excitatory FcγRs[231](H/M). Induces NETosis in interferon primed neutrophils[296].
C5aR2 (C5L2)	C5a, C5desarg	Predominantly intracellular. Activation results in β-arrestin binding and inhibition of C5aR mediated signalling pathways (H)[297]
C3aR	C3a	Not thought to cause neutrophils chemotaxis or degranulation of neutrophils (H) Negative regulator of neutrophil mobilisation [287](M)
CR1 (CD35)	C3b, iC3b	Co-factor for C3b and C4B cleavage to downstream inactive forms (H)[298]. Mediates phagocytosis of C3b coated immune complexes (H)[299]
CR3 (CD11b/18)	iC3b	Adhesion, chemotaxis, production of reactive oxygen species, phagocytosis (H)[299]
C1qR	C1q	Ligation may induce ROS production[300]

Neutrophils activate complement

Neutrophils are able to activate complement through the release of properdin in granules, thereby focusing complement activation at the site of degranulation. Properdin stabilises the C3 convertase and is a positive regulator of the alternative pathway. Properdin is released from the secondary granules in response to a variety of stimuli including C5a, TNF, and IL-8[301]. Stimulation of neutrophils by TNF or fMLP also leads to cell bound properdin which activates the alternative pathway leading to neutrophil surface C3 deposits[302]. The positive feedback loop of neutrophil activation, properdin and complement activation is partially driven by C5a signalling through the C5aR1 as C5aR1 inhibition diminishes neutrophil bound C3[302].

Neutrophils are also able to activate complement through MPO, via production of reactive oxygen species[303] and by binding to properdin in a C3 dependent mechanism with subsequent alternative pathway activation[304]. Other ways that neutrophils can activate complement include membrane microparticles [302, 305] and NETS, which induce complement activation through both alternative and non-alternative pathways[306].

Neutrophil surface complement regulators

Neutrophils are equipped with membrane bound complement regulators to prevent excessive complement mediated activation. These are delay accelerating factor (DAF, CD55) which inhibits the C3 convertase, membrane co-factor protein (CD46, MCP) a cofactor for factor I degradation of C3b/C4b and MAC inhibitory protein (CD59), which prevents MAC formation [307]. Membrane expression of these is variable and CD55 levels are increased during neutrophil activation but decrease in apoptosis[308, 309].

Summary and aims:

The ANCA associated vasculitides are autoimmune diseases with a complex pathogenesis. There is now a strong evidence base for complement C5a, signalling through the C5aR1 being a critical step in neutrophil activation by ANCAs and subsequent glomerulonephritis. Clinical trials of C5aR1 inhibition are currently ongoing. However, many questions surrounding the role of complement in this disease remain. This thesis presents work that addresses the following research aims.

-To investigate whether pathogenic role for C5aR1 in anti-MPO can be extended to its role in modulating nephritogenic anti-MPO autoimmunity using mouse models of disease. (Chapter 2)

-To determine whether the receptor for C3a, the C3aR influences anti-MPO autoimmunity or glomerulonephritis in murine models of disease. (Chapter 3)

-To investigate whether the alternative pathway of complement mediates anti-MPO autoimmunity or renal injury in a T cell mediated autoimmune model of anti-MPO glomerulonephritis. (Chapter 4)

-To determine whether ANCA from patients with AAV activate the classical pathway of complement, and if so, whether the classical pathway is a significant contributor to complement mediated activation of human neutrophils. (Chapter 5)

Chapter 2: The C5a receptor 1 promotes autoimmunity, neutrophil dysfunction and injury in experimental anti-myeloperoxidase glomerulonephritis

Jonathan Dick[1,2], Poh-Yi Gan[1], Sharon L. Ford[1,3]. Dragana Odobasic[1], Maliha A. Alikhan[1], Sven Loosen[1], Pam Hall[1], Clare L. Westhorpe[1], Anqi Li[1], Joshua D. Ooi[1], Trent M. Woodruff[4], Charles S R. Mackay[5], A. Richard Kitching[1,2,6], Michael J. Hickey[1], Stephen R. Holdsworth[1,2]

[1] Centre for Inflammatory Diseases, Monash University Department of Medicine, Clayton, Victoria, Australia

[2] Department of Nephrology, Monash Health, Clayton, Victoria, Australia

[3] Department of Nephrology, St Vincent's Hospital, Fitzroy, Victoria, Australia

[4] School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland, Australia.

[5] School of Biomedical Sciences- The Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia

[6] Department of Paediatric Nephrology, Monash Children's Hospital, Monash Health, Clayton, Victoria, Australia

Correspondence:

Prof. Stephen R. Holdsworth, Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, 246 Clayton Rd, Clayton, VIC 3168, Australia.

Phone: [redacted]

Email: [redacted]

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Running Title

C5aR1 promotes anti-MPO autoimmunity and GN.

Abstract:

The prospects for complement-targeted therapy in ANCA-associated vasculitis (AAV) have been enhanced by a recent clinical trial in which C5a receptor 1 (C5aR1) inhibition safely replaced glucocorticoids in induction treatment. C5aR1 primes neutrophils for activation by ANCA and is therefore required in models of glomerulonephritis induced by anti-myeloperoxidase (MPO) antibody. Although humoral and cellular autoimmunity play essential roles in AAV, a role for C5aR1 in these responses has not been described. In these studies, we use murine models to dissect the role of C5aR1 in the generation of anti-MPO autoimmunity and the effector responses resulting in renal injury. We show that that genetic absence or pharmacological inhibition of C5aR1 results in reduced autoimmunity to myeloperoxidase with an attenuated Th1 response, increased $Foxp3^{+}$ regulatory T cells and reduction in generation of MPO-ANCA. These changes are mediated by C5aR1 on dendritic cells, which promotes activation, and thus MPO autoimmunity and glomerulonephritis. We also use renal intravital microscopy to determine the effect of C5aR1 inhibition on ANCA induced neutrophil dysfunction. We observe that MPO-ANCA induce neutrophil retention and reactive oxygen species burst within glomerular capillaries. These pathological behaviours are abrogated by C5aR1 inhibition. Together, these data suggest C5aR1 inhibition ameliorates both autoimmunity and intra-renal neutrophil activation in AAV.

1499/1500 characters including spaces.

Keywords:

ANCA, glomerulonephritis, complement, autoimmunity, C5aR1, vasculitis

Introduction.

ANCA-associated vasculitis (AAV) is an autoimmune disease in which multiple immune participants contribute to the pathogenesis. A loss of self-tolerance results in ANCA production by B cells. These autoantibodies activate neutrophils, which induces their recruitment to glomeruli, degranulation and extracellular trap formation.[41, 50] This neutrophil activation results in both direct endothelial injury and extensive glomerular deposition of myeloperoxidase (MPO).[85, 119] In mouse models, the response of MPO specific effector T cells to glomerular MPO is a significant contributor to necrotising glomerulonephritis associated with MPO autoimmunity.[104, 310]

Complement plays an important role as a modulator and effector of immune responses. There are three activation pathways, the classical, lectin and alternative which converge to form C3 and C5 convertases. These result in the generation of multiple effector molecules including the pro-inflammatory C5 fragment, C5a, which binds to C5a receptor 1 (C5aR1) and C5a receptor 2 (C5aR2). The C5aR1 is expressed on myeloid cells including neutrophils, mast cells, monocyte/macrophages and dendritic cells (DCs).[219, 220] Therapeutic targeting of C5 has been shown to be a potent approach for treatment of diseases driven by dysregulation of complement such as atypical haemolytic uremic syndrome.

Previous pre-clinical studies have shown that complement amplified by the alternative pathway and signalling through C5aR1 plays a key role in the priming of neutrophils for activation by ANCA and in experimental glomerulonephritis induced by anti-MPO antibodies.[56, 185, 189] This data is supported by evidence of raised complement activation products in clinical samples from patients with active AAV.[191] The recently published phase II CLEAR study reported that the oral C5aR1 antagonist CCX168 (Avacopan) was non-inferior to standard dose prednisolone as a component of induction therapy for AAV.[207] Complement inhibition therefore has the

potential to be a paradigm changing therapy in AAV, a phase 3 clinical trial (NCT02994297) will further inform the validity of this approach.

Whilst therapeutic targeting of C5aR1 is in advanced clinical development, the importance of this receptor in the generation of the cellular and humoral anti-MPO autoimmunity that are required for this autoimmune disease has not been determined. Furthermore, how C5aR1 inhibition moderates ANCA induced neutrophil dysfunction in the glomerulus in vivo has not been described. In these studies we investigated the hypothesis that, independent of its effects on neutrophils, C5aR1 would promote autoreactivity to MPO and subsequent glomerulonephritis. In addition, we used confocal microscopy to define the effect of C5aR1 inhibition on neutrophil behaviour in the glomerular microvasculature.

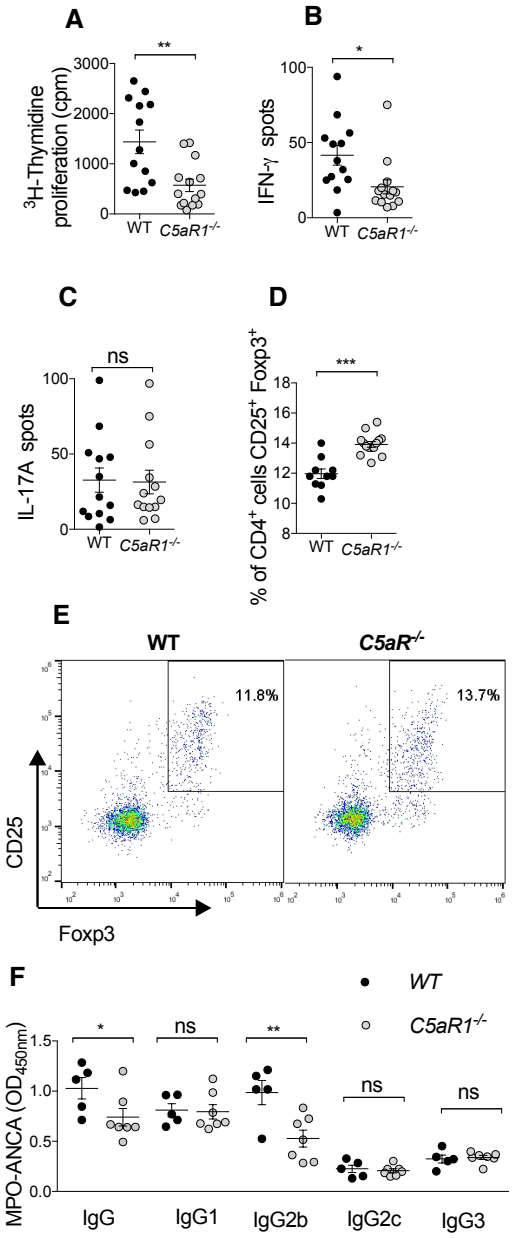
Results

C5aR1 promotes cellular and humoral autoimmunity to MPO

To determine whether endogenous C5aR1 promotes autoimmune responses to MPO, anti-MPO autoimmunity was studied in C5aR1 intact (WT) and *C5aR1*$^{-/-}$ mice 10 days after immunisation with MPO in Freund's complete adjuvant (FCA). In *C5aR1*$^{-/-}$ mice, there was a reduction in the proliferation of antigen restimulated lymphocytes, and a reduction in the MPO-specific Th1 response with a corresponding increase in the proportion of $CD4^+$ cells that were regulatory T cells (Treg). The Th17 response was not affected (Figure 1A-E). Humoral immunity was assessed 28 days after initial immunisation with MPO in FCA, followed by a boost dose of MPO in Freund's incomplete adjuvant on day 7. MPO-ANCA titres were decreased in *C5aR1*$^{-/-}$ mice, largely related to a reduction in the IgG2b subclass. There were no significant differences between groups in other IgG subclasses (Figure 1F). To assess whether inhibition, rather than genetic deletion would effect adaptive immunity we used the peptide inhibitor (Ac-Phe-[Orn-Pro-dCha-Trp-Arg], *PMX53*) to study the effect of C5aR1 inhibition on autoimmunity to MPO

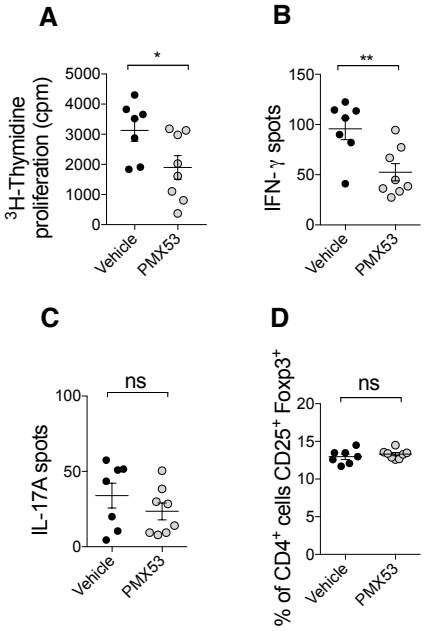
10 days after immunisation. Due to the drug's short life[311] it was administered continuously through an osmotic infusion mini-pump. Similar to the findings with *C5aR1*$^{-/-}$ mice, T cell proliferation and anti-MPO Th1 responses were reduced, although there was no significant difference in the proportion of CD4 cells with a Treg phenotype or Th17 response (Figure 2A-D).

Figure 1



Endogenous C5a promotes cellular and humoral immunity to MPO. *Cellular immune responses were measured 10 days after immunisation of mice with MPO in FCA. Compared to WT mice, C5aR1*$^{-/-}$ *mice showed (A) reduced proliferation of antigen restimulated cells from draining lymph nodes measured by* ^{3}*H-thymidine (B) reduced MPO specific Th1 response measured by IFN-γ ELISPOT. (C) Th17 response measured by IL-17A ELISPOT was similar. (D) The proportion of CD4*$^+$ *cells in the draining LN that were CD25*$^+$*Foxp3*$^+$ *Tregs was increased in C5aR1*$^{-/-}$ *mice. (E) Representative flow cytometry plot gated on CD4*$^+$ *cells showing CD25*$^+$*Foxp3*$^+$ *population. (F) Antibody responses were measured 28 days after MPO immunisation. Compared to WT mice, C5aR1*$^{-/-}$ *mice exhibited reductions in total MPO-ANCA and IgG2b MPO-ANCA, titres of other subclasses were similar. *P <0.05, **P< 0.01, ***P<0.001.*

Figure 2:



C5aR1 inhibition attenuates anti-MPO autoimmunity. *Cellular immunity was measured 10 days after immunisation with MPO in FCA in mice receiving the C5aR1 inhibitor PMX53 or vehicle delivered by osmotic infusion pump. C5aR1 inhibition resulted in (A) reduced proliferation of MPO restimulated cells (B) and reduced Th1 response. (C) There was no difference in Th17 response or (D) the proportion of CD4$^+$ cells that were CD25$^+$Foxp3$^+$. *P <0.05, **P< 0.01,*

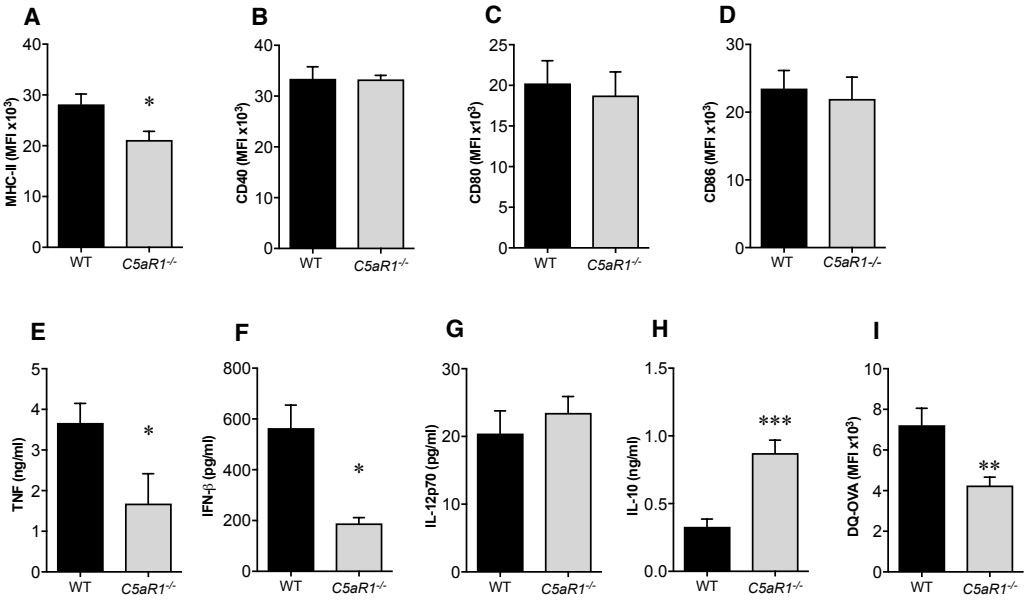
C5aR1 regulates DC activation and subsequent T cell response

C5aR1 has been reported to influence the generation of Th1, Th17 and Foxp3$^+$ Tregs through signalling within both antigen presenting cells (APCs) and T cells.[217, 244, 247, 252] We investigated expression of co-stimulatory molecules and the cytokine profile of bone marrow (BM) derived DCs from *C5aR1*$^{-/-}$ and WT mice. DCs from WT mice exhibited higher expression of MHC-II, whilst CD40, CD80 and CD86 were similar between groups (Figure 3 A-D). WT BMDCs secreted more TNF and IFN-β, and whilst IL-12p70 was similar between groups, *C5aR1*$^{-/-}$ DCs secreted more anti-inflammatory IL-10 (Figure 3 E-H). In addition, *C5aR1*$^{-/-}$ DCs were less efficient at internalising antigen measured by the fluorogenic substrate DQ-OVA (Figure 3I).

To determine whether the differences in DC activation and antigen uptake observed in *C5aR1*$^{-/-}$ DCs resulted in attenuated autoimmunity, we transferred MPO loaded BMDCs derived from either WT or *C5aR1*$^{-/-}$ mice into WT hosts to induce autoimmunity as previously described.[69] Draining lymph node cells were collected 10 days after BMDC immunisation for analysis of T cell responses. Compared to WT DCs, transfer of *C5aR1*$^{-/-}$ DCs resulted in a reduced Th1

response and increased proportion of Tregs without altering the Th17 response, mirroring results in mice globally deficient in the C5aR1 (Figure 4 A-D).

Figure 3



The C5aR1 on APCs modulates autoimmunity to myeloperoxidase.

*Bone marrow derived dendritic cells were grown from WT or C5aR1$^{-/-}$ mice. Day 8 BMDCs were stimulated for 24 hours with 25nM rC5a and 1 μg/ml LPS before surface expression of co-stimulatory molecules and supernatant cytokines were analysed. (A-D) WT BMDCs displayed higher expression of MHC-II, other co-stimulatory molecules were not different. (E-G) C5aR1$^{-/-}$ BMDCs secrete less TNF and IL-1β than WT DCs, levels of IL-12p70 were similar. (H) C5aR1$^{-/-}$ DCs secrete more IL-10 than WT DCs. (I) Day 8 BMDCs were incubated with 10μg/ml DQ-OVA for 120 minutes before antigen uptake was analysed by flow cytometry. *P <0.05, **P< 0.01, ***P<0.001.*

To explore the additional possibility for a functional effect of a T cell intrinsic C5aR1, we utilised *Foxp3*^{GFP} reporter mice with intact complement receptors or *Foxp3*^{GFP} mice deficient in both C5aR1 and C3aR (*C3aR*$^{-/-}$*C5aR1*$^{-/-}$*Foxp3*^{GFP}),[252] to isolate CD4^{+}Foxp3^{-} cells from naïve mice. These T cells were injected into *Rag1*$^{-/-}$ mice that were then immunised with MPO in

FCA. 10 days after immunisation there was no difference in the generation of splenic IFN-γ^+ or IL-17A$^+$ and, whilst, consistent with previous reports[312], generation of de-novo Foxp3$^+$ Tregs was minimal, this did not differ between groups (Figure 4 E-F).

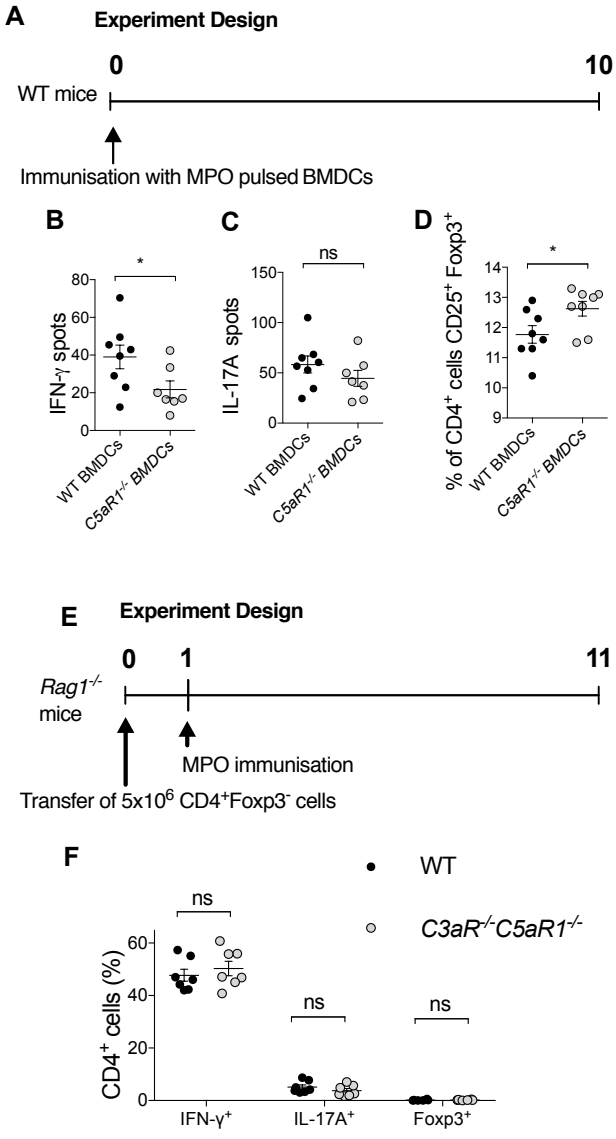


Figure 4: ***C5aR1 on DCs, but not T cells promotes anti-MPO autoimmunity.*** *(A) Day 8 BMDCs were incubated for 120 minutes with 10μg/ml rMPO before being matured for 18 hours with 1μg/ml LPS. The cells were extensively washed before 1x10^6 cells were injected into WT mice. Cellular immunity was measured 10 days after immunisation. (B) WT mice receiving C5aR1$^{-/-}$ MPO pulsed DCs had a reduced IFN-γ producing lymphocytes, (C) similar IL-17A producing lymphocytes and (D) an increased proportion of CD25$^+$Foxp3$^+$ T regulatory cells. (E) 5x10^6 CD4$^+$Foxp3$^-$ cells from Foxp3^{GFP} or C3aR$^{-/-}$C5aR1$^{-/-}$Foxp3^{GFP} mice were injected into Rag1$^{-/-}$ recipients. These were immunised with MPO in FCA and cellular immunity was measured 10 days later. (F) There was no difference in the proportion of CD4$^+$ IFN-γ$^+$, IL-17A$^+$ or Foxp3$^+$ cells. *P <0.05.*

C5aR1 on DCs induces T cell mediated anti-MPO glomerulonephritis

We have previously shown that when autoimmunity to MPO is induced in C57BL/6 mice by immunisation, the humoral response generated is insufficient to cause glomerulonephritis. However, when glomerular neutrophil recruitment and MPO deposition is induced with low dose sheep anti-mouse anti-glomerular basement membrane (GBM) globulin, mice develop antigen specific T cell mediated necrotising glomerulonephritis.[104, 120] Importantly, previously published controls including the lack of injury in mice immunised with OVA or M*po*$^{-/-}$ mice and the induction of injury by MPO but not OVA specific T cell clones, confirm that glomerular injury is caused by MPO specific effector T cells.[34, 104, 120] This model is dependent on glomerular neutrophil recruitment by anti-GBM globulin, and as glomerular neutrophil recruitment by immune complexes has been reported to be complement dependent,[313] we first ascertained whether neutrophil recruitment to the glomerulus was C5aR1 dependent. Glomerular neutrophil recruitment in *C5aR1*$^{-/-}$ mice was significantly less than that observed in WT mice one hour after injection of anti-GBM globulin (WT: 1.26±0.64 vs. *C5aR1*$^{-/-}$: 0.40±0.13 neutrophils/GCS p=0.019). Therefore, to dissociate the role of C5aR1 on immunity from its effects on neutrophils, we induced MPO autoimmunity in WT mice with MPO-pulsed BMDCs and 14 days later, triggered glomerulonephritis with low dose anti-GBM globulin. Compared with transfer of MPO-pulsed WT DCs, transfer of MPO-pulsed *C5aR1*$^{-/-}$ BMDCs resulted in attenuated glomerulonephritis with reduced albuminuria and glomerular necrosis, accompanied by reductions in glomerular leukocytes (Figure 5A-G). Serum MPO-ANCA titres were also reduced in mice receiving *C5aR1*$^{-/-}$ BMDCs (Figure 5H).

Figure 5

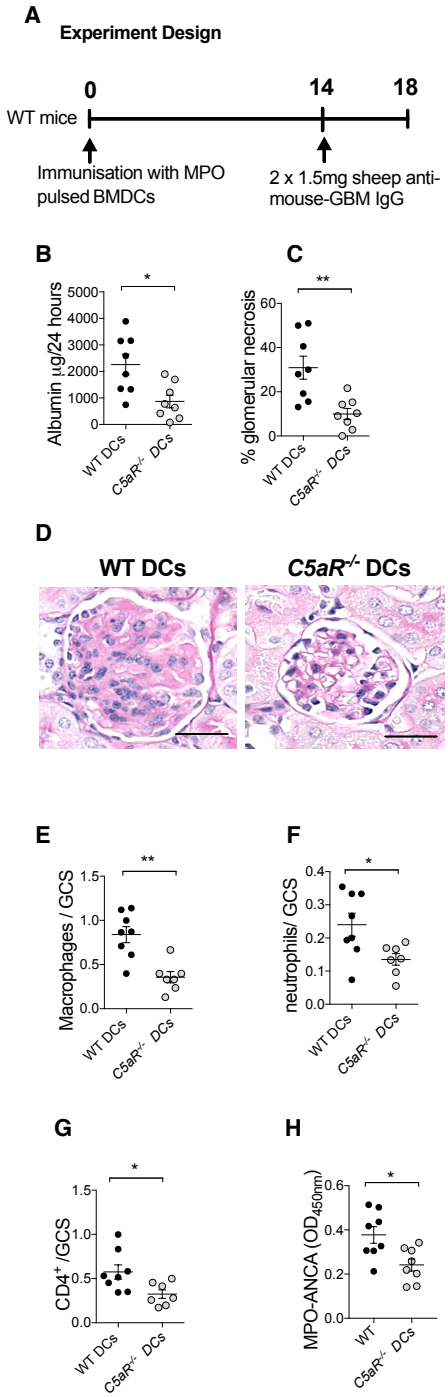


Figure 5:

Autoimmunity transferred by C5aR1$^{-/-}$ DCs results in attenuated anti-MPO glomerulonephritis. *(A) MPO autoimmunity was induced by immunisation of WT mice with MPO pulsed WT or C5aR1$^{-/-}$ BMDCs. Glomerulonephritis was triggered by a sub-nephritogenic dose of anti-GBM IgG. (B-C) Mice receiving C5aR1$^{-/-}$ BMDCs developed significantly less albuminuria and glomerular necrosis. (D) Segmental glomerular necrosis in mice receiving WT DCs. Magnification 400x, scale bar 30µm. (E-G) The accumulation of CD4^{+} T cells, neutrophils and macrophages in glomeruli was attenuated in mice receiving C5aR1$^{-/-}$ BMDCs. (H) MPO-ANCA titres were reduced in mice receiving C5aR1$^{-/-}$ DCs. *P <0.05, **P< 0.01.*

C5aR1 mediates ANCA induced neutrophil retention and ROS production in the glomerulus

We have previously used multiphoton imaging to demonstrate that MPO-ANCA causes retention of neutrophils in glomeruli.[314] Here, we examined the role of C5aR1 alongside MPO-ANCA induced neutrophil behaviour and dysfunction in glomeruli. Mice were co-administered LPS and either MPO-ANCA or anti-OVA IgG as control. Compared to anti-OVA IgG, MPO-ANCA did not affect the total number of neutrophils adhering in glomeruli, but increased neutrophil dwell time in glomeruli. This was due to an increase in the dwell time of crawling (but not static) neutrophils (Figure 6A-C and Supplementary Videos S1 & S2). In MPO-ANCA treated mice administration of an anti-C5aR1 neutralising monoclonal antibody limited the increase in dwell time of crawling neutrophils (Figure 6D).

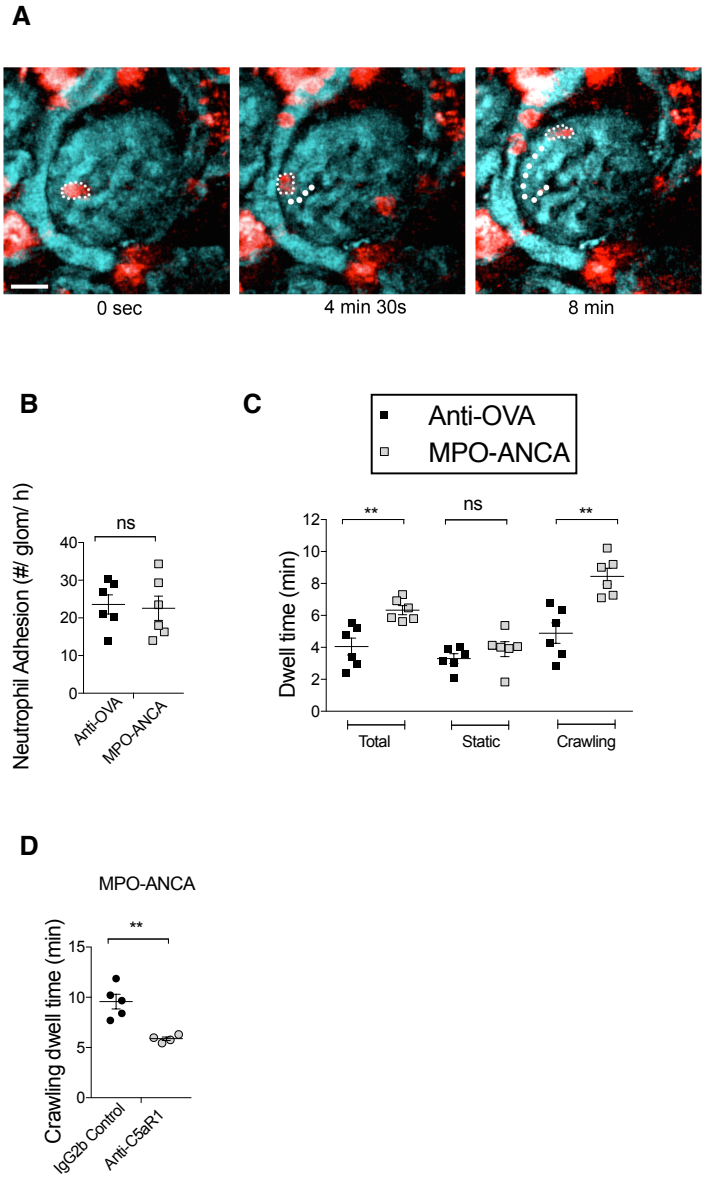


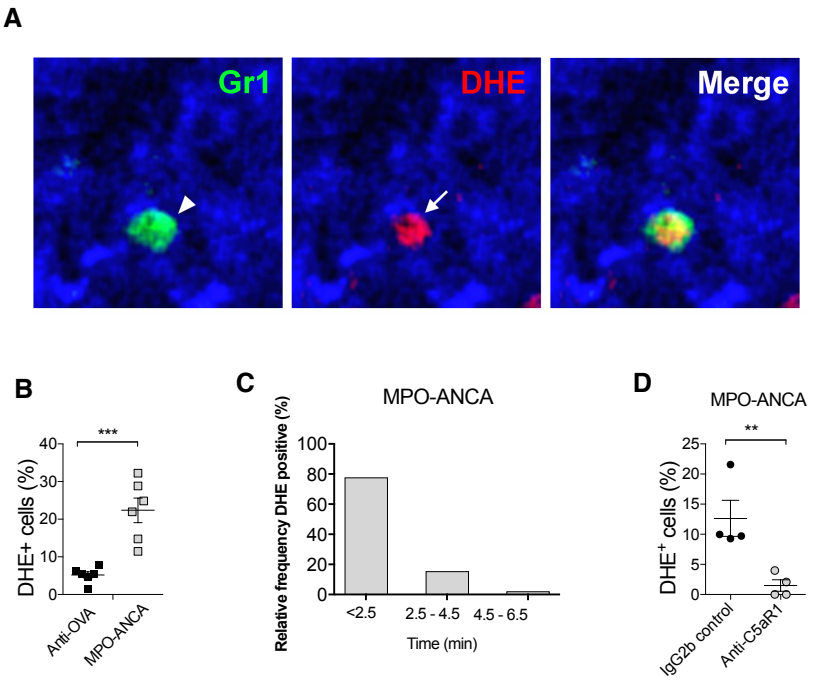
Figure 6

MPO-ANCA induces neutrophil retention in the glomerular capillaries which is attenuated by C5aR1 inhibition. *Multiphoton microscopy was used to analyze neutrophil recruitment in wild-type mice treated with either MPO-ANCA or anti-OVA IgG (as control), following priming with systemic LPS. (A) Sequence of images showing an anti-Gr-1-PE stained neutrophil (red, outlined by fine dotted line) undergoing prolonged intravascular migration in glomerulus following administration of anti-MPO*

*IgG. (Dotted line denotes migration path, time stamp shown below images). Vasculature (blue) is labeled by Pacific Blue dextran (see also Supplementary Videos S1 & S2). (B) MPO-ANCA does not increase the number of adherent neutrophils in the glomerulus. (C) MPO-ANCA causes an increase in glomerular dwell time due to prolonged retention of crawling, but not static neutrophils. (D) C5aR1 inhibition reduces glomerular crawling time compared to control. **P< 0.01.*

As ANCA induce neutrophil production of reactive oxygen species (ROS) *in vitro*, we examined whether MPO-ANCA induced ROS by neutrophils *in vivo* within glomerular capillaries. ROS production was measured by dihydroethidium (DHE) associated fluorescence. In mice treated with LPS and MPO-ANCA, approximately 20% of adherent neutrophils displayed detectable DHE fluorescence. This ROS generation was rapid, occurring within 2.5 minutes of adhesion in the majority of neutrophils (Figure 7A-C). C5aR1 inhibition reduced the number of DHE-positive neutrophils, so that ROS-producing neutrophils were almost entirely absent (Figure 7D). Together, these findings demonstrate that C5aR1 plays a key role in both MPO-ANCA induced neutrophil retention and ROS production in the glomerular microvasculature, thus promoting ANCA induced glomerular injury.

Figure 7



MPO-ANCA induced glomerular neutrophil ROS production is abrogated by C5aR1 inhibition.

(A) Multiphoton intravital microscopy image showing neutrophil ROS generation in mouse following administration of MPO-ANCA. Neutrophils were detected using anti-Gr-1 (green, arrowhead), while ROS generation was detected using the oxidant-sensitive fluorochrome dihydroethidium (DHE) (red, arrow) (B) MPO-ANCA induces ROS production by intraglomerular neutrophils. (C) Histogram showing time-course of ROS production in DHE positive adherent neutrophils. (D) MPO-ANCA induced ROS production in glomerular neutrophils is abrogated by C5aR1 inhibition.

***P< 0.01, ***P<0.001.*

Discussion

Previous studies showing a role for C5aR1 in mediating *in vitro* neutrophil activation and experimental glomerulonephritis induced by MPO-ANCA have led to clinical trials in humans. The studies described here suggest an additional, potentially beneficial consequence of targeting the C5aR1 in AAV, is to dampen the underlying cellular and humoral autoreactivity to MPO that drives this disease.

T cells play multifaceted roles in AAV. These include; provision of help to B cells to facilitate ANCA production[315], a direct contribution to renal injury by T effector cells[104] and the suppression of autoimmunity and inflammation by Tregs.[34] To date, studies investigating the role of C5aR1 in experimental anti-MPO vasculitis have not investigated the influence of C5aR1 signalling on these behaviours. We observed attenuation of Th1 immunity, an important mediator of glomerular injury in established crescentic glomerulonephritis,[316] in both *C5aR1*$^{-/-}$ mice and mice treated with a C5aR1 inhibitor. This was accompanied by an increased proportion of Tregs in *C5aR1*$^{-/-}$ mice. These findings are consistent with previous studies in which signalling through the C5aR1 has been found to induce Th1 responses and inhibit induction of regulatory T cells.[246, 252, 253]

It is likely that signalling through C5aR1 also influences T cell immunity in humans as C5a has been reported to up-regulate costimulatory molecules on human DCs, induce Th1 polarisation and inhibit the formation of human Tregs.[243, 253, 254] The potential clinical relevance of this is that C5aR1 inhibition may have beneficial effects on the enhancement of Th1[102] and diminution of Treg [101] cell functions that are associated with AAV.

An area of recent controversy has been the relative contribution of T cell intrinsic and DC expressed C5aR1 to its effects on T cell polarisation. A role for T cell intrinsic C5aR1 in inducing Th1 and Treg polarisation has been proposed,[217, 218, 252] however, two studies utilising

different C5aR1-GFP reporter mice have not confirmed T cell expression.[219, 220] In keeping with other studies[244, 247, 317], we found that DCs from *C5aR1*$^{-/-}$ mice had a less activated phenotype with reduced MHC-II expression and antigen processing alongside increased secretion of IL-10. Transfer of *C5aR1*$^{-/-}$ DCs into WT mice resulted in attenuated anti-MPO autoimmunity. In contrast, we did not find a difference in T cell polarisation when cells were transferred into *Rag1*$^{-/-}$ hosts, although under these conditions, generation of de-novo Foxp3^{+} cells was minimal. This suggests, at least in mice, that effects of C5aR1 on immunity are mediated by the receptor on APCs.

C5aR1 signalling has previously been reported to exacerbate immune mediated renal injury[255, 256], we investigated whether the observed reduction of autoimmunity would translate to reduced glomerular disease independent of any effect of C5aR1 on neutrophils. The attenuated disease observed in mice receiving *C5aR1*$^{-/-}$ BCMDCs confirms that C5aR1 induced nephritogenic autoimmunity to myeloperoxidase is a significant contributor to glomerular injury. In addition to the generation of autoimmunity, signalling through C5aR1 may also contribute to the perpetuation of nephritogenic autoimmunity that occurs in active disease after myeloperoxidase deposition in the kidney, which is a site enriched for C5aR1 expressing APCs.[318]

The production of ANCA is essential for the pathogenesis of AAV. We observed that MPO-ANCA titres were reduced in immunised C5aR1$^{-/-}$ mice, this supports the role for the C5aR1 in promoting humoral immunity to other antigens seen in previous studies.[319-321] As murine B cells do not express the C5aR1,[219, 220] and the reduction was observed when immunity was transferred by MPO loaded *C5aR1*$^{-/-}$ DCs into WT mice, C5aR1 is likely to promote humoral immunity by APC modulation of the T cell response.

Whilst the C5aR1 has previously been shown to be important for neutrophil priming for ANCA activation *in vitro*, how it affects glomerular neutrophil behaviour *in vivo* has not previously been defined. We have previously used intravitral microscopy to visualise MPO-ANCA induced glomerular neutrophil adhesion in the glomerulus and to demonstrate that this process that is dependent on the adhesion molecules lymphocyte function associated antigen-1 (LFA-1) and α4-integrin.[84, 314] Here we extend these observations by showing that MPO-ANCA also rapidly induce glomerular ROS production by neutrophils, and that both this, and the increased intravascular migration observed in MPO-ANCA treated mice are abrogated by C5aR1 inhibition. Ligation of C5aR1 has been shown to induce the translocation and surface expression of autoantigens which is required for neutrophil activation by ANCA.[56, 322] Additional biological effects of C5a which may be contribute to its ability to prime neutrophils for activation by ANCA include the induction of FcγIIa and suppression of FcγRIIb transcription as well as the enhancement of LFA-1 mediated adhesion.[323, 324]

In summary, this work builds on previous studies and extends the role for the C5aR1 in ANCA associated vasculitis beyond its role in neutrophil priming for activation by ANCA. We propose that the C5a plays multiple roles in this disease. These include activation of DCs facilitating the loss of tolerance to the auto-antigens and the development and maintenance of Th1 polarised anti-MPO autoimmunity. C5a is also likely to attenuate the generation of immunomodulatory Tregs and have an indirect effect on B cell production of ANCA through APC polarisation of T cell autoimmunity. At the level of the neutrophil, signalling through the C5aR1 is required for the aberrant activation, migration and ROS production induced by ANCA and may also mediate tissue factor release with resulting thrombosis.[325] Remaining questions that have not been addressed include the role of the C5aR1 on glomerular macrophages, the vascular endothelium, and its potential role in moderating the tubulointerstitial fibrosis

associated with adverse renal survival in AAV.[326, 327] The generation of a lineage selective C5aR1 knockout mouse will facilitate examination of some of these issues.[220]

In the context of previous studies in murine models of anti-MPO glomerulonephritis and the early results of efficacy of small molecule inhibitors of C5aR1 in clinical trials, these results strengthen the argument for the C5aR1 as a therapeutic target by defining its multiple roles in the generation of anti-MPO autoimmunity and effector responses in the kidney.

Concise Methods

Induction and Assessment of systemic immune response

C57BL/6J and *C5aR1*$^{-/-}$ mice[235] (gift of Prof Rick Wetsel, University of Texas) were bred at the Monash University Animal Research Platform. Native and recombinant murine myeloperoxidase were produced as previously described.[328] Mice were immunised subcutaneously with 20μg MPO in FCA (Sigma-Aldrich, St Louis, MO), To inhibit C5aR1, PMX53 (synthesized as previously described[311]) was dissolved in 5% dextrose and infused through an osmotic infusion mini-pump (Azlet, Cupertino, CA) at a rate of 24μg/day. Draining lymph nodes were harvested ten days after immunisation and a single cell suspension was obtained. IFN-γ and IL-17A ELISPOT was performed according to manufacturer's instructions (eBioscience, San Diego, CA) with $5x10^5$ cells per well. Cells were incubated for 18 hours at 37 °C with 10μg/ml heat inactivated rMPO. MPO-specific cell proliferation was measured by culturing lymphocytes at 5×10^5 cells/well in 96-well round-bottom plates re-stimulated with or without 10 μg/ml MPO and incubated for 72 hours. During the last 16 hours of culture, 0.5 μCi of ^{3}H-thymidine (PerkinElmer, Waltham, MA) was added. To quantify Tregs, FoxP3 staining kit (eBioscience), anti-CD4 (RM4.5), anti-CD25 (PC61) (both BD Biosciences) and anti-Foxp3 (FKJ-16s) were used. Flow cytometry was performed on the Beckman Coulter Navios platform and analyzed using FlowJo software (TreeStar, Ashland, OR). Total anti-MPO IgG was

measured by ELISA on MPO coated plates with IgG detected with sheep-anti mouse IgG-HRP (Sigma-Aldrich). Antibody subclasses were measured using subclass specific goat anti-mouse IgG-HRP (Southern Biotech, Birmingham, AL).

BMDCs Culture and BMDC immunization

Bone marrow cells were harvested from the tibia and femurs of WT or *C5aR1*$^{-/-}$ mice and cultured in RPMI media containing 10% FCS, 2mM L-Glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 50µM 2-ME and 10ng/ml GM-CSF (Invitrogen Life Tech). Media was changed on day 3 and 6. On day 8 non-adherent cells were collected and cultured with 25nM recombinant murine C5a (Hycult Biotech) 10ng/ml GM-CSF and with LPS 1µg/ml. Expression of surface markers was measured by flow cytometry using propidum iodide, anti-CD11c (N418), anti-CD86 (GL-1) anti-CD80 (16-10A1), anti-MHC-II (M5/114.15.2) (all Biolegend, San Diego, CA) and anti-CD40 (3/23) (BD Biosciences) Supernatant cytokines were measured by multiplex bead array (Biolegend) with the exception of IL-10 which was measured by ELISA with amplification: coating mAb JESS 2A-5, detection mAb SXC-1 (both BD Biosciences), amplification with sequential extravidin, biotin anti-avidin and extraavidin peroxidase (all Sigma Aldrich). DQ-OVA uptake was measured by incubating day 8 BMDCs in complete media containing 10 µg/ml DQ-OVA (ThermoFisher Scientific). For DC immunization day 8 BMDCs were incubated with 50 µg/ml recombinant murine MPO in RPMI media with 2.5% FCS for 120 minutes, Cells were extensively washed prior to maturation with 1 µg/ml LPS. 1x10^{6} washed BMDCs were injected s/c into wild type mice. 10 days after DC immunization mice were culled for measurement of the immune response or glomerulonephritis was triggered at day 14 by two doses of 1.5mg sheep anti-GBM as previously described.[120]

Transfer of Foxp3^{-} T Cells into Rag1$^{-/-}$ recipients

Naive Foxp3^{GFP} or *C3aR*$^{-/-}$*C5aR1*$^{-/-}$Foxp3^{GFP} mice (Gift of Prof Peter Heeger, Icahn School of Medicine) mice were culled by CO_2 inhalation. The spleens and LN were harvested and single

cell suspension obtained. Cells were CD4 enriched using L3T4 CD4 microbeads (Miltenyi Biotec), Enriched cells were CD4 stained (GK1.5) and sorted by flow cytometry to obtain a $CD4^{+}Foxp3^{-}$ve population. $5x10^{6}$ viable T cells were injected into naïve *Rag1*$^{-/-}$ mice, which were immunised with 20µg MPO in FCA 20 hours later and culled on day 10. Splenocytes were analysed by flow cytometry for formation of de-novo $Foxp3^{+}$ve cells or stimulated with brefeldin A (10µg/ml), ionomycin (500ng/ml) and PMA (100ng/ml) for 4 hours prior to intracellular cytokine staining with anti-IFN-γ (XMG1.2, BD Bioscience) and anti-IL-17A (eBio17B7, eBioscience).

Assessment of Renal Injury

Glomerular segmental necrosis was assessed on formalin fixed, paraffin embedded, periodic acid-Schiff (PAS) stained, 4µm sections. Leukocyte infiltrate was assessed in periodate lysine paraformaldehyde fixed 5 µm section stained with Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and DAB Brown using the following detection antibodies: anti-CD4 (GK1.5), anti-CD68 (FA/11; gift from Gordon L. Koch, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom), and anti-Gr-1 (RB6-8C5; DNAX, Palo Alto, CA). Albuminuria was measured by ELISA (Bethyl Laboratories, Montgomery, TX).

Multiphoton Microscopy

Multiphoton microscopy was performed as previously described.[314] Briefly, mice underwent unilateral ureteric ligation at 4-5 weeks of age. 12 weeks was allowed for the kidney to undergo hydronephrosis before mice were used. Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (150 mg/kg, Troy Labs, Glendenning, Australia) and xylazine (10mg/kg, Pfizer), and the jugular vein cannulated for administration of fluorescent dyes and additional anaesthetic. Body temperature was maintained with a heat pad. The hydronephrotic kidney was exteriorized through a lateral incision and drained of urine. The kidney was

fastened onto a viewing platform, superfused with saline and covered with a coverslip. The renal microvasculature was observed with a Leica SP5 multiphoton microscope (Leica Microsystems) using a 20X 1.0 NA WI objective lens and a SpectraPhysics MaiTai pulsed infrared laser. Three to five glomeruli were imaged at 30 sec intervals during a 1-hour recording period. Images (6 μm z step size) were collected to a depth of ~ 125 μm.

Preparations were imaged using 810 nm excitation, neutrophils were labelled with anti-Gr-1-PE and the vasculature was labelled with Pacific Blue-dextran (conjugated in-house). To detect reactive oxygen species, DHE 2 mg/kg (Sigma-Aldrich) was administered i.v. 20 min before imaging, neutrophils, labelled with Gr-1-AF488, and the vasculature labelled with non-functionalised eFluor650 nanocrystals (eBioscience). Recordings were analysed using Imaris software (Bitplane). Leukocytes were defined as adherent to the endothelium if they remained arrested in the glomerulus for at least two consecutive images (≥30 sec), and dwell time (duration of adhesion) and crawling behaviour were recorded.

MPO-ANCA and Anti-OVA IgG were generated by immunising *Mpo*$^{-/-}$ mice and purified as using protein G column as previously described.[104] To examine MPO-ANCA induced glomerular leukocyte recruitment, mice were primed with LPS (0.1 μg, i.p.), and 4 h later received either MPO-ANCA or anti-OVA IgG (50 μg i.v.). Intravital imaging of glomeruli was performed either 0-60 min or 60-120 min after IgG administration. Anti-C5aR1 mAb (20/70, 100 μg i.v) was administered 15 min before anti-MPO IgG.

Ethics Statement

All experiments were approved by Monash University Animal Ethics Committee and performed in accordance with National Health and Medical Research Council guidelines for animal experimentation.

Statistics

Data are presented as mean+/- SEM with each dot representing a mouse. Prism 6 (Graphpad, San Diego, CA) software was used for analysis with an unpaired two-tailed *t*-test for normally distributed data and Mann–Whitney *U*-test for non-normally distributed data. . * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Chapter 3:The C3aR promotes macrophage infiltration and regulates ANCA production but does not affect glomerular injury in experimental anti-myeloperoxidase glomerulonephritis.

JONATHAN S.C. DICK[1,2], POH-YI GAN[1], A. RICHARD KITCHING[1,2,3], STEPHEN R. HOLDSWORTH[1,2]

[1] Centre for Inflammatory Diseases, Monash University Department of Medicine, Clayton, Victoria, Australia

[2] Department of Nephrology, Monash Health, Clayton, Victoria, Australia

[3] Department of Paediatric Nephrology, Monash Children's Hospital, Monash Health, Clayton, Victoria, Australia

Abstract:

The anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides are autoimmune diseases associated with significant morbidity and mortality. They often affect the kidney causing rapidly progressive glomerulonephritis. While signalling by complement anaphylatoxin C5a though the C5a receptor 1 is important in this disease, the role of the anaphylatoxin C3a signalling via the C3aR is not known. Using two different murine models of anti-myeloperoxidase (MPO) glomerulonephritis, one mediated by passive transfer of anti-MPO antibodies, the other by cell-mediated immunity, we found that the C3aR did not alter histological disease severity. However, it promoted macrophage recruitment to the inflamed glomerulus and inhibited the generation of MPO-ANCA whilst not influencing T cell autoimmunity. Thus, whilst the C3aR modulates some elements of disease pathogenesis, overall it is not critical in effector responses and glomerular injury caused by autoimmunity to MPO.

Introduction:

The anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV) are diseases in which autoimmunity to the neutrophil granule proteins myeloperoxidase (MPO) or proteinase-3 (Pr3) can cause multi-organ injury, including rapidly progressive glomerulonephritis. The pathogenesis of AAV involves multiple steps. T and B cell tolerance to MPO or Pr3 is lost, resulting in the secretion of autoantibodies (ANCA). ANCA can bind to their cognate autoantigen on primed neutrophils, inducing them to activate and lodge in the glomerulus. These intraglomerular neutrophils degranulate, producing reactive oxygen species and causing direct glomerular injury[329]. Degranulation results in extensive glomerular deposits of non-leukocyte associated MPO in patients with AAV[119]. Murine models suggest that MPO-specific effector T cells recognize glomerular MPO and contribute to glomerular injury[104, 120].

The complement system is an important component of innate immunity. Three pathways can activate complement, the classical, alternative and lectin pathways, which all converge on generation of a C3 convertase. C3a is a bioactive split product of C3 produced, along with C3b, by the action of the C3 convertases. C3a is rapidly inactivated by cleavage of the C-terminal arginine to form C3a-desArg. The cellular receptor for C3a, the C3aR, is a G-protein coupled receptor with 7 trans-membrane domains and high homology to the human C5aR1. Activation of the receptor leads to intracellular calcium mobilisation [260, 261].

Complement has emerged as an important mediator of disease in AAV. Murine studies revealed that complement, activated via the alternative pathway and signalling through C5aR1 is required for ANCA-induced neutrophil activation and glomerulonephritis[56, 185, 188, 189]. Supporting evidence from human cohorts include elevated circulating complement activation products in active disease[191], the association of low serum C3 levels with adverse outcomes[193, 194], and evidence of complement deposition in biopsies of patients with AAV[197, 198]. The proof of concept phase 2 CLEAR study showed that the small molecule C5aR1 inhibitor CCX168

(Avacopan) was non-inferior to glucocorticoids for induction therapy in AAV[207]. This strategy is currently the subject of a phase 3 clinical trial (NCT02994297) in acute AAV.

Although circulating levels of C3a are elevated in patients with active AAV [191], whether C3a is pathogenic in this disease is not known. The only relevant published work to date in AAV has been the finding that C3a does not prime isolated neutrophils for activation by ANCA *in vitro*[56]. This is consistent with the inability of C3a to cause chemotaxis or degranulation in neutrophils[330]. However, AAV is a disease with the complex participation of multiple innate and adaptive immune components. Thus, as signalling through C3aR has been implicated in several relevant processes, including neutrophil mobilisation[287], the generation of T cell [331] and B cell [266]responses, macrophage recruitment[291] and mast cell degranulation[261] there are multiple potential mechanisms by which the C3aR may participate in AAV. We therefore examined the role of signalling through the C3aR in anti-MPO autoimmunity and renal injury, by studying *C3ar*$^{-/-}$ mice[286] in two complementary models of anti-MPO glomerulonephritis.

Results

Endogenous C3a does not exacerbate glomerular injury induced by passive transfer of anti-MPO IgG but promotes glomerular macrophage recruitment.

Passive transfer of anti-MPO IgG, with or without a priming stimulus into mice induces a neutrophil mediated necrotising glomerulonephritis [137, 138]. We used this model incorporating lipopolysaccharide (LPS) to investigate the role of the endogenous C3a in the effector phase of anti-MPO IgG induced glomerulonephritis. WT and *C3ar*$^{-/-}$ mice received anti-MPO IgG and LPS. Renal injury was studied after 7 days. Both groups of mice developed glomerulonephritis with a similar degree of histological glomerular injury (Fig 1A-C). However, fewer glomerular macrophages were observed in *C3ar*$^{-/-}$ mice with glomerulonephritis (Fig 1 D-F). Numbers of glomerular neutrophils were not different between groups and, consistent with histological findings, albuminuria was similar between groups (Fig1 G-H). Therefore, in this model, the C3aR is not required for the development of anti-MPO IgG induced glomerular injury, but does promote macrophage infiltration to the inflamed glomerulus.

Figure 1

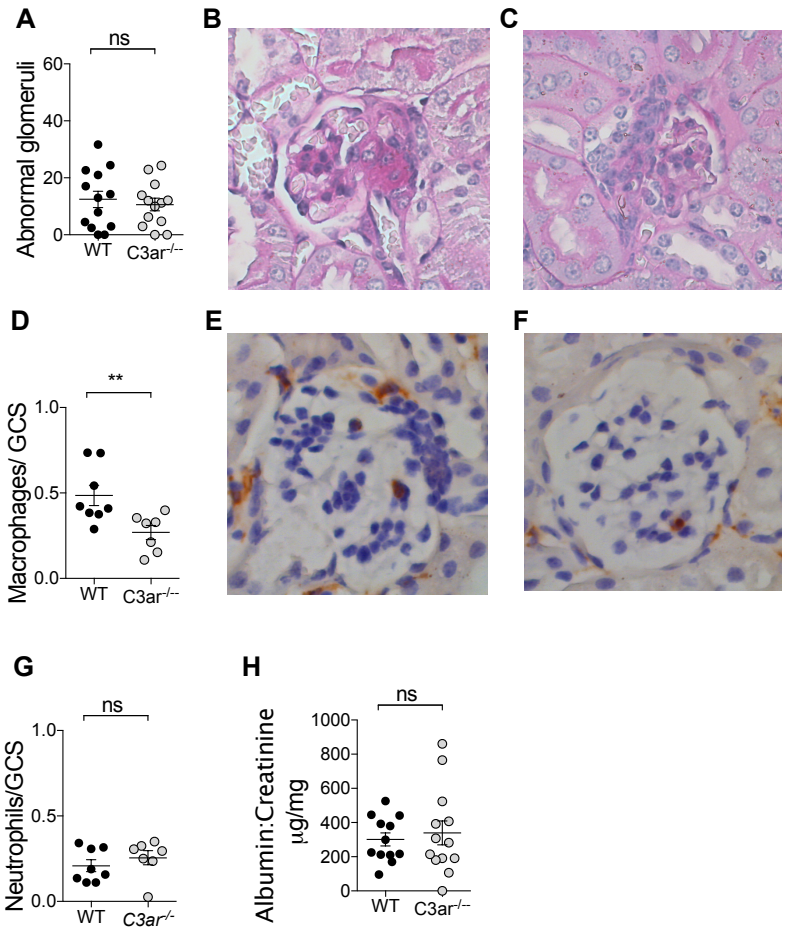


Figure 1: Endogenous C3a does not promote anti-MPO glomerulonephritis but supports macrophage recruitment to the inflamed glomerulus.

Anti-MPO glomerulonephritis was induced in WT and C3ar$^{-/-}$ mice (n=14/group) by injection of 100µg/g anti-MPO IgG and 0.5µg/g LPS. On day 7 histological glomerular injury was assessed. Both groups of mice developed glomerulonephritis including segmental necrosis. There was no difference in degree of injury (A) between WT (B) and C3ar$^{-/-}$ (C) mice. Glomerular leucocyte influx was assessed by immunohistochemistry (n=7/group). Compared to WT mice (D-E), the number of glomerular macrophages was reduced in C3ar$^{-/-}$ mice (F). There was no difference in glomerular neutrophils (G), albuminuria, measured by urinary albumin: creatinine ratio was not different between groups (H).

The C3aR does not promote injury in experimental autoimmune anti-MPO glomerulonephritis.

We then examined the role of C3aR in an autoimmune model of anti-MPO glomerulonephritis that is mediated by the T cell effector response to glomerular MPO[120]. In mice, autoimmunity to MPO induced by immunisation does not result in ANCA of sufficient pathogenicity to cause disease. However, injection of a sub-nephritogenic dose of anti-glomerular basement membrane (GBM) globulin induces glomerular neutrophil recruitment and deposition of MPO. Glomerular MPO is recognised by antigen specific effector T cells with resulting necrotising glomerulonephritis[104, 120]. Several strands of evidence confirm that this disease is due to MPO-specific T cell effectors. These include lack of injury in *Mpo*$^{-/-}$ mice or OVA immunised mice, a similar degree of injury in B cell deficient mice and the induction of glomerulonephritis by transfer of MPO-specific, but not OVA-specific CD4+ T cell clones [34, 104, 120]. As this model is dependent on glomerular neutrophil recruitment by anti-GBM globulin, we first confirmed that this parameter was not affected by the absence of the C3aR (150 minutes after anti-GBM IgG, WT 1.17±0.17 vs. *C3ar*$^{-/-}$1.13±0.16; mean ± SEM, neutrophils/glomerular cross section). Having excluded this potential confounder, we induced autoimmune anti-MPO glomerulonephritis in WT and *C3ar*$^{-/-}$ mice. Both groups had similar severity of histological injury and albuminuria, with no differences in the numbers of neutrophils, macrophages or CD4^{+} T cells between WT and *C3ar*$^{-/-}$ mice (Fig 2A-G).

Figure 2

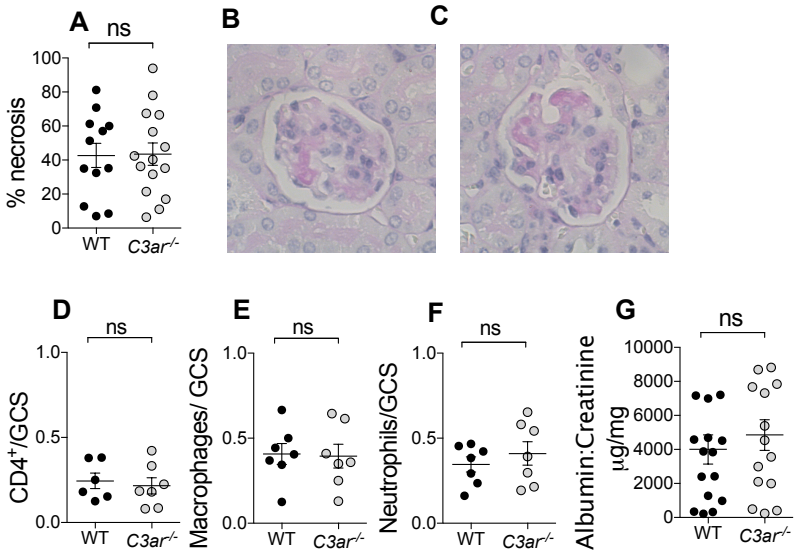


Figure 2: Endogenous C3a does not promote autoimmune anti-MPO glomerulonephritis.

Autoimmunity to MPO was induced in WT and C3ar$^{-/-}$ mice (n=13-15/group) by immunisation with 20µg MPO in FCA followed 7 days later by 10µg MPO in FIA. Disease was triggered on day 16 by i.v. injection of 0.12mg/g sheep anti-mouse GBM globulin in two divided doses and glomerular injury was assessed on day 21. Mice developed glomerulonephritis with focal areas of segmental necrosis, but there was no difference in the degree of injury (A) between WT (B) and C3ar$^{-/-}$ (C) mice. Glomerular leucocytes were assessed by immunohistochemistry. The number of glomerular CD4^{+} cells (D), macrophages (E) and neutrophils (F) were similar between groups. Functional renal injury measured by urinary albumin: creatinine ratio was similar between groups (G).

C3a supresses humoral autoimmunity to MPO.

As ANCA production is important in the pathogenesis of AAV, we measured the development of anti-MPO humoral autoimmunity in this disease model. MPO-ANCA IgG titres were increased in *C3ar*$^{-/-}$ mice (Figure 3A). Levels of anti-MPO IgG1 and IgG2b isotypes, but not IgG2c and IgG3 were significantly higher in *C3ar*$^{-/-}$ than wild-type mice (Fig3 B).

Figure 3

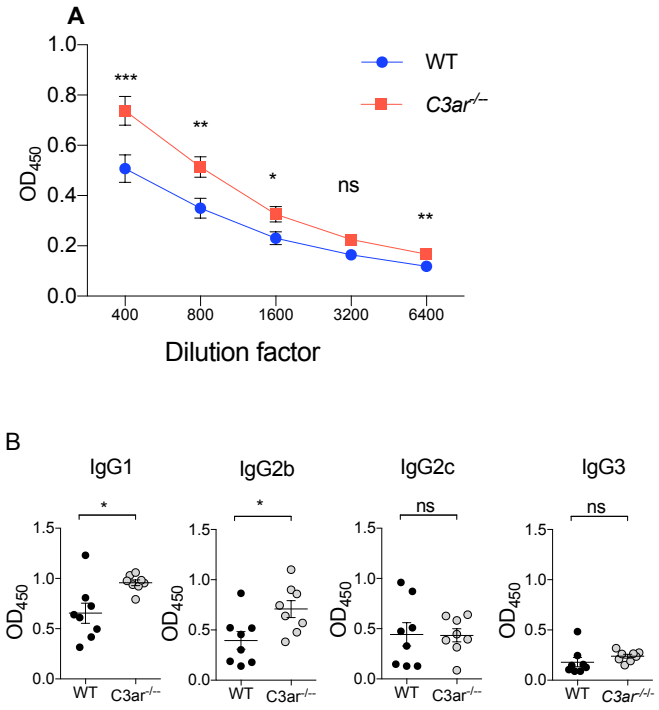


Figure 3: C3a suppresses anti-MPO humoral immunity.

Anti-MPO IgG titres and anti-MPO IgG subclass titres were measured by ELISA in WT and C3ar$^{-/-}$ *mice (n=8/group). Anti-MPO IgG titres were higher in C3ar*$^{-/-}$ *mice compared to WT, due to elevated anti-MPO antibodies of the IgG1 and IgG2b subclasses. MPO-specific IgG2c and IgG3 were not different between groups (B).*

To further investigate the differences in humoral autoimmunity, we analysed B cell development in the bone marrow and spleen. In naïve mice, the proportion of bone marrow B cells that were proB-preB ($B220^{+}IgM^{low}IgD^{low}$), transitional ($B220^{+}IgM^{+}IgD^{low}$) or mature ($B220^{+}IgM^{int}IgD^{+}$) was not different between WT and *C3ar*$^{-/-}$ mice. In mice immunised with MPO in FCA, there was no difference in the total number of splenic $B220^{+}$ B cells, Follicular B cells ($B220^{+}CD21^{+}CD23^{+}$), marginal zone B cells ($B220^{+}CD21^{+}CD23^{low}$) or plasma cells ($B220^{low/int}$ $CD138^{+}$) between groups (Fig 4). B cell activating factor (BAFF), expressed by myeloid and bone marrow stromal cells is elevated in AAV[332] and is important in B cell development and differentiation. Levels of serum BAFF in immunised WT and *C3ar*$^{-/-}$ mice were not significantly different between groups.

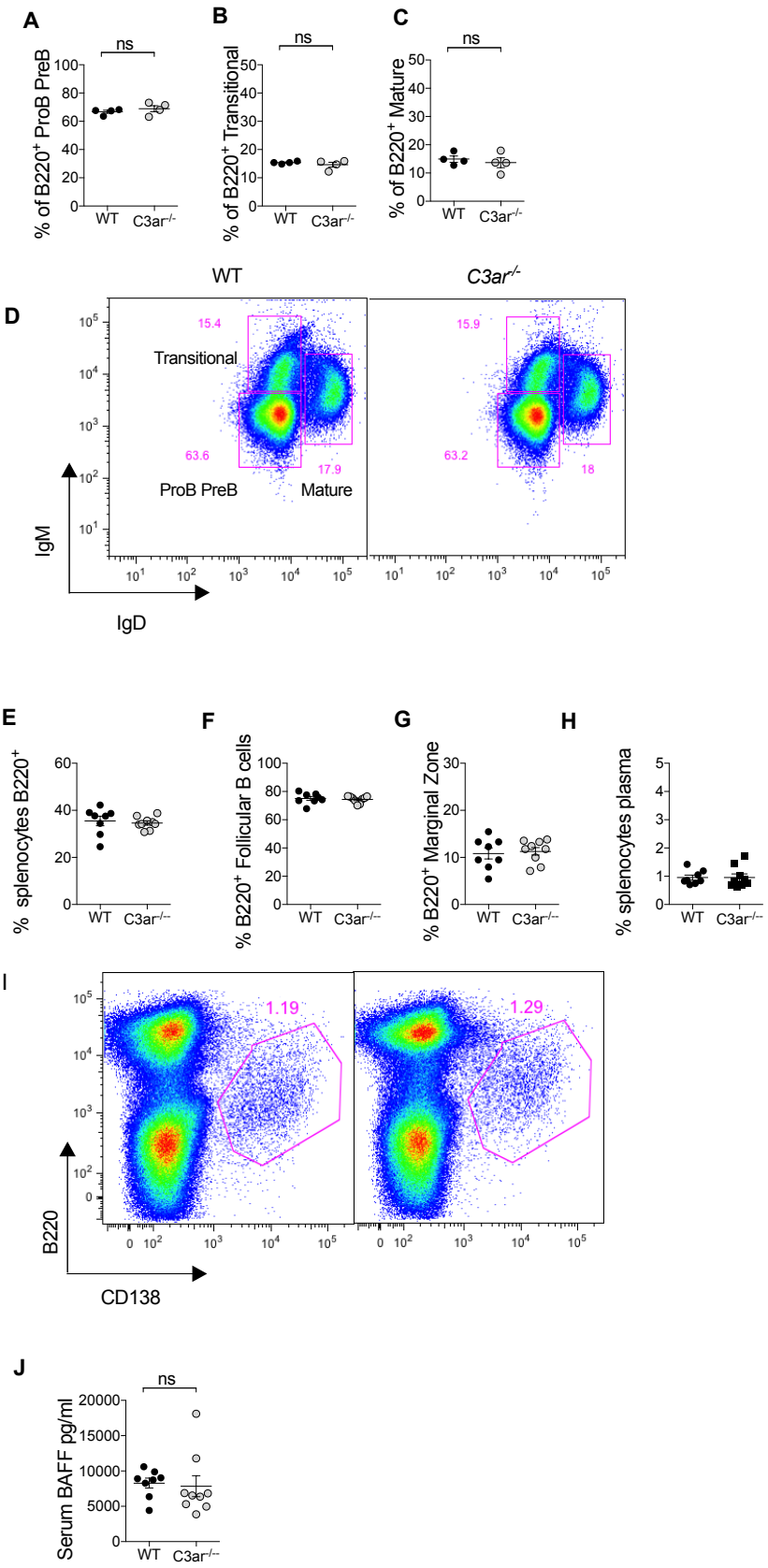


Figure 4: C3ar$^{-/-}$ mice have normal B cell development in the spleen and bone marrow.

Bone marrow cells were extracted from tibiae of naïve WT and C3ar$^{-/-}$ mice (n=4/group) and surface markers analysed by flow cytometry. (A-C)The proportion of B220^{+} cells that were preB or proB (IgM^{low}IgD^{low}), transitional (IgM^{+}IGD^{low}) or mature (IgM^{+}IgD^{+}) did not differ between groups. (D) Represntive flow cytometry plot gated on B220^{+} cells in the bone marrow showing gating strategy. The splenic B cell compartment was analysed in WT and C3ar$^{-/-}$ mice (n=8/group) at the end of the autoimmune anti-MPO glomerulonephritis model. There was no difference in (E) the proportion of splenocytes that were B220^{+} B cells, (F) the proportion of B220^{+} cells that were CD21^{+}CD23^{+} follicular B cells, or (G) CD21^{+}CD23^{low} marginal zone B cells was similar between groups. (H) The proportion of splenocytes that were B220$^{low/int}$CD138+ plasma cells also did not differ between groups. (I) Representative flow cytometry

plot of splenocytes showing gating of plasma cells. (J) Serum B cell activating factor (BAFF) was similar between groups.

C3a does not promote cellular autoimmunity to myeloperoxidase.

$CD4^+$ T cells are not only required for humoral immune response to protein antigens, including MPO, they also effect glomerular injury in crescentic glomerulonephritis via Th1 and Th17 responses.[125, 316, 333]. *In vitro*, C3aR ligation on dendritic cells results in increased activation and functional capacity [249, 275], and T cell expressed C3aR may be important in Th1 responses and in inhibiting the generation of $Foxp3^+$ T regulatory cells (Tregs)[252, 253, 269]. We therefore analysed cellular immune responses in mice at two time points: early (10 days) and later (21 days) after immunisation. Ten days after MPO immunisation there were no differences in MPO-stimulated Th1 or Th17 responses measured by IFN-γ and IL-17A ELISPOT (Figure 5A-B). Furthermore, proportions of $CD4^+$ cells that were $CD44^{Hi}$ (activated T cells) (Figure 5C), or $CD25^+Foxp3^+$ Tregs (Figure 5D-E) were similar between groups. T follicular helper cells (TFH) are important for the formation of germinal centres and subsequent antibody response, the proportion of CD4 cells with $CXCR5^{hi}PD\text{-}1^{hi}$ TFH phenotype were also similar between groups (Figure 5F-G).

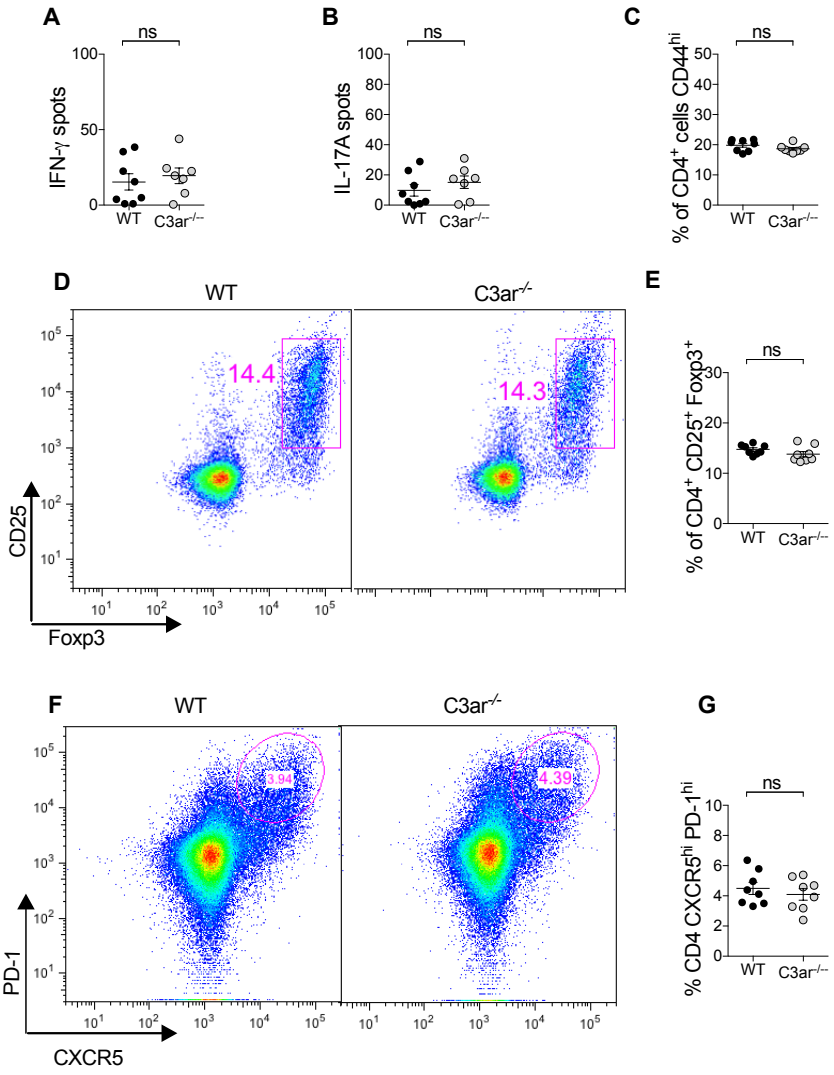


Figure 5: Endogenous C3a does not promote early cellular immunity to myeloperoxidase.

To assess the early cellular immune response lymphocytes from the draining lymph nodes of WT and C3ar$^{-/-}$ mice (n=8/group) were studied 10 days after immunisation with 20 µg MPO in FCA. (A-B)There was no difference between groups in Th1 and Th17 response measured by IFN-γ and IL-17A ELISPOT. (C) The proportion of activated CD4^{+}CD44^{+} T cells was similar between groups. (D) Representative flow cytometry plots gated on CD4^{+} cells showing CD25^{+}Foxp3^{+} Tregs. (E) The proportion of CD4 cells that were Tregs did not differ between groups. (F) Representative flow cytometry plots gated on CD4^{+} cells showing PD-1^{hi} CXCR5^{hi} T follicular helper cells. (G) There was no difference between groups in the proportion of T cells that had a TFH phenotype.

Similar to findings at day 10, at day 21 there was no difference in MPO-stimulated IFN-γ and IL-17A producing cells measured by ELISPOT (Fig 6A-B). Additionally at this time point, the proportion of CD4 T cells that were $CD44^+$ activated or $CD25^+Foxp3^+$ Tregs were similar, both in the draining lymph nodes and spleen(Fig6 C-E).

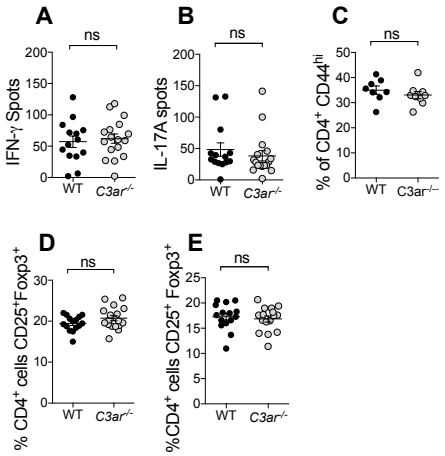


Figure 6: Endogenous C3a does not promote later cellular immunity to MPO in autoimmune anti-MPO glomerulonephritis.

Cellular autoimmunity to MPO was assessed on day 21. Draining lymph nodes and spleen were removed from mice and a single cell suspension obtained. (A-B)There was no difference between groups in Th1 or TH17 response as measured by IFN-γ and IL-17A ELISPOT. (C) There was no difference between groups in the proportion of CD4 cells that had a $CD44^+$ activated phenotype. There was also no difference in the proportion of CD4 cells that had a $CD25^+Foxp3^+$ regulatory phenotype in either the draining lymph nodes (D) or spleen (E).

Discussion

A significant body of evidence implicates complement in the pathogenesis of AAV. To date, the C5aR1 has been identified as a key mediator of neutrophil activation and glomerular injury. Here, we assessed whether the signalling though the C3aR mediated autoimmunity or glomerular injury, and thus had potential as a therapeutic target. C3aR has previously been described to play both pathogenic and protective roles in experimental inflammatory diseases. Animal models in which the C3aR is pathogenic include antibody-induced arthritis[240], adriamycin induced nephropathy[290], complement mediated tubulointerstitial injury[291] and renal ischemia-reperfusion injury[237]. However, the C3aR is protective in lupus-like disease in the MRL/lpr mouse[293], endotoxic shock[286] and intestinal ischemia-reperfusion injury[287]. In the current studies, although we found that the C3aR had biological effects, there were no net effects on glomerulonephritis and renal injury in the two complementary disease models that we studied.

Passive transfer of anti-MPO IgG with LPS models pathological neutrophil activation with glomerular injury being caused by neutrophil degranulation, and the consequent release of inflammatory mediators and reactive oxygen species[137]. Whilst absence of the C3aR did not influence overall injury it was associated with an attenuation of glomerular macrophage accumulation, suggesting a role in this context for local C3a. *In vitro* and *in vivo* evidence supports a role for the C3aR in renal macrophage recruitment. *In vitro*, C3a acts as a chemotaxin in a murine macrophage cell line [274]. *In vivo* evidence derives from a model of complement induced renal injury in which $Crry^{-/-}C3^{-/-}$ kidneys are transplanted into syngeneic complement sufficient recipients, resulting in unrestricted renal complement activation with a inflammatory cell influx. When $Crry^{-/-}C3^{-/-}$ kidneys were transplanted into $C3ar^{-/-}$ recipients, there was a marked attenuation of the monocyte-macrophage influx, suggesting a role for the C3aR in monocyte trafficking to areas of complement activation[291].

Whilst AAV is classically described to be "pauci-immune" with minimal glomerular complement deposition, detailed analysis of kidney biopsies from humans with AAV reveals

evidence of local complement activation with detectable C3c, C3d and C5b-9 deposition[192, 198, 200], additional evidence for renal complement activation in AAV is the elevated urinary C3a observed in patients with active disease[192]. This complement activation, which is likely to be initiated by neutrophil secreted factors such as properdin[302], MPO [334] and neutrophil extracellular traps[306] may be one of the early signals contributing to macrophage recruitment to the inflamed glomerulus.

While passive transfer of anti-MPO antibodies allows study of the role of effector pathways responding to autoantibodies, it cannot be used to examine the contribution of the adaptive immune system to glomerular injury. AAV is an autoimmune disease in which both B and T cells play essential roles in the pathogenesis. B cells are required for the production of ANCAs. T cells are required for B cell response to protein antigens and also contribute to glomerular injury through an injurious Th1 and Th17 response to the autoantigen that has been deposited in the glomerulus[104, 120, 121]. We used the model of experimental autoimmune anti-MPO glomerulonephritis to model these processes and found that absence of C3aR did not affect albuminuria, histological injury or glomerular leucocyte influx. The discrepancy between results for glomerular macrophage infiltration between passive transfer of anti-MPO IgG and autoimmune anti MPO glomerulonephritis is likely to be because the dominant signals driving glomerular macrophage recruitment in the model of autoimmune anti-MPO GN are T helper cell derived costimulatory molecules, such as CD154-CD40 interactions[335] and cytokines, such as IL-17A[125] and IFN-γ[336]

C3ar$^{-/-}$ mice had an increased humoral immune response to MPO with higher MPO-ANCA titres. Elevated autoantibody titres associated with accelerated renal injury have also described in *C3ar*$^{-/-}$ mice when backcrossed to the MRL lupus-prone strain[293]. A direct effect of C3a acting on B and T lymphocytes to suppress humoral immunity has previously been reported [266, 337]. *C3ar*$^{-/-}$ deficient mice have also been reported to have an enhanced Th2 response and higher

anti-Ova IgG titres in response to epicutanteous sensitisation [285]. In contrast, other models of infection have found that absence of the C3aR results in unaltered[338] or attenuated[339] humoral immunity. The enhanced humoral immunity in *C3ar*$^{-/-}$ mice is not likely to be due to elevated C3 levels (and enhanced generation of C3d which is a potent B cell adjuvant), as serum C3 in this strain has previously be described to be similar to that of WT mice[240, 293].

Because of the observed differences in anti-MPO IgG titres we investigated whether *C3ar*$^{-/-}$ mice had any numerical or developmental deficit in the B lymphocyte compartment. However, B cell number and development in the spleen and bone marrow appeared similar to WT mice.

Given the previous descriptions of the important role that C3aR plays in influencing adaptive immunity we investigated the effect of absence of this receptor on the generation of T cell mediated immunity. We found no difference in the generation of Th1 or Th17 effector responses, nor in the proportion of T cells that had a $CD25^{+}Foxp3^{+}$ regulatory phenotype. This is in contrast to previous reports of elevated T regulatory cells in *C3ar*$^{-/-}$ mice[252, 281]. The model of autoimmune anti-MPO glomerulonephritis relies on immunisation with MPO in Freund's complete adjuvant to break immune tolerance and generate autoimmunity. Other investigators who have reported increased T regulatory cells in *C3ar*$^{-/-}$ mice used animal models that either require no adjuvant or use incomplete Freund's adjuvant. It is possible that differences in the additional immune signals provided by the adjuvant may account for this discrepancy. Additionally, whilst C3aR expression in the mouse has previously been reported to be extensive in both myeloid and lymphoid lineages, this has been called into questions by recent findings using a C3aR reporter mouse in which expression of C3aR was evident on macrophages and some dendritic cell subsets but not bone marrow neutrophils, B or T cells[340, 341]. In contrast, neutrophil, macrophage and T cell as well as renal expression have been reported in humans[263, 267, 269, 270, 342]. Potential species differences in C3aR distribution should therefore be considered when interpreting studies using murine models.

In summary these data suggest a role for C3a in driving glomerular macrophage recruitment and suppressing humoral immunity in these pre-clinical models of AAV. However, the lack of attenuation of immune mediated glomerular injury in either model does not support the C3aR as a putative therapeutic target in this disease.

Methods

All mice were on a C57BL/6 background. *C3ar*$^{-/-}$ mice[286] were kindly provided by Professor Rick Wetzel, University of Texas. Mice were bred at Monash Medical Centre, Clayton. Experiments were approved by Monash University Animal Ethics committee.

Genotyping

DNA was isolated from mouse tail clippings by ispropanol precipitation. The isolated DNA was used as a template for PCR with 0.5µM of each of the primers C1 (TACAATATAGTCAGTTGGAAGTCAGCC), NeoA (TGGGCTCTATGGCTTCTGAGGCGGAAAG), and A201+ (GAGAATCAGGTGAGCCAAGGAGAAG). GoTaq Green Master Mix (Promega) was used for the PCR reaction. The PCR was run at 95°C for 1 minute, followed by 40 cycles of 95°C for 15sec, 57°C for 15sec, 72°C for 30sec, then a final elongation step at 72°C for 30sec and a holding step of 4°C. The primers C1 and NeoA yield a fragment of 537 bp denoting the *C3ar*$^{-/-}$ allele. Primers C1 and A201+ yield a fragment of 726 bp, denoting WT allele. All mice tested had the C3ar$^{-/-}$ genotype (Supplementary data 1).

Induction of glomerulonephritis and assessment of autoimmunity

Anti-MPO IgG was generated by immunising *Mpo*$^{-/-}$ mice with native murine MPO generated as previously described[328]. Briefly mice were immunised sub-cutaneously with 15mcg MPO in FCA (Sigma-Aldrich, St Louis, MO) on day 0 followed by 10mcg in Freund's incomplete adjuvant on days 7 and 14. On day 24 mice were humanely killed and blood was obtained. IgG fractions were obtained by Protein G affinity chromatography (GE Healthcare) and then extensively dialysed against PBS.

To induce glomerulonephritis age matched 6-10 week old C57B/L6 mice and *C3ar*$^{-/-}$ mice received 100μg/g body weight anti-MPO IgG by tail vein injection. 1 hour later 0.5 μg/g bacterial lipopolysaccharide (LPS) (Sigma-Aldrich) was administered by a single intra-peritoneal injection. After 5 days, 24 hours before the end of experiments, mice were placed in metabolic cages for urine collection.

To induce autoimmune anti-MPO glomerulonephritis mice were immunised s.c. with 20μg MPO in FCA followed by 10μg MPO in FIA on day 7. On day 16 glomerulonephritis was triggered by injection of 0.12mg/g sheep anti-mouse GBM globulin in two divided doses. Mice were placed in metabolic cages for on day 20 for assessment of proteinuria before being euthanased on day 21 by CO_2 inhalation.

Assessment of renal injury

Glomerular abnormalities were assessed on periodic acid-Schiff (PAS) stained, 4μm, formalin fixed, paraffin embedded sections using coded slides. Abnormalities scored included mesangial proliferation, capillary wall thickening, glomerular necrosis (defined as accumulation of PAS positive material combined with hypocellularity) and crescent formation (defined as two or more cells visible in Bowman's space). A minimum of 30 glomeruli per mouse were examined. To evaluate leukocyte infiltrate kidneys were fixed in periodate lysine paraformaldehyde for four hours then washed with 20% sucrose. 5 micrometre sections were stained with a three

layer immunoperoxidase technique [343] using GK1.5 (anti-CD4), FA11 (anti-CD68) or Gr1 (anti Ly6g/c). The secondary antibody was rabbit anti-rat biotin (DAKO). A minimum of 30 glomeruli per section were scored. Albuminuria was measured by ELISA (Bethyl . Montgomery, TX). Creatinine was measured using standard methods at the biochemistry laboratory, Monash Medical Centre.

Assessment of immunity:

The spleen and draining lymph nodes were harvested and a single cell suspension was obtained. IFN-γ and IL-17A ELISPOT was performed according to manufacturers instructions (Ebioscience, San Diego, CA) with $5x10^5$ cells per well. Cells were incubated for 18 hours at 37 with 10μg/ml heat inactivated rMPO. Total anti-MPO IgG was measured by ELISA on MPO coated plates with IgG detected with sheep-anti mouse IgG-HRP (Sigma-Aldrich). Antibody subclasses were measured using subclass specific goat anti-mouse IgG-HRP (Southern Biotech, Birmingham, AL). Serum BAFF was measured by ELISA (RnD Systems, Minneapolis, MN). For assessment of B cell development, bone marrow was flushed from the tibia and femur of naïve mice and analysed by flow cytometry.

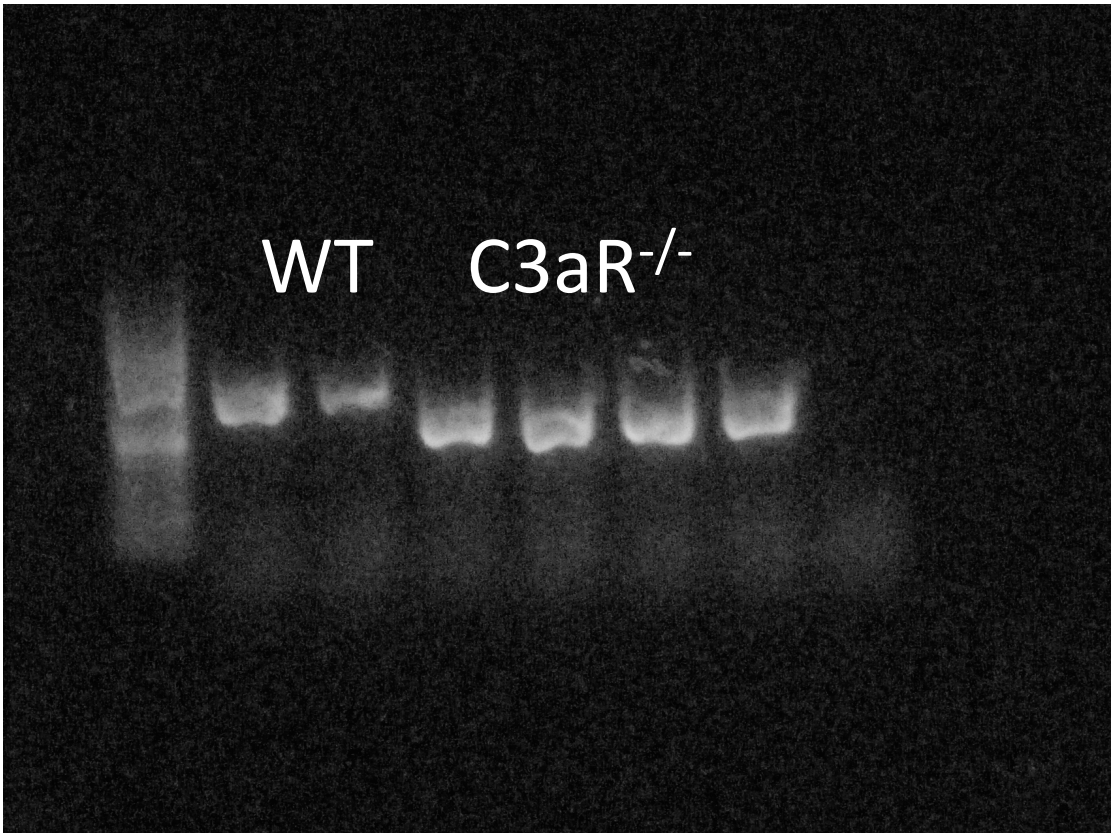
Antibodies:

The following antibodies were used for flow cytometry. Anti-CD4(RM4.5) anti-CD25 (PC61), anti-CD44 (IM7), Anti-IFN-γ (XMG1.2), anti-CXCR5 (2G8), all BD. Anti-IL-17A (eBio17B7) Anti-FOXP3 (FJK-16s), anti-PD-1 (J43) all Ebioscience. Anti-B220 (RA2-6B2), anti-CD23 (B3B4), anti-CD21 (7E9), anti-IgM (RMMM-1), anti-IgD (11-26c.2a), anti-CD138 (281-2) all Biolegend. Fixable viability stain 450 or propidium iodide (BD Biosciences) were used to exclude dead cells. Flow cytometry was performed on the Beckman Coulter Navios platforms and analysed using FlowJo software (TreeStar,Ashland, OR).

Statistics

Data are presented as mean+/- SEM with each dot representing a mouse. Prism 6 (Graphpad, San Diego, CA) software was used for analysis with an unpaired two-tailed *t*-test for normally distributed data and Mann–Whitney *U*-test for non-normally distributed data. . * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Supplementary Data 1



Primers C1 and A201+ yield a fragment of 726 bp, denoting WT allele.

Primers C1 and NeoA yield a fragment of 537 bp denoting the C3ar$^{-/-}$ allele.

Chapter 4: The alternative pathway of complement does not mediate glomerular injury or autoimmunity in experimental autoimmune anti-MPO glomerulonephritis

Introduction

The alternative pathway (AP) of complement is an important meditator of host immune defence and inflammation. The alternative pathway is continuously active. Spontaneous C3 hydrolysis leads to the generation of C3b(H_20), which after association with FB and activation by factor D (FD) results in a active fluid phase C3 convertase: C3b(H_20)Bb. The alternative pathway is also an amplification loop that potentiates C3 convertase generation initiated by all three initiation pathways. C3b produced by any pathway associates with FB, which after activation by FD results in the amplification C3 convertase: C3bBb, which, further activates C3, an action that is stabilised by properdin.

The AP amplification loop is the dominant producer of complement activation fragments, even when the cascade is initiated by other pathways of complement. A quantification of the importance of the AP is that inhibition of FD in classical pathway triggered complement activation reduces C5a and C5b-9 production by around 80%[344].

The AP has shown to be injurious in multiple murine models of autoimmune disease including anti-collagen antibody induced arthritis, collagen induced arthritis, experimental autoimmune encephalomyelitis and bullous phemphigoid[345-348]. In models of immune mediated renal disease, $fB^{-/-}$ mice crossed onto the MRL/lpr strain have reduced glomerular histological injury, proteinuria and glomerular IgG deposition without differences in serum anti-dsDNA levels[349]. As the $fB^{-/-}$ mice used in this study had a different MHC haplotype to their wild-type littermates

the importance of the alternative pathway has subsequently been confirmed in *fD*$^{-/-}$ mice backcrossed to the MRL/lpr strain, which also developed attenuated renal injury[350].

The AP appears to be less important in models of anti-GBM antibody induced renal injury (nephrotoxic serum nephritis). In the heterologous model of early injury, two studies have reported no difference between *fB*$^{-/-}$ and WT mice in albuminuria or glomerular neutrophil recruitment after injection of anti-GBM globulin[351, 352]. In the accelerated model, in which mice are pre-sensitized with sheep IgG, *fB*$^{-/-}$ mice had similar histological injury by both light and electron microscopy, although glomerular C3 deposition, macrophage infiltration and albuminuria were all moderately reduced.[352]

The first report of the complement dependence of anti-MPO IgG induced glomerulonephritis demonstrated that *fB*$^{-/-}$ mice were completely protected from disease[185], implicating the AP as the key mechanism for generating the C5a that is required for neutrophil priming. Subsequently, the importance of the AP has been question by the observation that properdin deficient mice, which have drastically reduced AP activity[353] were not protected from disease induced by anti-MPO IgG, G-CSF and LPS[187]. This suggests that stabilisation of the AP C3 convertase by properdin is not a significant contributor to either neutrophil activation or subsequent tissue damage. Activation of the AP in AAV has been corroborated by clinical data from patients in whom the level of of serum, glomerular and urinary AP products associate with disease activity and renal damage [191, 192, 197, 200].

Whilst investigation into the role of the AP in the murine model of anti-MPO IgG induced glomerulonephritis has produced conflicting data, the importance of the AP in the loss of immune tolerance to MPO and the anti-MPO specific T cell mediated effector stage of

glomerulonephritis has not been investigated. The model of autoimmune anti-MPO glomerulonephritis allows study of these parameters.

There are several ways in which the alternative pathway could influence disease in this model. First, absence of a functioning alternative pathway may result in the decreased generation of pro-inflammatory molecules such as C3a, C3b and C5a. This may lead to reduced APC activation and less robust autoimmunity. Whilst humoral immune responses in *fB*$^{-/-}$ mice have been reported to be either the same, or slightly increased compared to WT mice[349, 354], an effect of the AP on the T cell response has not been published. Mice deficient in the regulatory protein DAF (with consequentially increased C3 and C5 convertase activity) have enhanced T cell response, it is possible that the absence of a functioning AP will have the converse effect[355].

A second mechanism by which AP activity may influence disease severity in autoimmune anti-MPO GN is though the glomerular neutrophil activation and subsequent MPO deposition triggered by anti-GBM globulin. Although numbers of glomerular neutrophils recruited by anti-GBM globulin has been reported to be similar in WT and *fB*$^{-/-}$ mice, given the importance of the AP in other models of type II hypersensitivity reactions, other more subtle changes such as glomerular deposition of myeloperoxidase may affected.

An additional possibility is that once activated neutrophils have lodged in the glomerulus, they induce AP mediated complement activation via degranulation and release of properdin. The resulting complement activation products may either cause direct injury or augment the influx of inflammatory cells to the glomerulus.

Concise Methods

Complement factor $B^{-/-}$ mice on a C57BL/6 background [354] were a kind gift of Michael Holers (University of Colorado, Denver, USA). Genotype was confirmed using polymerase chain reaction by detection of FB exon 6 by PCR: DNA was isolated from mouse tail clippings by isopropanol precipitation. The isolated DNA was used as a template for PCR with 0.5μM of each of the primers. GAGAACAGCAGAAGAGGAAGATTGTCCTAG and CTTCTCAATCAAGTTGGTGAGGCACCGCTT (Thermo Fisher Scientific). GoTaq Green Master Mix (Promega, Madison, WI) was used for the PCR reaction. The PCR was run at 95°C for 3 minutes, followed by 40 cycles of 95°C for 30sec, 55°C for 30sec, 72°C for 1 minutes, then a final elongation step at 72°C for 10 minutes and a holding step of 4°C. WT mice exhibit a band representing the Exon 6 sequences which was not amplified in tail DNA of $fB^{-/-}$ mice.

Experimental autoimmune anti-MPO glomerulonephritis was by immunizing WT or $fB^{-/-}$ mice with 20μg MPO in FCA s.c. on day 0 followed by 10μg MPO in FIA s.c. on day 7. On day 16, glomerulonephritis was triggered by injection of 2.5mg sheep anti-mouse GBM globulin in two divided doses. Dermal delayed type hypersensitivity was induced on day 20 by injection of 20μg MPO in PBS into the right footpad. Mice were placed in metabolic cages for on day 20 for assessment of proteinuria before being humanely euthanased on day 21 by CO_2 inhalation.

Assessment of renal pathology

Glomerular abnormalities were assessed on periodic acid-Schiff (PAS) stained, 4 μm, formalin fixed, paraffin embedded sections using coded slides. A minimum of 30 glomeruli per mouse were examined. To evaluate leukocyte infiltrate kidneys were fixed in periodate lysine paraformaldehyde for four hours then washed with 20% sucrose. 5μm sections were stained with a three layer immunoperoxidase technique [343] using GK1.5 (anti-CD4), FA11 (anti-CD68) or Gr1 (anti Ly6g/c). The secondary antibody was rabbit anti-rat biotin (DAKO). A minimum

of 30 glomeruli per section were scored. Albuminuria was measured by ELISA (Bethyl, Montgomery, TX). Creatinine was measured using standard methods at the Biochemistry Laboratory, Monash Medical Centre.

Assessment of immunity

The spleen and draining lymph nodes were harvested and a single cell suspension was obtained. IFN-γ and IL-17A ELISPOT was performed according to manufacturers instructions (Ebioscience) with $5x10^5$ cells per well. Cells were incubated for 18 hours at 37°C with 10µg/ml heat inactivated rMPO. To measure anti-MPO specific IgG ELISA plates (Nunc Maxisorb) were coated overnight with 5µg/ml recombinant MPO in 50mM carbonate/bicarbonate buffer, pH 9.6. After washing and blocking with 2% casein in PBS for one hour, serial dilutions of serum in PBS were incubated on the plate for one hour at 4°C before washing and detection with sheep anti-mouse IgG-HRP (Sigma Aldrich) and incubated with the chromogenic substrate 3,3',5,5'-Tetramethylbenzidine. The reaction was stopped with the addition of 0.19M H_2SO_4 and absorbance read at 450nm.

Antibodies and flow cytometry

The following antibodies were used for flow cytometry. anti CD4 (RM4.5) anti CD-25 (PC61), anti-CD44 (IM7), anti-CD62L (MEL-14), all BD Biosciences and anti-FOXP3 (FJK-16s) (Ebioscience). Foxp3 Fix/perm kit (Ebioscience) was used for intracellular staining. Flow cytometry was performed on the Beckman Coulter Navios platforms and analysed using FlowJo (Tree star) software

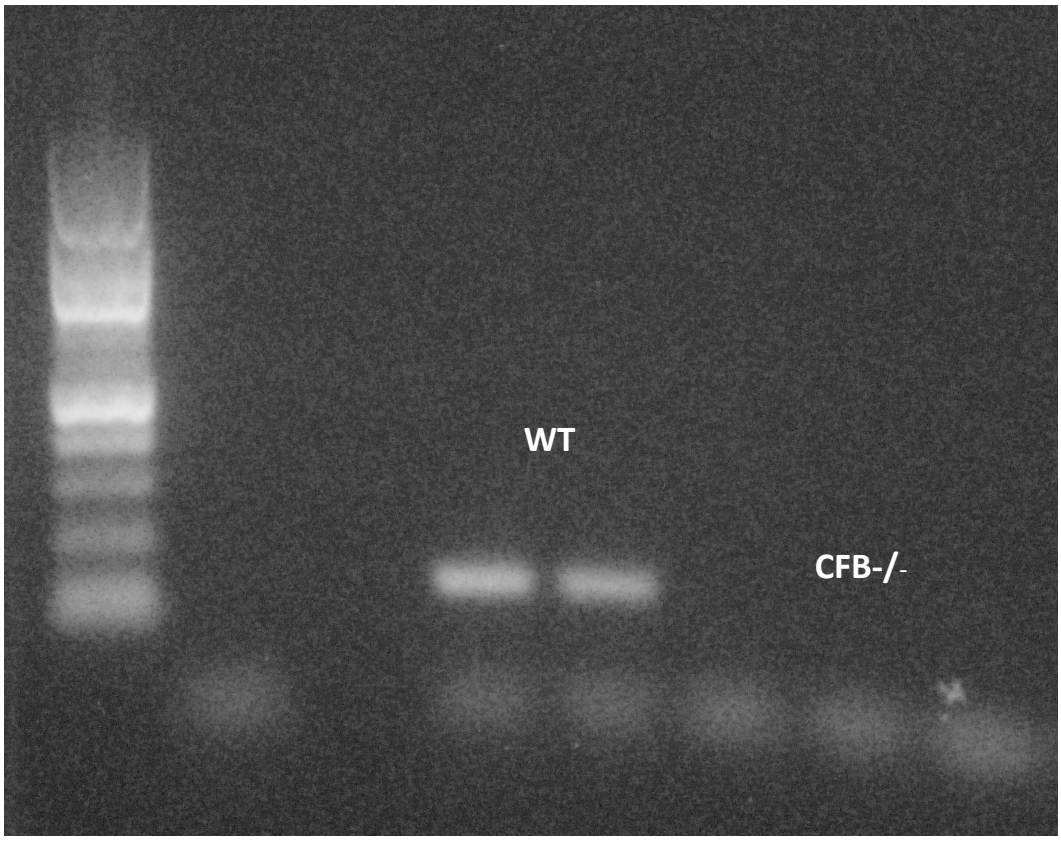
Statistics

Data are shown as mean ±SEM. The students unpaired T test was used to compare groups.

Results:

Confirmation of *fB*$^{-/-}$ genotype by PCR.

PCR confirmed band representing the Exon 6 sequence in WT mice which was not amplified in tail DNA from *fB*$^{-/-}$ mice in all mice tested.



fB$^{-/-}$ mice do not develop reduced renal injury in autoimmune anti-MPO GN

After induction of autoimmune anti-MPO glomerulonephritis, mice were culled on day 21 for evaluation of renal injury. Both WT and *fB*$^{-/-}$ mice developed severe glomerular necrosis. The proportion of glomeruli affected and the degree of albuminuria did not differ between groups. The influx of glomerular CD4^{+} T cells, macrophages and neutrophils was evaulated by immunohistochemistry and also did not differ between groups (Figure 4.2).

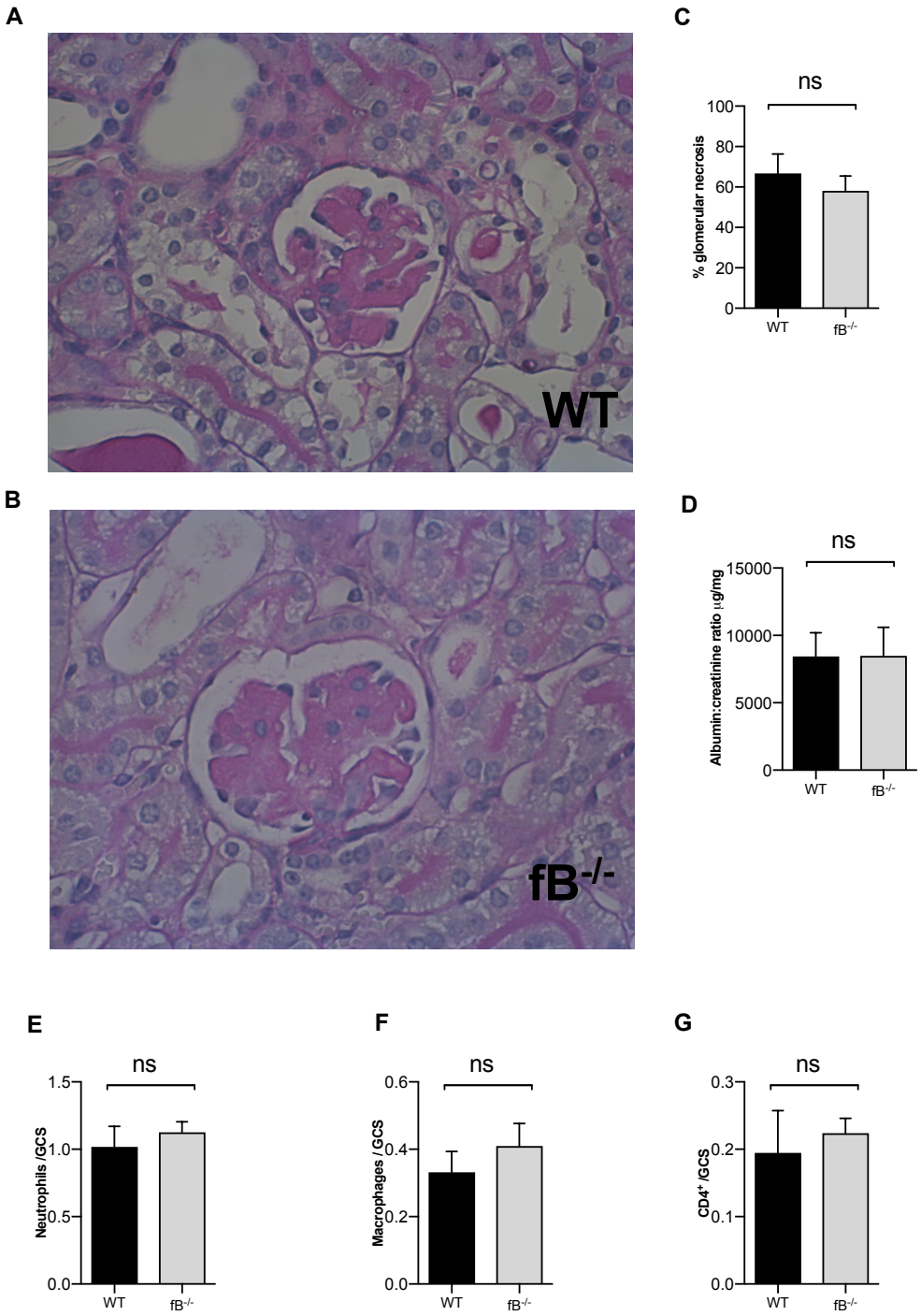


Figure 4.2 fB-/- mice are not protected from autoimmune anti-MPO glomerulonephritis

Glomerular injury was assessed at the end of the autoimmune anti-MPO glomerulonephritis model. (A) WT and (B) fB$^{-/-}$ mice developed similar degrees of glomerular necrosis (C). (D) albumin:creatinine ratio was similar between groups. The number of glomerular (E) neutrophils, (F) macrophages and (G) CD4^{+} T cells did not differ between groups.

The alternative pathway does not promote cellular immunity to MPO

MPO specific Th1 and Th17 response as measured by IFN-γ and IL-17A ELISPOT were similar between groups. Dermal delayed type hypersensitivity, a classic Th1 response was also not reduced in *fB*$^{-/-}$ mice. Similarly, measured by flow cytometry, the proportion of splenic CD4^{+} cells that were CD25^{+}Foxp3^{+} T regulatory cells or CD44^{+}CD62L^{low} T effector memory cells were similar between groups (Figure 4.3).

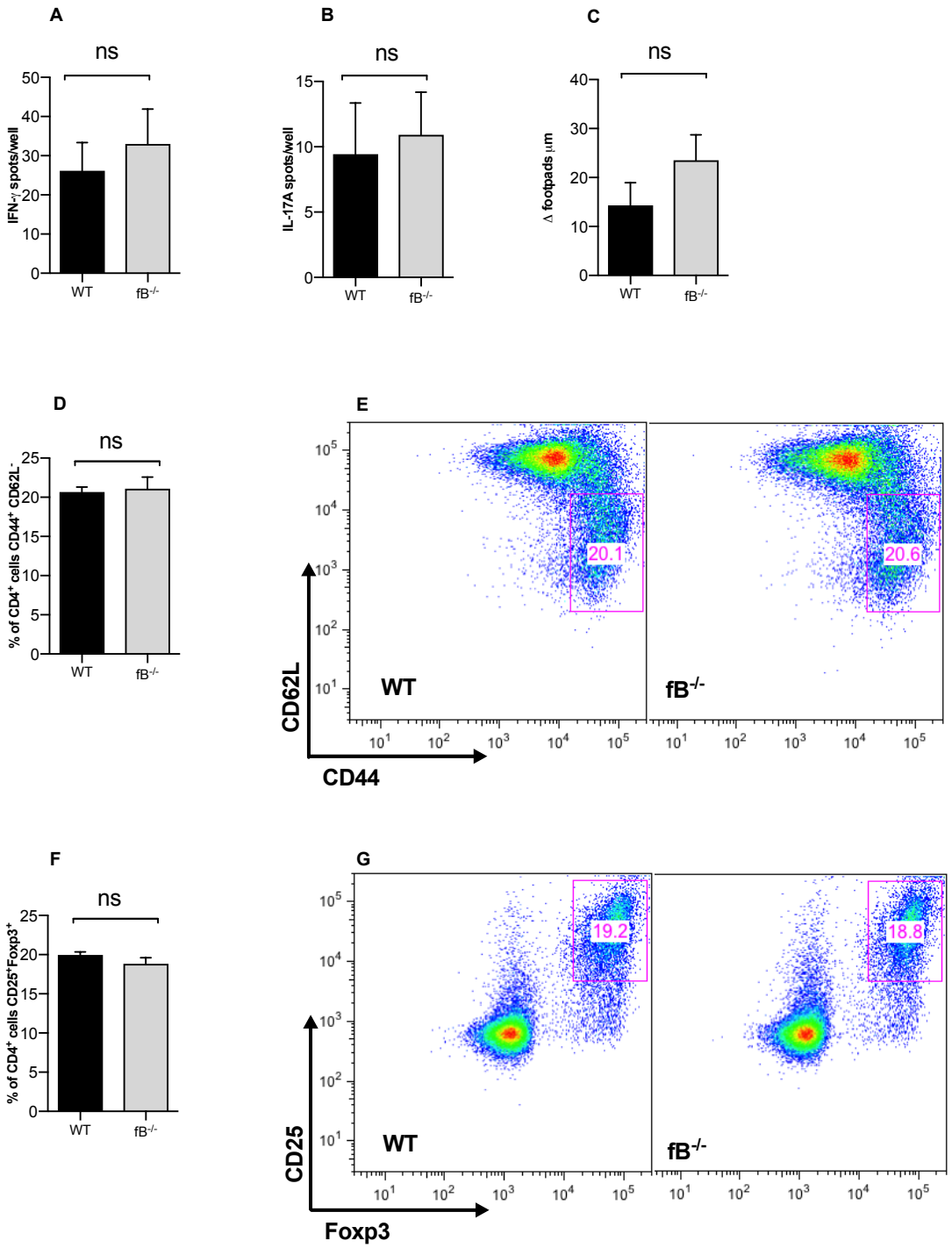


Figure 4.3 Cellular immunity to MPO is unaffected by the absence of the alternate pathway.

Th1 and Th17 response measured by (A) IFN-γ and (B) IL-17A ELISPOT was similar between groups as was (C) dermal delayed type hypersensitivity to MPO. (D) The proportion of splenic $CD4^+$ cells that were $CD44^+CD62^{low}$ T effector memory cells was similar between groups. (E) Representative flow cytometry plot gated on $CD4^+$ T cells showing CD44 and CD62L staining. (F) The proportion of splenic $CD4^+$ cells that were $CD25^+Foxp3^+$ T regulatory cells was similar between groups. (G) Representative flow cytometry plot gated on $CD4^+$ T cells showing CD25 and Foxp3 staining.

Absence of the alternative pathway may increase humoral immunity to MPO

MPO specific IgG titres were measured in serial dilutions at the end of the experiment. These were significantly elevated in *fB*$^{-/-}$ mice at lower dilutions (1:500 and 1:1000) although these differences were not statistically significant at higher dilutions.

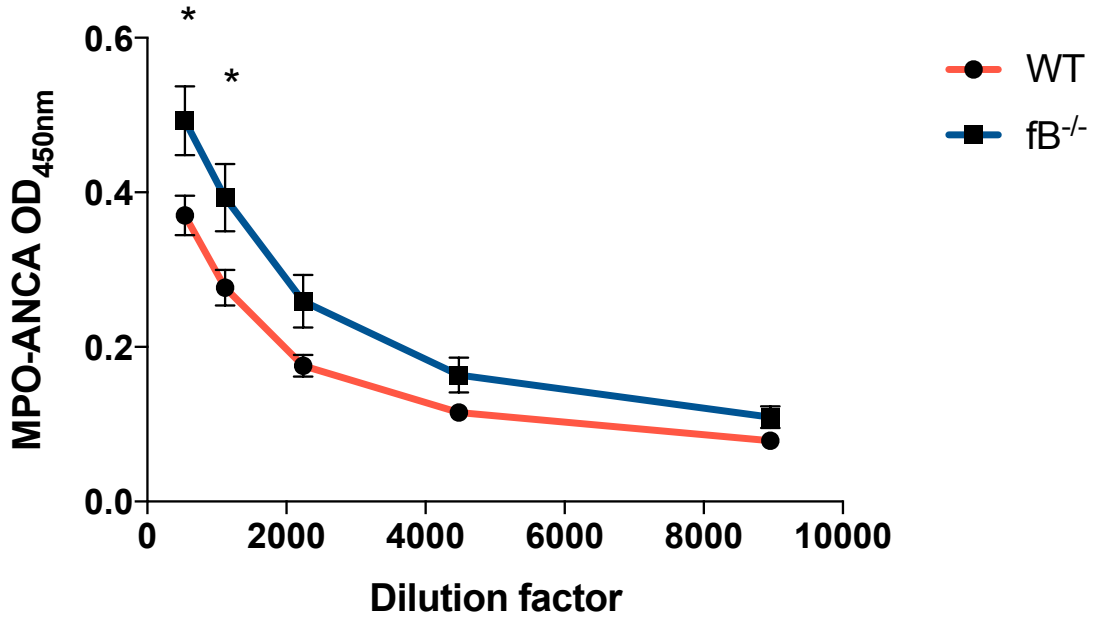


Figure 4.4 fB$^{-/-}$ mice develop higher MPO-ANCA titers after immunsation with MPO

Serum samples from WT and fB$^{-/-}$ mice were serially diluted in PBS before being analysed by for MPO specific IgG by ELISA. At 1:500 and 1:1000 dilutions anti-MPO specific IgG titres were higher in fB$^{-/-}$ mice. Data represents mean ±SEM.=P<0.05.*

Discussion:

The AP is thought to play a critical role in the generation of active complement fragments that prime neutrophils for activation by ANCA. In this thesis, potential additional roles of the AP in the generation of anti-MPO autoimmunity and subsequent T cell directed renal injury were investigated using the model of autoimmune anti-MPO glomerulonephritis. The AP was not found to play a significant injurious role in this model. Whilst the absence of protection observed in $fB^{-/-}$ mice was surprising given the pro-inflammatory effect described for the AP in a number of disease models it is not entirely inconsistent with the previous reports in the literature. $FB^{-/-}$ mice have been reported to be as susceptible as WT mice to the acute, neutrophil and complement mediated heterologous phase of anti-GBM antibody induced renal injury with similar accumulation of glomerular neutrophils. It is therefore likely that, in the first stage of the model, - the recruitment of glomerular neutrophils by anti-GBM globulin- glomerular deposition of myeloperoxidase was unaffected by the AP.

Whilst complement has been shown to be an important mediator in the generation of adaptive T cell immunity, surprisingly, there are no published reports of adaptive cellular immune responses in alternative pathway deficient mice. The lack of difference in both renal injury and measured cellular immune response would suggest that isolated AP deficiency does not sufficiently suppress complement activation through other pathways to produce a measurable effect on adaptive immunity. The model of autoimmune anti-MPO glomerulonephritis has similarities to accelerated anti-GBM disease/ serum nephrotoxic nephritis in that they are both models of a Freund's complete adjuvant enhanced immune response to a planted glomerular antigen. The modest reduction in albuminuria, though not glomerular injury previously reported in this model[352] would be consistent with at most a minor contribution of the alternative pathway to the immune response to a glomerular planted antigen.

The observed differences in humoral immunity in this model are challenging to interpret. Since the 1970s complement has been recognized to be an important promoter of antibody response[283]. The primary driver of this is the interaction of the complement receptor 2 (CD21) on B cells and follicular dendritic cells with C3d. The reduced C3 activation associated with lack of AP activity might therefore be expected to result in impaired humoral immunity. Previous reports on humoral immune response in *fB*$^{-/-}$ mice to T dependent antigens mice have suggested broadly similar levels of IgG response [352, 354], although the production of rheumatoid factor IgG in the MRL/lpr mouse with coexisting factor D deficiency was elevated[350].

It is possible that this observation has an underlying immunological basis. The AP cleavage product Ba has previously been reported to inhibit human B lymphocyte proliferation[356], alternatively, the observation in this thesis that *C3ar*$^{-/-}$ mice also had elevated anti-MPO specific IgG levels after immunisation suggests that this effect could be mediated through AP generation of C3a. Anti-inflammatory roles of complement are less well described than their pro-inflammatory counterparts, but these include negative immune regulation by CD46[357], CR1[358, 359], and C5aR2[254]. Interestingly, like *MRL/lpr C3*$^{+/-}$ heterozygotes, *MRL/lpr fB*$^{+/-}$ heterozygotes have been reported to develop more severe renal disease than either their *fB*$^{+/+}$ or $^{-/-}$ littermates, emphasising the importance of the balance of signals through the large number of complement receptors in determining net immune response. An additional possibility is that whilst the generation of humoral immunity is unchanged by the absence of a functional AP the metabolic clearance of immunoglobulin is somehow affected.

Alternatively, a type I error, either due to experimental technique or other potential confounders such as incomplete backcrossing of the *fB*$^{-/-}$ mouse (derived from a 129xC57BL/6) background leading to the presence of other susceptibility haplotypes in the *fB*$^{-/-}$ mice could explain the observed minor difference in antibody response.

In summary this work suggests that the both the generation of anti-MPO autoimmunity and the T cell mediated effector response to glomerular MPO do not require participation of the AP. The role for the alternative pathway of complement in AAV is therefore likely to be confined to a role in ANCA-induced neutrophil activation.

Chapter 5: ANCA activate Classical pathway *in vitro* on single antigen beads and induce C3 deposition *in vitro* on human neutrophils

Introduction

The classical pathway is predominantly activated by antibody. The Fc portion of two immunoglobulin in close proximity bind to C1q, leading to the formation of the C1 complex and the generation of the enzyme C1s esterase. This cleaves C4 and C2 to form the classical pathway C3 convertase (C4bC2a) that activates C3 to C3a and C3b. C3b can go on to form a C5 convertase or an additional alternative pathway C3 convertase.

The first description of the complement dependence of anti-MPO IgG induced glomerulonephritis in mice demonstrated that the alternative pathway was required for disease but that *C4*$^{-/-}$ mice –which do not have a functional classical pathway- had similar disease severity to wild-type mice[185]. Based on this evidence, the classical pathway is not thought to play a pathological role in AAV. There is however, significant inter-species variation in the potency of the classical pathway. The classical pathway in murine serum, measured by lysis of antibody targeted cells has less than <10% of the activity of human serum.[360, 361] This may be due to a factor(s) present in mouse serum that inhibits the classical pathway[362]. Caution should therefore be exercised when interpreting how findings relating to the classical pathway of complement in murine models translate to humans.

After ANCA bind to their cognate auto-antigen on the neutrophil surface, subsequent interaction of the Fc region with excitatory FcγRs on the neutrophil is thought to mediate activation *in vitro* and *in vivo*. The possibility that ANCA could also activate complement via the classical pathway has however not been explored. It is possible that the classical pathway

could mediate early complement activation, with subsequent activation and amplification being provided by the alternative pathway amplification loop.

Neutrophils are protected from surface complement activation by the membrane regulatory proteins delay accelerating factor (DAF) and membrane cofactor protein (MCP) as well as the plasma regulators factor H and C4 binding protein. However, the effect of these can be surmounted, as antibody targeted neutrophils have been reported to have detectable surface membrane attack complex[363].

Serum containing ANCA has previously been shown to lead to C3c and C5b-9 generation when incubated on smeared, ethanol fixed neutrophils, [364] however the mechanism of this observation and whether ANCA trigger classical pathway activation in more physiological systems remains to be determined. Some evidence for classical complement pathway activation exists in clinical samples from patients with AAV. Elevated urinary C1q has been reported in patients with active AAV compared to patients in remission and healthy controls[192] In addition, serum C4d is elevated both in patients with active AAV than healthy controls, although levels did not reduce in remission[191]. Interpretation of these data is however complicated by the role of the classical pathway in the clearance of apoptotic and cells and cellular debris.

The aim of this study was therefore to determine whether complement activation via the classical pathway could be involved in the pathogenesis of AAV.

1) To measure whether ANCA bind C1q in an *in vitro* bead based system.

2) To assess the ability of ANCA to induce local complement activation on the surface of neutrophils and to determine whether this was mediated by the classical pathway.

Methods:

Conjugation of MPO functional beads for flow cytometry:

To prepare beads for coupling, functional beads (BD Bioscience) were placed into a microcentrifuge tube and sonicated for one minute before being incubated with 1M 1,4-Dithiothreitol (DTT) (Sigma-Aldrich) for 1 hour at room temperature on an orbital shaker. 1ml of functional bead coupling buffer (BD Biosciences) was added before beads were pelleted by centrifugation at 900g for 3 minutes. Beads were resuspended with 20µl coupling buffer.

To modify myeloperoxidase for coupling, 2µl of 2g/L sulfosuccinimyidyl 4-N-maleimidomethyl cyclohexane 1-carboxylate (Sulfo-SMCC, Sigma Aldrich) was added to 90µl of human myeloperoxidase (Calbiochem) 1mg/ml in PBS and the solution incubated for one hour at room temperature. To remove unreacted components, a Spin column (Bio-Rad) was primed with coupling buffer before the MPO/Sulfo-SMCC solution was added. The column was placed in a 12x75mm test tube and centrifuged at 1000g for 2 minutes. The eluted MPO/Sulfo-SMCC solution was added to the prepared functional beads and incubated on an orbital shaker for one hour at room temperature.

The reaction was stopped by adding 2µl N-Ethylmaleimide 2g/L in DMSO (both Sigma Aldrich) and beads were washed in functional bead storage buffer and pelleted by centrifugation three times before being resuspended in storage buffer. To confirm successful conjugation of MPO on the beads, an aliquot was incubated with anti-MPO-PE (BD Biosciences) or IgG1-PE isotype control prior to analysis by flow cytometry.

Conjugation of anti-C1q antibody:

Sheep anti-human C1q IgG (Abcam, Cambridge, UK)was extensively ultra-filtered in PBS using a Vivaspin 10,000 MWCO concentrator (Vivascience, Littleton, MA, USA) to remove storage proteins and subsequently conjugated to R-PE using a R-PE conjugation kit (Abcam)

Serum samples

Serum stored at Monash Medical Centre at -20°C from patients with biopsy confirmed necrotising pauci-immune glomerulonephritis and positive MPO (n=26) or Pr3 (n=6) was analysed. All patients were anti-GBM IgG negative. An investigator blinded to the studies results reviewed the patient's medical records and categorised patients into active disease or remission. Serum samples from Groningen University were kindly provided by Dr Peter Heeringa and Dr Jan-Stephan Sanders.

C1q fixation detection on custom-made beads.

The assay to detect C1q fixing IgG on single antigen beads was based on a previously published method[365]. In the modified method, 4µl of single antigen beads were added to 30µl of patient serum and incubated for 30 minutes at room temperature on an orbital shaker then washed twice with 1% BSA/PBS. Beads were resuspended in 1% BSA/PBS and 4.5µl of custom conjugated sheep anti-human C1q IgG-PE for 30 minutes.

After 30 minutes, beads were washed twice in 1% BSA/PBS before data were acquired on a Navios flow cytometer. Beads were gated by characteristic FS/SS profile. A minimum of 300 events was collected for the bead population.

C1q fixation detection on commercially available beads.

This was performed as described above using FIDIS vasculitis beads (Theradiag, Marne La Valee, France) Beads were gated by characteristic FS/SS profile before separating bead populations by FL6/FL8 fluorescence characteristics. The anti-GBM bead served as an internal negative control for non-specific binding. A minimum of 300 events was collected for each bead population. To de-complement serum it was heated to 56°C for 30 mins in a water bath. De-complemented serum was reconstituted with human C1q (Sigma-Aldrich) to a final concentration of 150 µg/ml. Total anti-MPO IgG was measured using the FIDIS Vasculitis bead kit according to manufacturer's instructions. To measure C1q fixation by purified IgG samples these were incubated with beads at a concentration of 1mg/ml at 4°C for 30 minutes, the beads were then washed and incubated with normal human serum at room temperature on an orbital shaker for 30 minutes before three further wash steps and staining with C1q detection antibody.

Isolation of neutrophils from healthy human donors.

Blood was taken directly into a syringe containing EDTA 0.5M solution to a final concentration of 10mM. In experiments in which C4d was measured, blood was drawn in to EDTA 10mM/0.05g/L nafamostat mesilate (Futhan, BD Bioscience), a serine protease inhibitor which prevents lectin pathway mediated C4 cleavage in vitro[366]. The anti-coagulated blood was laid onto Polymorphprep (Axis-Shield, Dundee, UK) and centrifuged at 500g for 30 minutes without application of the brake at the end of centrifugation. The band corresponding to neutrophils was carefully aspirated and washed twice in PBS with 0.5mM EDTA/0.5% BSA. Contaminating erythrocytes were not lysed to avoid sample activation. Neutrophils were counted using a haemocytometer. Viability measured by trypan blue exclusion was routinely in excess of 98%.

Purification of immunoglobulin from patients with AAV and controls.

Clinical grade disposal plastic was used for all steps; where this was not possible, autoclaved laboratory glassware was heated in an oven at 250°C for one hour to destroy endotoxin. Dialysis tubing was sterilised by boiling in 10mM EDTA and stored in sterile water containing 0.1% sodium azide. All processing steps were carried out using aseptic non-touch technique. All chemicals used were tissue culture grade. All reagents tested negative for endotoxin using the limulus amebocyte lysate assay (Sigma-Aldrich)

ANCA IgG was purified from plasma exchange effluent from patients with severe, active AAV. Controls were purified from plasma exchange effluent of a patient with anti-GBM disease or from the serum of healthy controls. To precipitate IgG a saturated solution of ammonium sulphate (Sigma-Aldrich) was prepared by dissolving in sterile water. An equal volume of serum was added to ammonium sulphate and the solution was left to precipitate for three hours. The precipitate was pelleted by centrifuging at 3500RPM for 45 minutes at 4°C. The pellet was redissolved in sterile water and then dialysed for 36 hours with three exchanges in phosphate buffered saline (PBS).

To avoid possible endotoxin contamination from the fast liquid protein chromatography (FLPC) system, a closed system using only clinical grade plastic/tubing was used. Using a syringe infusion pump, tilted to minimise the risk of any air bubbles being expelled from the syringe, protein precipitate in PBS was infused at 2mls/minute through a new protein G column (GE healthcare). After rinsing the column with PBS at 5ml/minute for 15 minutes, IgG was eluted using 0.1M glycine/HCL buffer (pH 2.7) in 2ml aliquots into sterile tubes containing 120 all 1M trisaminomethane –HCL (Tris-HCL, pH 9.0). Based on previous experience with FLPC measured ultraviolet absorbance the 4^{th} -6^{th} aliquots have high IgG concentrations and these were retained. The solution was then dialysed for 24 hours with three exchanges in PBS.

Light absorbance at 280nm was measured using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo-Fisher) and IgG concentration calculated using a standard curve derived in the laboratory from the absorbance of bovine IgG.

Human serum.

Normal human serum and factor b depleted human serum (Comptech, TX, USA) was stored at -70°C and defrosted just before use to be diluted to 33% in TAE buffer. Serum aliquots were not re-frozen.

Detection of complement breakdown products on neutrophil surface.

All incubation and wash steps were performed in a 96 well sterile polypropylene plate (Cornig, NY, USA). The plate was centrifuged at 1200rpm for 3 minutes to pellet neutrophils with the supernatant removed by flicking the plate. Neutrophils were Fc blocked (Truestain Fcx, Biolegend) at 4 degrees for 10 minutes before surface staining with anti C3b/iC3b/C3d C3 mAb; 1H8 (Cedarlane, Burlington, Canada), anti-iC3b/C3d/C3dg; 053A-514.3.1.4 or anti-C4dneo; 057-51.5.1.6 (BioRad, Herculues, CA, USA) at a dilution of 1:100. Isotype controls were mouse IgG2a MG2a-52 and mouse IgG1 MOPC-21 (Biolegend). After three wash steps secondary staining was performed with affinity purified, cross-adsorbed goat anti mouse Ig2a-PE or IgG1-PE (Thermo-Fisher). In experiments in which TNT003 was used, biotinylated anti-C3 (1H8, Cedarlane) was used as a primary antibody followed by detection with APC-streptavidin at a final concentration of 0.5µg/ml.

Sensitisation of neutrophils with IgM

To sensitise with IgM, neutrophils were incubated with varying concentrations of mouse-anti-CD15 IgM mAb (clone HI98, Biolegend) diluted in PBS/0.5%BSA/0.5mM EDTA at 4 °C for 30 minutes.

Priming of neutrophils with TNF

Purified neutrophils were incubated with varying concentrations of recombinant human TNF (Biolegend) in Hank's balanced salt solution (Thermo Fisher) at 37°C for 20 minutes.

Exposure of neutrophils to serum

Cells were washed twice and then incubated in 33% NHS in TAE-CHB buffer for 30 minutes at room temperature, except for experiments in which neutrophil activation markers were measured in which incubation temperature was 37 °C. TAE-CHB buffer was preferred to veronal buffered saline due to difficulties importing this buffer into Australia.

Measurement of neutrophil surface MPO

$1x10^6$ neutrophils per well were resuspended in PBS containing 0-1000ng/ml human MPO (Calbiochem). The neutrophils were incubated for 30 minutes at 4°C before being washed 4 times in FACS buffer. Primary antibody detection was with rabbit-anti MPO IgG (1:75 dilution) (Thermo-Fisher), secondary antibody chicken-anti-rabbit Alexa Flour 677 (1:200 dilution)(Life Technologies)

Incubation of neutrophils with ANCA

Neutrophils were exposed to 500ng/ml MPO in PBS for 30 minutes at 4°C. ANCA and control IgG were centrifuged at 10,000 RPM for 10 minutes prior to use to remove aggregates. Neutrophils were exposed to 500µg/ml ANCA or control IgG in PBS/0.5% human albumin solution (Sigma-Aldrich) for 30 minutes at 4 °C. The classical pathway was inhibited by adding mouse anti- human C1s $(Fab)_2$ (TNT003) or isotype control (both provided by True North Therapeutics, CA, USA.) to serum at 50µg/ml.

Assessment of Neutrophil apoptosis

To assess apoptosis neutrophils were incubated with Annexin V-FITC (Biolegend) at a final concentration of 3.6µg/ml in 100µl Annexin V binding buffer (Biolegend) for 15 minutes at room temperature. A further 100µl of Annexin V binding buffer was then added with propidium iodide (BD Bioscience) to a final concentration of 1µg/ml and the samples were immediately acquired by flow cytometry.

Buffers

Wash Buffer: Phosphate buffered saline was made using sterile water for irrigation (Baxter), PBS bioperformance sachets, 0.5% BSA and 0.5mM EDTA (all Sigma-Aldrich). The solution was filter sterilised before use.

FACS Buffer: As for wash buffer with the addition of 0.05% sodium azide (Sigma-Aldrich)

TAE-CHB Buffer: Was made as previously described[367]. 5x stock solution was made by adding 2.5mls of 1M $MgCl_2$ and 0.3M $CaCl_2$ solution to 997.5mls of 0.73M NaCl and 0.02M trietholanamine hydrochloride in sterile water. This solution was sterile filtered and made up to 1x solution with sterile water before use.

Flow cytometry

Flow cytometry was performed on the Beckman Coulter Navios platform, and analysed using FlowJo software (TreeStar, OR, USA).

Statistical Analysis.

For parametric data involving comparison of two variables unpaired Student's T-test was performed. For non-parametric data the Mann-Witney U Test was used.

Multiple comparisons were compared by one-way ANOVA followed by Tukey's multiple comparison test. The Pearson product-moment correlation coefficient was used to measure association between two variables. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.00$

Results:

Validation of human MPO conjugation to functional beads

In order to determine the ability of ANCA to activate the classical pathway, an assay to measure C1q deposition on single antigen beads was developed. This assay was based on a previous previously published method to detect complement fixing donor specific antibodies in human allotransplantation[365]. Functional beads for flow cytometry were conjugated in-house to purified human MPO. Successful conjugation of human MPO to functional beads was confirmed by staining beads with an anti-MPO mAb (Figure 5.1).

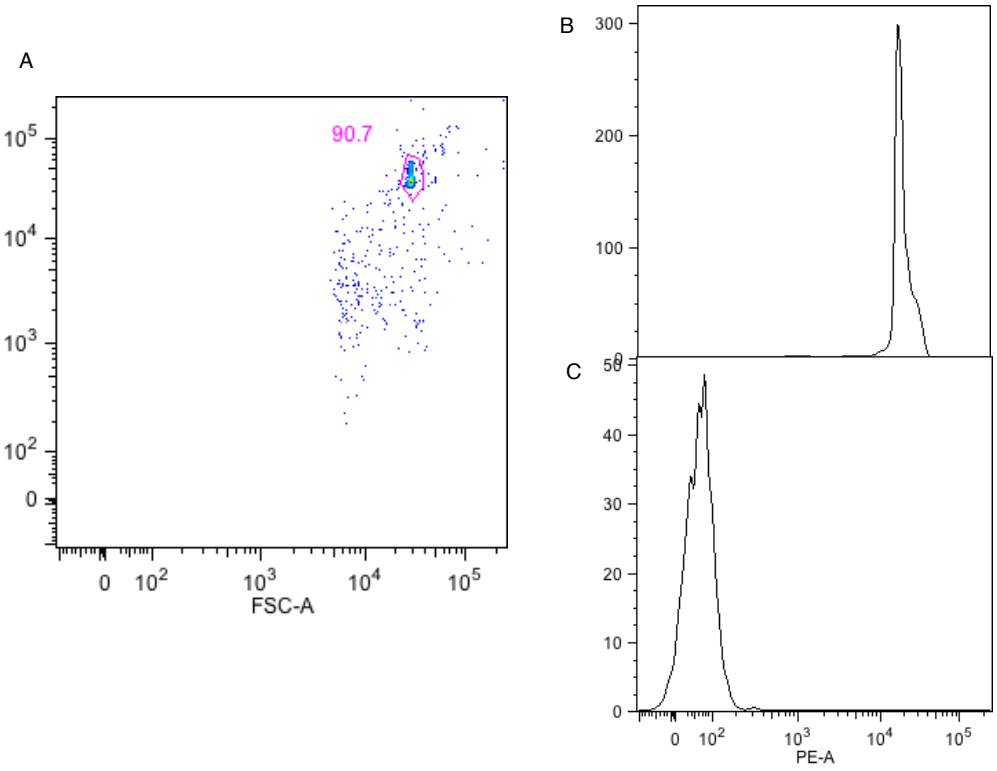


Figure 5.1 Confirmation of conjugation of human MPO to functional beads

(A) Custom conjugated beads were acquired by flow cytometry and gated by characteristic forward/side scatter properties. (B) Beads were incubated with anti-MPO-PE mAb or (C) IgG1-PE isotype control.

MPO-ANCA from patients with active disease fixes C1q: pilot study

MPO coupled beads were incubated in serum from patients with AAV or controls then washed prior to detection of C1q with a sheep anti-human C1q polyclonal antibody conjugated to phycoerythrin. The beads were then acquired by flow cytometry with measurement of C1q associated fluorescence. Active disease was associated with significantly higher C1q fixation was observed either patients in remission. Minimal C1q associated fluorescence was seen in healthy controls (Figure 5.2).

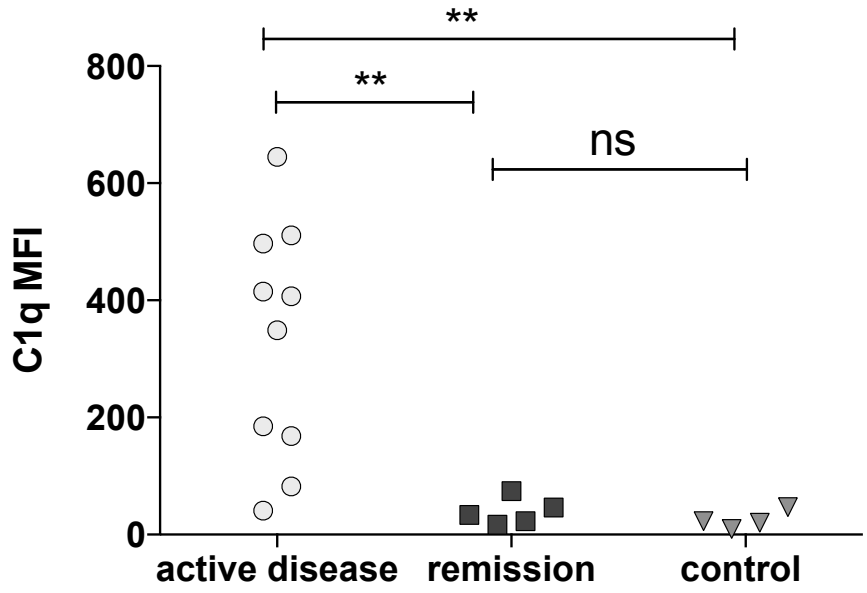


Figure 5.2 Active anti-MPO disease is associated with C1q fixing ANCA

*Median fluorescence intensity (MFI) of C1q fixation on MPO couple beads from patients with active anti-MPO disease (Mean±SEM 330±63 MFI, n=10), anti-MPO disease in remission (Mean±SEM 38±10, n=5) and healthy controls (Mean±SEM 24±7, n-5) was measured by flow cytometry. C1q fixation was higher in active disease than remission or healthy controls **=p<0.01 Statistical analysis by one way ANOVA followed by Tukey's multiple comparison test.*

Commercially available beads can be adapted to perform C1q fixation assay

To permit further exploration of the C1q fixing ability of ANCA, additional experiments were performed using a commercially available preparation of single antigen beads (Theradiag Fidis Vasculitis Panel). This is a U.S FDA approved diagnostic test for semi-quantitative measurement of ANCA and anti-GBM antibodies. The kit contains beads covalently bound to MPO, Pr3 and type IV collagen. The bead populations can be separated by their size and fluorescence characteristics using flow cytometry (Figure 5.3).

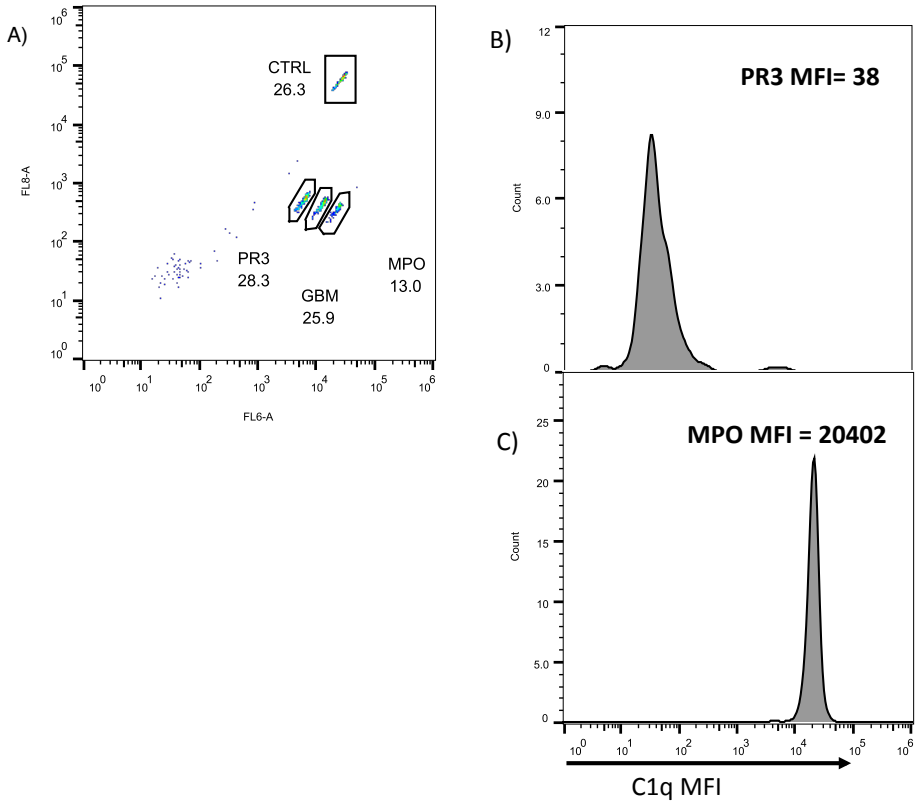


Figure 5.3 Measurement of C1q fixing ANCA on commercial single antigen beads

(A) Beads were incubated in sera and washed prior to staining with anti-C1q PE. Samples were analysed by flow cytometry. Beads were discriminated from debris by their forward scatter/side scatter profile before individual bead populations were gated based on their fluorescence in the FL6 (650-750nm) and FL8 (>755nm) channels. Binding of anti-C1q-PE was measured on all bead populations. (B-C) Representative histograms of C1q fixing MPO sera with minimal signal on Pr3 conjugated bead and fluorescence on MPO conjugated bead populations.

Specificity of Anti-C1q antibody preparation

To confirm that the custom conjugated anti-C1q antibody bound specifically to C1q without any significant cross-reactivity, a series of experiments was performed. Serum was decomplemented by warming to 56°C and, in some samples, C1q was restored by adding autologous human C1q to the heat depleted serum. Heat inactivation of serum abrogated C1q detection in positive samples, C1q signal was restored by addition of C1q, confirming the specificity of the antibody preparation (Figure 5.4).

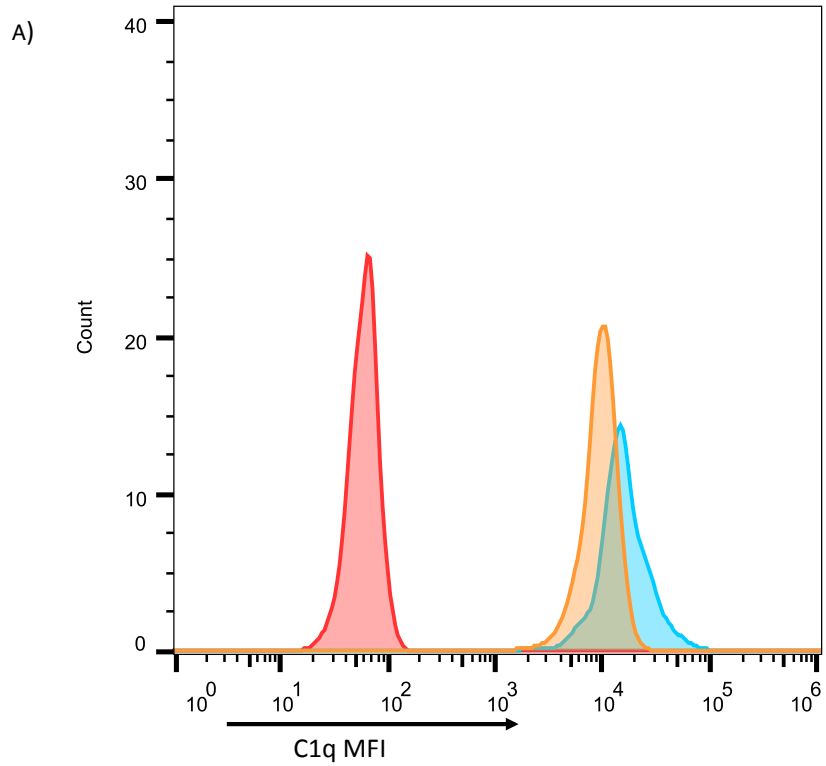


Figure 5.4 Anti-C1q antibody specifically binds to bead associated C1q
Histograms of anti-C1q associated fluorescence in a single sample of sera. Beads were incubated in heat decomplemented serum (Red), heat decomplemented serum restored with exogenous human C1q (Blue) unmodified human serum (Orange) before C1q was detected using sheep anti-human C1q-PE.

MPO-ANCA from patients with active disease fix C1q

Serum samples from patients at Monash Medical Centre with anti-MPO and anti-Pr3 disease in both active disease and remission states were analysed for ANCA C1q fixation using the Fidis Vasculitis beads. The majority of patients with active anti-MPO disease demonstrated ANCA C1q fixation that was significantly higher than those from patients in remission (Figure 5.5).

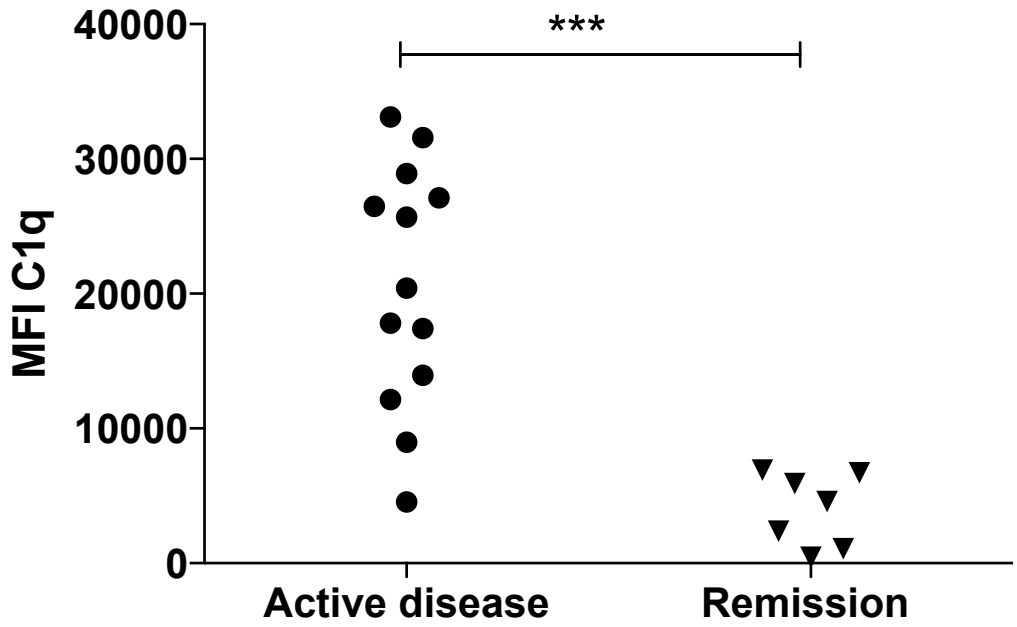


Figure 5.5 MPO-ANCA from patients with active disease fixes C1q.

*C1q fixation of sera from patients with anti-MPO disease. Single antigen beads were incubated in serum before C1q fixation was detected with anti-C1q-PE. C1q fixation was significantly higher in samples from patients with active disease (Mean±SEM 20628±2499) than those in remission (4009±2689) Statistical analysis by Mann-Whitney U Test. ***=p<0.001.*

C1q fixing ability of MPO-ANCA correlates to MPO-ANCA titre.

To determine whether C1q fixation by ANCA was correlated to total MPO-ANCA titres, MPO bead bound human IgG was measured. Serum samples were diluted 1:100 in PBS before being incubated with the beads for 40 minutes. The beads were washed before IgG was detected with anti-human IgG-PE. C1q fixation was strongly associated with anti-MPO IgG titres (Pearson r= 0.77 95% CI 0.53-0.90) (Figure 5.6)

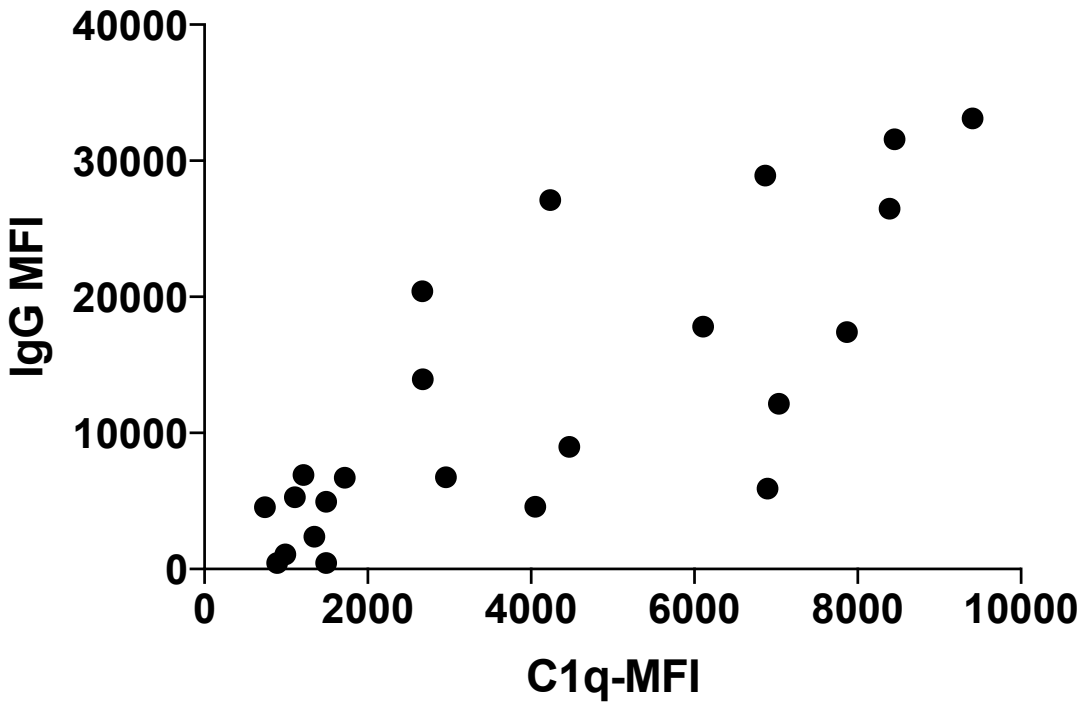


Figure 5.6 C1q fixation correlates to MPO-ANCA titre.

MPO specific IgG was measured by incubating a 1:100 dilution of serum with single antigen beads. Beads were washed before incubation with anti-human IgG-PE. Samples were analysed by flow cytometry. Pearson r= 0.77 95% CI 0.53-0.90.

Pr3 C1q fixation is elevated in active disease

The C1q assay was repeated for the small set of samples with active anti-Pr3 disease (n=3) and disease in remission (n=3). In this small cohort, C1q fixation was numerically higher in samples from patients with active disease, although due to the small sample size, meaningful statistical analysis for non-parametric data was not possible (Figure 5.7).

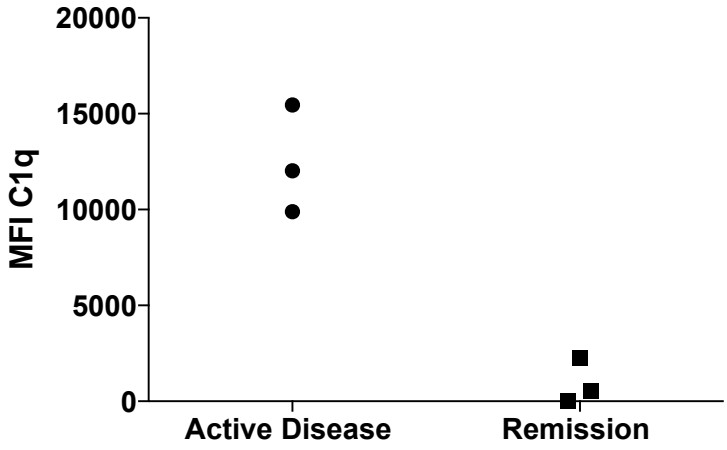


Figure 5.7

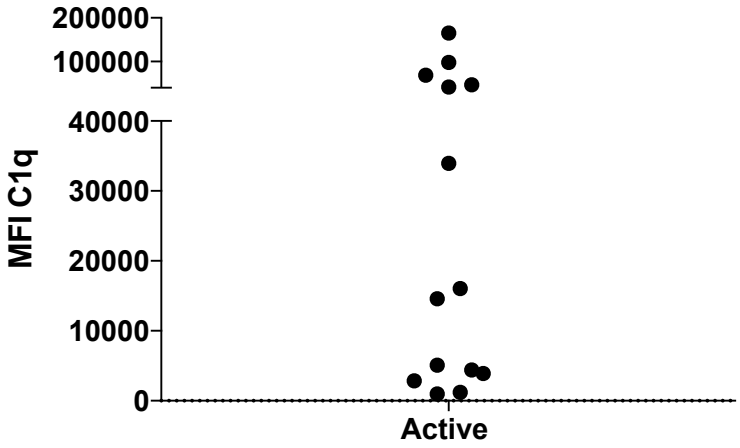
C1q fixation of sera from patients with active anti-Pr3 disease (n=3) is numerically higher (Mean±SEM 12033±1619) than anti-Pr3 disease in remission (947±675, n=3)

At the time of analysis, there were a limited number of sera samples available in the Monash ANCA cohort, particularly paired samples from the same patient in active disease/remission and samples from patients with anti-Pr3 disease. Therefore, a further set of samples was obtained through collaboration with the University of Groningen, Netherlands. This consisted of serum from patients with active Pr3 disease (n=25) paired anti-Pr3 remission samples (n=10) and active anti-MPO disease (n=14). These were analysed using the same protocol, including the same voltage settings on the flow cytometer. A different batch of both FIDIS vasculitis beads and batch of anti-C1q-PE produced by a separate conjugation was used in these experiments.

MPO-ANCA data Groningen cohort

Analysis of C1q fixation by serum samples from patients with MPO-ANCA from the Groningen cohort showed that 8/16 demonstrated high C1q fixation with an MFI>10,000 whilst in the remainder C1q fixation was low. Correlation between MPO-IgG and C1q fixation approached, but did not reach statistical significance, (Pearson r=0.54, 95% CI -0.04 to 0.83)

A



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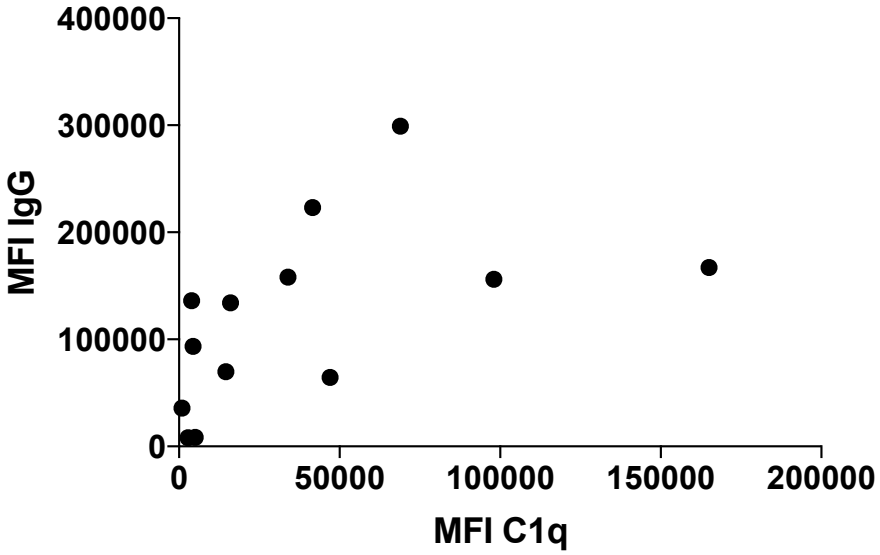

Figure 5.9 C1q fixation by MPO-ANCA from Groningen cohort.

(A) C1q fixation in sera from patients with active anti-MPO disease (n=14) from Groningen cohort (Mean±SEM =37977±12646)

*(B) Correlation of anti-MPO IgG and C1q fixation (*Pearson r=0.54, 95% CI -0.04 to 0.83)

C1q fixing anti-Pr3 is not associated with active disease: Groningen cohort

Serum samples from patients with active anti-Pr3 disease (n=25) and disease in remission (n=10) were analysed for C1q fixation. Whilst the level of Pr3 specific C1q fixation was higher in active disease, only a minority of samples (5/25) exhibited strong C1q fixation with a MFI>10,000.

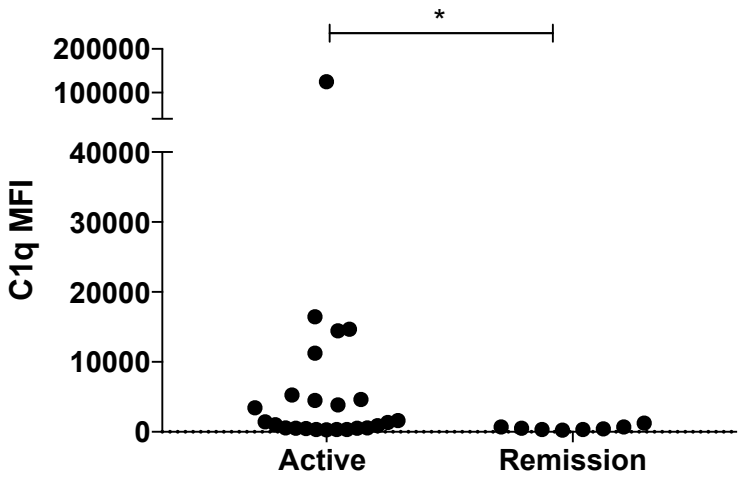

Figure 5.10 Strong C1q fixing anti-Pr3 antibodies are not common in active anti-Pr3 disease.

*C1q fixation was higher in active anti-Pr3 disease vs. remission (Mean±SEM 8910±5151 vs.14068±13492). However, anti-Pr3 autoantibodies that strongly fix Pr3 were uncommon in this cohort. Data analysed by Mann-Whitney U Test *=P<0.05*

Paired samples from 10 patients taken during active anti-PR3 disease and remission were further analysed. Both C1q fixation and total anti-Pr3 titres were measured. C1q fixation of anti-Pr3 IgG did not statistically differ between disease states. This was because of a single outlier in which both C1q fixation and anti-Pr3 titre increased between active disease and remission (Figure 5.11)

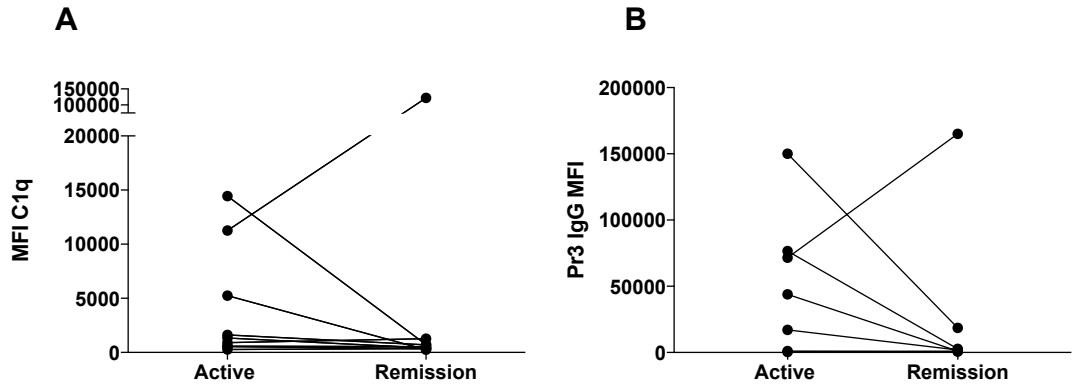

Figure 5.11 Analysis of paired samples from patients with anti-Pr3 disease.

(A) C1q fixation in paired anti-Pr3 patient samples from active disease and remisison did not statistically differ between states. (B) Anti-Pr3 IgG in paired anti-Pr3 samples.

C3 breakdown products can be detected on human neutrophils

Whilst these data suggest that in this *in vitro*, bead based system some ANCA (predominantly MPO-ANCA from patients with active disease) are able to fix C1q and therefore activate the classical pathway of complement, differences in antigen density and the presence of complement regulatory proteins means that ANCA binding and subsequent complement activation on human neutrophils is likely to be very different to that observed on beads. The ability of ANCA to induce detectable complement activation on human neutrophils was therefore investigated.

The amplifying mechanism of the complement cascade results in around 200 cell bound C3 molecules for every antibody bound C1q molecule[368]. C3 breakdown products can therefore be detected on the surface of antibody-targeted cells in the absence of detectable C1q and C4d[369]. To optimise an experimental protocol to detect C3 breakdown products on the surface of neutrophils, an IgM anti-human CD15 mouse monoclonal antibody was used to sensitise neutrophils, as this is a subclass that strongly activates the classical pathway.

Neutrophils from human healthy donors were purified using Polymorphprep before being sensitised with anti-CD15 mAb followed by incubation in 33% human serum in TAE-CHB buffer at 37°C for 30 minutes to induce complement deposition. After extensive washing neutrophils were stained with monoclonal antibodies against C3 breakdown products, which were then detected with an isotype specific, cross-adsorbed, PE-conjugated secondary antibody. Samples were acquired by flow cytometry with neutrophils identified by characteristic FS/SS properties. At high concentrations of anti-CD15 1-10μg/ml there was significant lysis of neutrophils. The experiment was therefore repeated with anti-CD15 mAb concentrations in the ng/ml range. At lower concentrations of IgM, neutrophil bound C3 was clearly detectable using two monoclonal antibodies: 1H8 specific for C3b/iC3b/C3d[370], and 053A-514.3.1.4 specific for iC3b/C3d/C3dg (Figure 5.12). This established that an assay to measure neutrophil surface C3

breakdown product was viable. Since both antibody clones performed similarly, 1H8 was selected for future use on the basis of cost.

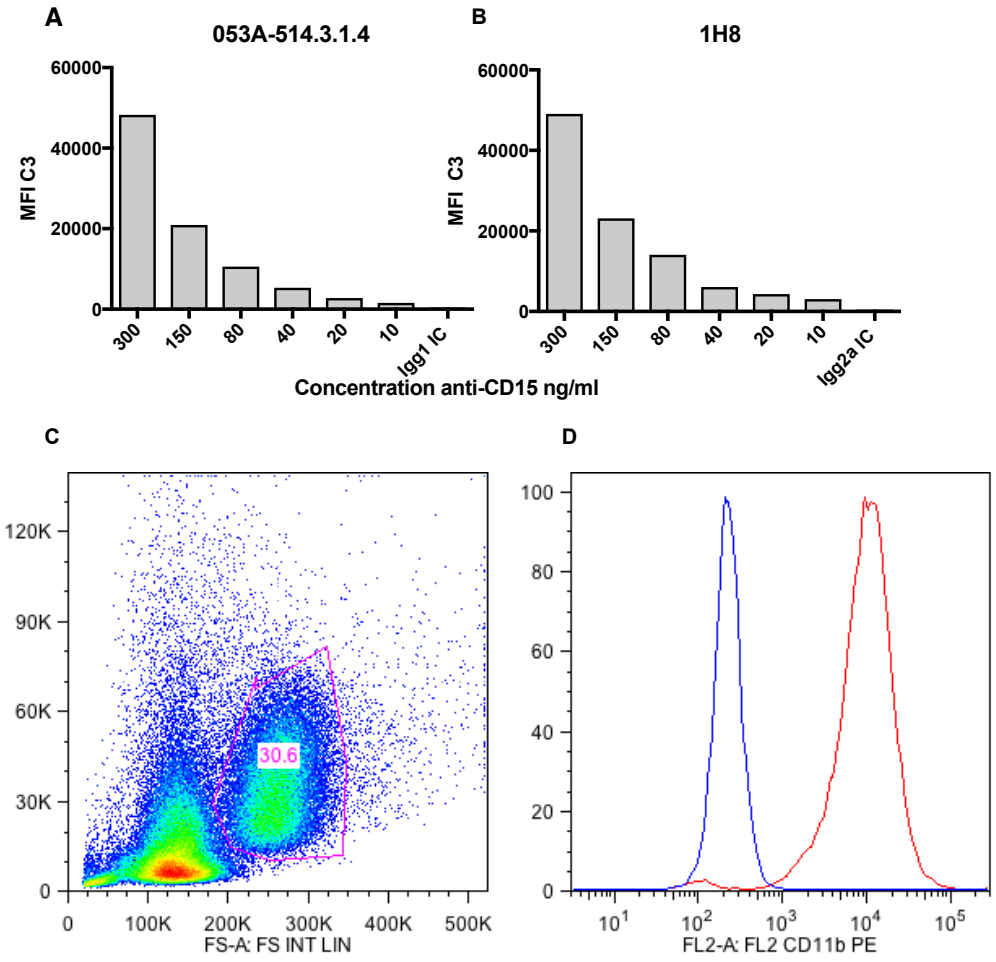

Figure 5.12 C3 Cleavage products can be detected on the surface of antibody targeted neutrophils.

MFI of C3 breakdown products on neutrophils sensitised with varying concentrations of anti-CD15 mAb and incubated in 33% NHS. (A) C3 breakdown products as detected with the monoclonal antibody 053A-514.3.1.4 (B) C3 breakdown product detected with monoclonal antibody 1H8. (C) Representative flow cytometry plot showing granulocytes gated by characteristic FS/SS. (D) Histogram of C3 surface breakdown product on neutrophils incubated with 80ng/ml anti-CD15, followed by incubation in serum and detection with ether IgG2a isotope control (blue line) or mAb 1H8 (red line).

Purification of MPO-ANCA and Control IgG

To investigate whether ANCA was capable of activating complement on the surface of neutrophils, IgG was purified from the plasma exchange effluent of 5 patients with anti-MPO AAV by ammonium sulphate precipitation followed by protein G column purification. As the indications for plasma exchange in AAV are limited to those with severe active disease, ANCA from these patients was assumed to be highly pathogenic. To act as controls, IgG was purified from the plasma exchange effluent of one patient with anti-GBM disease and the serum of 5 healthy volunteers. The final measured concentrations of purified IgG are tabulated below:

Sample	**Final IgG Concentration (mg/ml)**
MPO-ANCA 1	3.6
MPO-ANCA 2	9.4
MPO-ANCA 3	9.2
MPO-ANCA 4	22.2
MPO-ANCA 5	9.4
Anti-GBM (control)	3.45
Healthy Control 1	9.97
Healthy Control 2	12.3
Healthy Control 3	13.5
Healthy Control 4	12.12
Healthy Control 5	9.4

Purified IgG samples were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue under non-reducing conditions to ensure that there was a protein band consistent with immunoglobulin without evidence of significant aggregates (Figure 5.13).

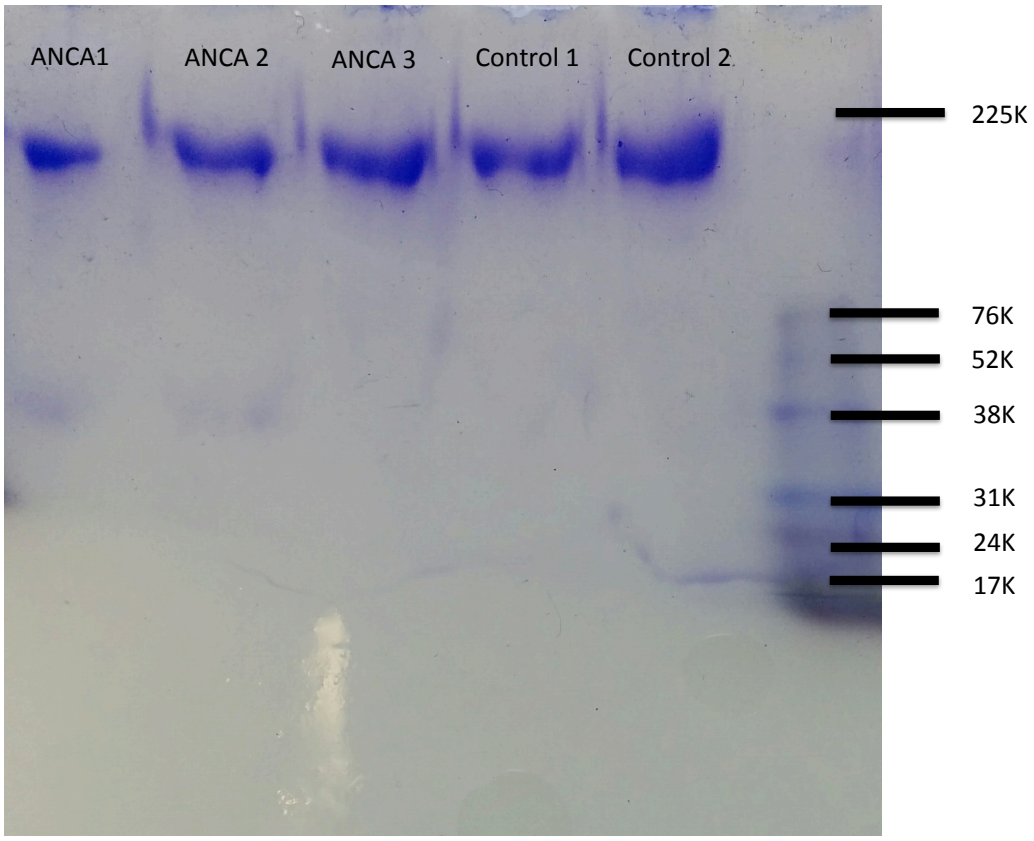

Figure 5.13: SDS-PAGE analysis of IgG preparations

Purified IgG from patients with MPO-ANCA and healthy controls was run alongside a high range protein ladder on SDS-PAGE followed by staining with Coomassie brilliant blue. Image demonstrates a band consistent with the 150-170 kDa molecular weight of human IgG without evidence of large protein aggregates.

TNF but not ANCA induces C3 activation on the surface of human neutrophils

MPO is stored in the intracellular granules of neutrophils. *In vitro*, TNF is commonly used to prime neutrophils for ANCA mediated activation. Mechanistically, TNF is thought to prime neutrophils by increasing MPO surface expression[50]. Neutrophils were primed with concentrations of TNF varying from 0-10ng/ml before simulation with MPO-ANCA IgG or control IgG followed by incubation in 33% normal human serum. The deposition of C3 activation products on the neutrophil surface was then analysed. There was no difference in C3 deposition on neutrophils between MPO-ANCA IgG and control IgG at any concentration of TNF. However, neutrophil surface C3 deposition dramatically increased with rising concentrations of TNF. This suggests that the concentration of TNF used to prime neutrophils (which is in several fold excess of that measured in patients with AAV[371]) causes changes that result in robust surface complement activation. To determine the role of the alternative pathway in this TNF induced complement activation, neutrophils were incubated in complement factor b depleted serum, this resulted in attenuation of TNF induced C3 deposition suggesting that the alternative pathway initiation and/or amplification loop was a significant contributor to neutrophil surface complement activation. (Figure 5.14, overleaf)

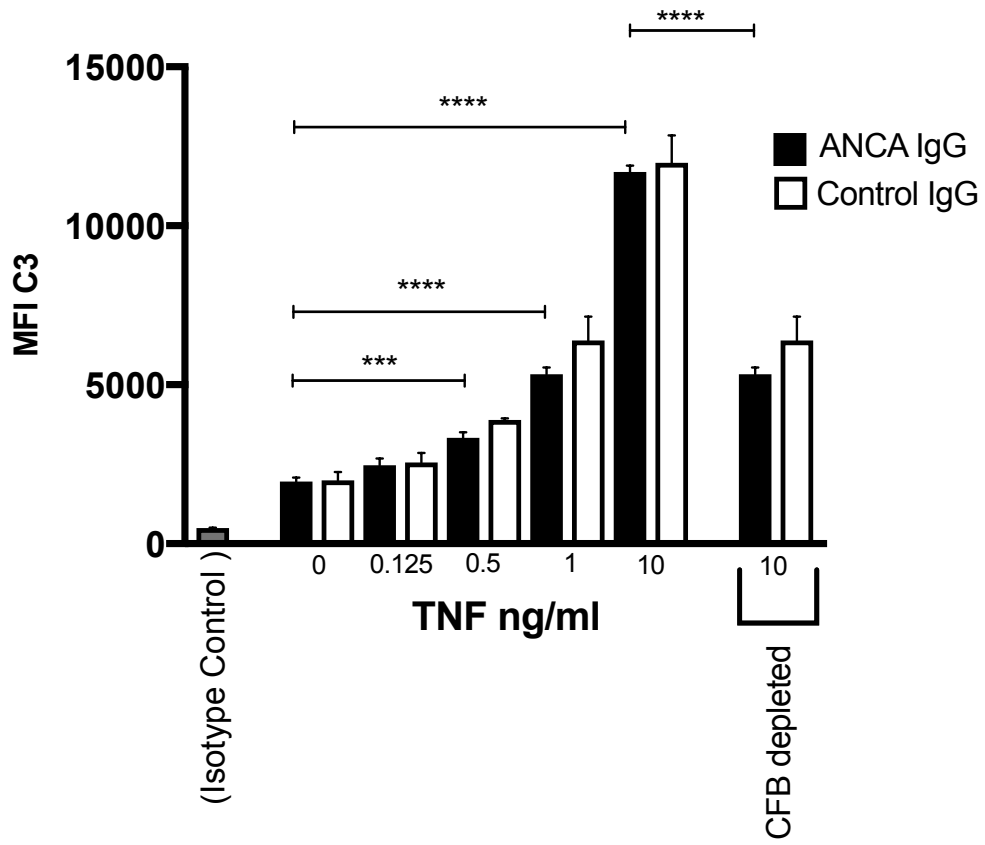

Figure 5.14 TNF, but not MPO-ANCA induces C3 deposition on the surface of neutrophils.

*Neutrophil surface C3 was measured after priming neutrophils with varying concentrations of TNF, followed by incubating them with ANCA (n=5) or control IgG (n=6) then 33% NHS. Neutrophil surface C3 increased in a dose dependent manner with increasing TNF concentrations but was not altered by the presence of ANCA IgG. C3 deposition on neutrophils primed with 10ng/ml TNF was significantly reduced when they were incubated in complement factor B depleted serum. Data show mean±SEM with statistical analysis by one way ANOVA followed by Tukey's multiple comparison test. ***=P<0.001, ****=P<0.0001*

Neutrophils acquire exogenous MPO on their surface.

As stimulating neutrophils with TNF induced significant complement activation, an alternative approach to priming neutrophils for activation by ANCA was investigated. Although surface expression of MPO by neutrophils is conventionally thought to result from the externalisation of endogenous MPO from granules, exogenous myeloperoxidase can also bind to neutrophils via a CD11b/CD18 dependent mechanism[372]. Mean MPO levels in healthy individuals are approximately 400pmol/l with a 5-fold increase in inflammatory conditions such as sepsis[373]. Levels of neutrophil membrane associated MPO are increased in inflammation[372]. Incubation of neutrophils with purified MPO results in those neutrophils being responsive to activation by ANCA[374], suggesting that exogenous MPO bound to the neutrophil surface can be an alternative source of antigen for MPO-ANCA to target.

To confirm this, purified neutrophils were incubated with purified human MPO at concentrations comparable to those seen in human inflammatory disease before measuring surface MPO binding by flow cytometry. Increasing concentrations of MPO resulted in a dose dependent increase in neutrophil membrane associated MPO measured by flow cytometry. (Figure 5.15, overleaf)

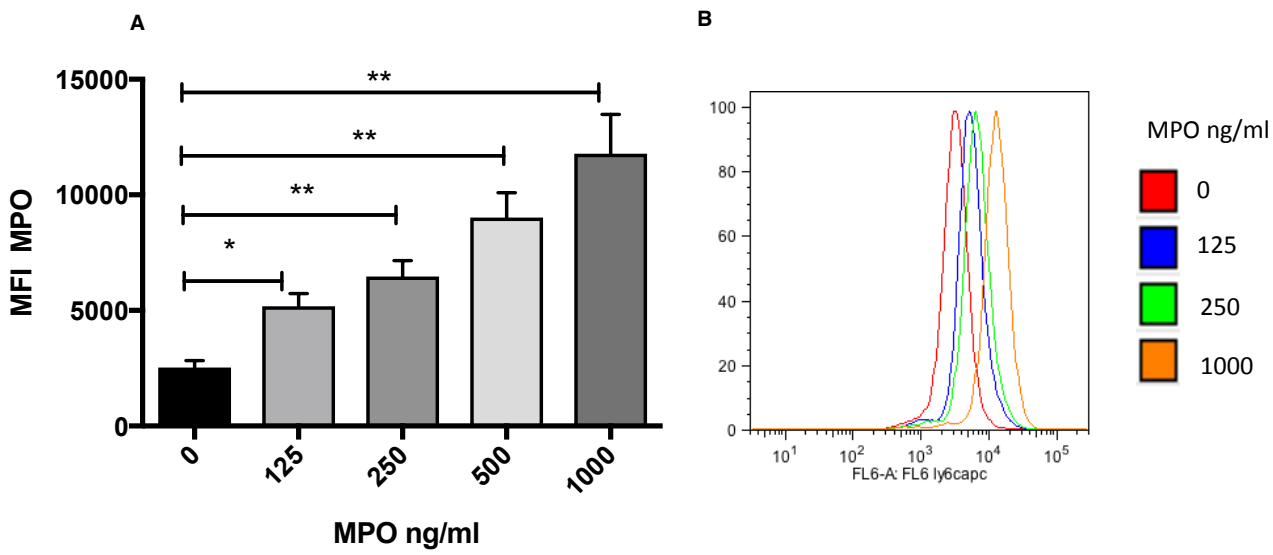

Figure 5.15. Neutrophils acquire exogenous MPO on their surface.

*(A) Incubation of neutrophils with purified MPO results in dose-dependent expression of MPO on the neutrophil surface. (B) Representative histogram of surface MPO signal on neutrophils incubated with increasing concentrations of purified MPO. Data show mean±SEM. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparison test. *=p<0.05, **=p<0.01.*

MPO-ANCA induces C3 deposition on MPO exposed neutrophils

The capacity of MPO-ANCA to bind to surface MPO resulting in antibody induced complement activation was then investigated. Purified neutrophils were sequentially incubated with MPO, MPO-ANCA then 33% normal human serum. C3 split product deposition was then measured by flow cytometry. MPO-ANCA induced significantly greater neutrophil C3 deposition than control IgG including inducing a population of neutrophils with high levels of surface bound C3 (Figure 5.16).

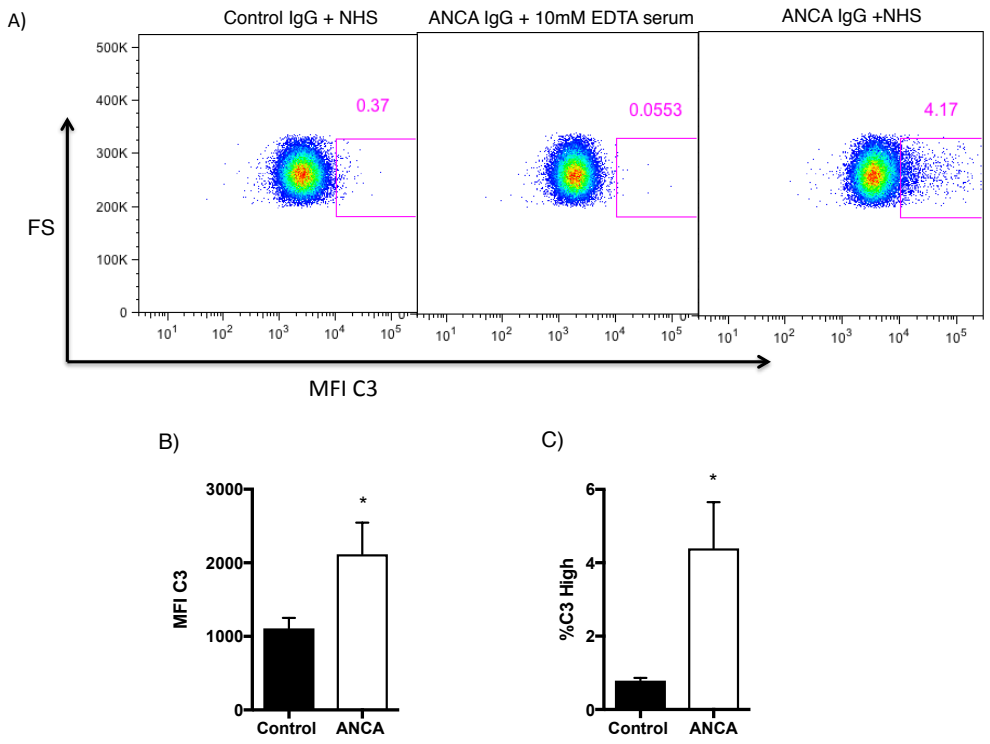

Figure 5.16: MPO-ANCA induces C3 deposition on surface of neutrophils

*(A) Representative flow cytometry plots showing effect of incubating MPO primed neutrophils with control IgG followed by 33% NHS, ANCA IgG followed by 33% NHS containing 10mM EDTA (to inhibit complement) and ANCA IgG followed by 33% NHS. (B) ANCA IgG (n=5) induces significantly more C3 surface deposition on neutrophils than control IgG (n=6). (C) A C3 high population of neutrophils is induced by incubation with ANCA IgG which was not seen in controls. Data presented as Mean±SEM. *=p<0.05*

C3 deposition correlates with C1q binding of purified IgG on single antigen beads

To provide supportive evidence that the increased surface C3 deposition induced by ANCA IgG correlated with complement fixing properties of the MPO-ANCA preparation, the ability of the purified MPO-ANCA preparations to fix C1q on single antigen beads was measured, and compared to the C3 deposition induced by the same MPO-ANCA preparation on human neutrophils. Neutrophil surface C3 induced by MPO-ANCA IgG preparations correlated with C1q fixation on MPO-coupled beads (*Pearson r= 0.86 95% C.I 0.07 to 0.99)* (Figure 5.17).

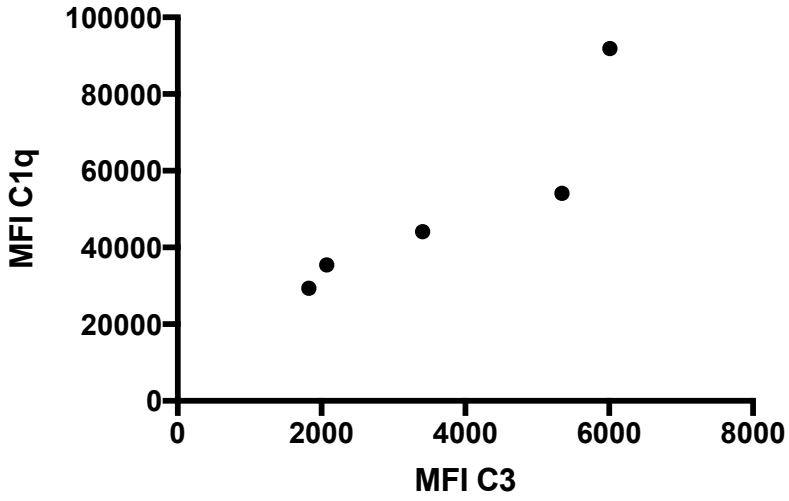

Figure 5.17 The ability of ANCA to induce C3 deposition on human neutrophils correlates with C1q fixation on MPO coupled beads.

MPO-ANCA IgG C1q fixation on single antigen beads correlates with induced C3 deposition on neutrophils. Single antigen beads were incubated with MPO-ANCA, washed and then incubated in 33% NHS. C1q deposition was measured with polyclonal anti-C1q antibody and the samples were analysed by flow cytometry. Pearson r= 0.86 95% C.I 0.07 to 0.99

Analysis of ANCA induced C4d deposition on neutrophils

To investigate whether MPO-ANCA induced complement activation and C3 deposition was initiated by the classical pathway, the deposition of C4d on neutrophils was assessed. C4d is a C4 cleavage product generated by the classical and lectin, but not alternative pathways. Preliminary studies showed that using anti-CD15 IgM (40ng/ml) targeted neutrophils as a positive control, C4d was detectable (MFI 1083 vs isotype control 406). However the magnitude of signal was a fraction of that seen when staining for neutrophil C3 breakdown products under the same conditions (MFI 6064).

Neutrophils were sequentially exposed to MPO, MPO-ANCA and 33% NHS before surface staining for C4d. Whilst there was a trend towards increased C4d staining in the MPO-ANCA exposed group, this did not reach statistical significance (Figure 5.18)

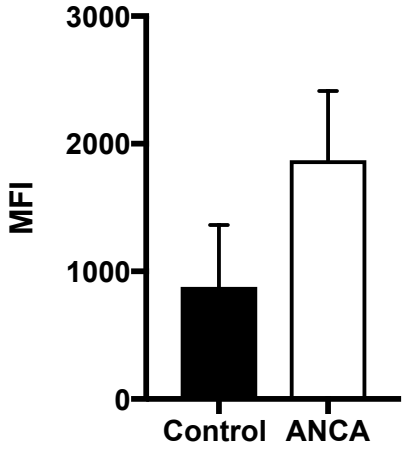

Figure 5.18 ANCA does not induce significant C4 deposition on the surface of neutrophils.

Neutrophil surface C4d was stained in neutrophils sequentially exposed to MPO, MPO ANCA or control IgG and 33% NHS. C4d staining was not significantly greater in the MPO-ANCA group ((Mean±SEM 1871±543, n=5) than the control IgG group (876±486, n=6, Statistical analysis by Mann-Whitney U Test. p=0.25). Data represent Mean±SEM values from two independent experiments.

Classical pathway inhibition reduces neutrophil surface C3 and activation markers

The amplification loop of the alternative pathway means that the production of C3 cleavage products is several orders of magnitude more than that of C4d. As an alternative method to assess the contribution of the classical pathway to ANCA induced complement activation, the deposition of C3 breakdown products on neutrophils was measured in the presence of a specific classical pathway inhibitor: the mouse IgG2a anti-C1s monoclonal antibody TNT003. C1s is a serine protease that, after complexing with C1q cleaves C4, resulting in the eventual formation of the classical pathway C3 convertase. TNT003 does not prevent the complexing of C1s to C1q and C1r, but inhibits its subsequent enzymatic activity. Because of a theoretical concern that the TNT003 mAb bound to C1s at the neutrophil surface could itself induce neutrophil activation, purified $F(ab')_2$ fragment of TNT003 was used.

Neutrophils were incubated with MPO, followed by MPO-ANCA and 33%NHS containing either 50μg/ml TNT003 $F(ab')_2$ or an IgG2a isotype control $F(ab')_2$. This was a concentration of TNT003 that had previously been shown to result in total inhibition of the classical pathway in human serum (personal communication, Dr Sandip Panicker, True North Therapeutics). Neutrophil activation markers CD11b, CD63 and CD66b, chosen on the basis of increased expression in active disease in previous studies in AAV[375] were also measured.

As previously observed, levels of neutrophil surface C3 were higher in MPO-ANCA treated neutrophils than those exposed to control IgG. Classical pathway inhibition resulted in a profound decrease in neutrophil membrane associated C3 in both ANCA exposed and control IgG treated neutrophils. The level of the activation markers CD11b, CD63 and CD66b did not differ between neutrophils treated with MPO-ANCA and control IgG, activation markers were reduced in both groups exposed to serum containing classical pathway inhibitor (Figure 5.19).

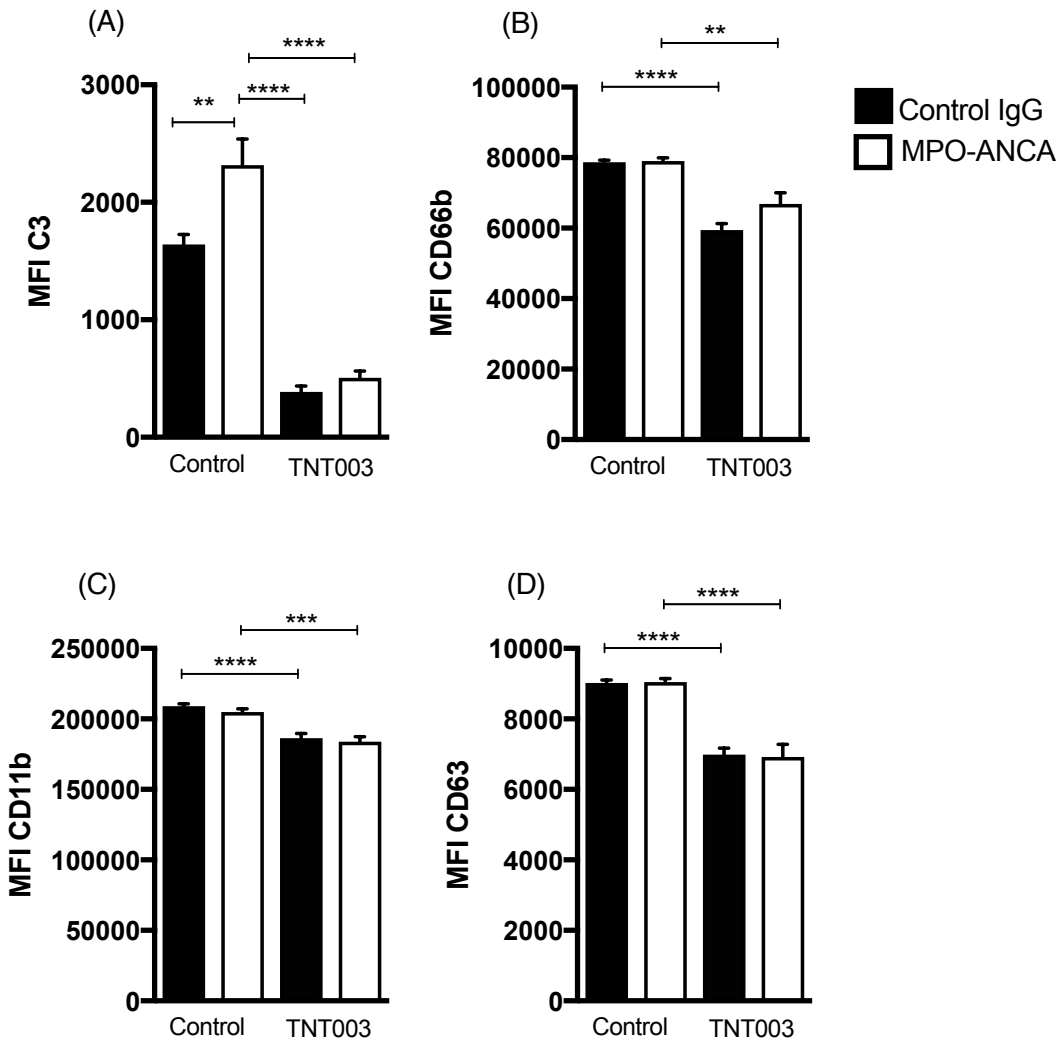

Figure 5.19 Inhibition of the classical pathway significantly reduces C3 deposition and activation markers in both MPO-ANCA and control IgG treated neutrophils.

*Neutrophils were incubated with MPO, then sensitised with MPO-ANCA or control IgG before being exposed to 33% NHS containing anti-C1s mAb TNT003 or F(ab')$_2$ isotype control. (A) MPO-ANCA exposed neutrophils had higher levels of surface C3 compared to control IgG exposed neutrophils when incubated in serum containing isotype control. Inhibition of the classical pathway resulted in a large decrease in neutrophil bound C3 in both MPO-ANCA and control IgG groups. (B) There was no difference in activation markers CD11b, CD63 and CD66b between MPO-ANCA and control IgG treated groups, however, classical pathway inhibition resulted in significant decreases in these activation markers in both groups. **=p<0.01, ***=p<0.001, ****=p<0.0001.*

Neutrophil apoptosis is not induced by the experimental protocol.

As C1s inhibition resulted in a significant reduction in surface C3 deposition in both neutrophils treated with ANCA and control IgG, the possibility that the experimental protocol induced classical pathway activation through neutrophil apoptosis was investigated. Neutrophils are short-lived cells and apoptosis is an essential physiological aspect of their life-cycle as it allows for the resolution of inflammation. An early event in apoptosis is the externalisation of phosphatidylserine from the intracellular leaflet of the plasma membrane. C1q is able to bind to phosphatidylserine, initiating the classical pathway, which plays an essential role in the clearance of apoptotic cells[376]. Whilst efforts were made to minimise the potential of the experimental protocol to induce apoptosis by performing the majority of steps at 4°C, factors such as the total ex-vivo time of 5 hours and the multiple centrifugation steps could potentially induce apoptosis[377]. To determine whether the experimental protocol induced significant neutrophil apoptosis, neutrophils were stained with annexin V. Controls analysed in parallel included neutrophils immediately after isolation with Polymorphprep, neutrophils incubated for 3 hours at 37°C in complete RPMI, and as a positive control neutrophils incubated for 3 hours at 37°C with10ng/ml TNF and 2.5µg/ml cyclohexamide; a protocol that potently induces apoptosis[55]. The proportion of neutrophils that were apoptotic at the end of the experimental protocol as measured by annexin V staining was 1.16±0.1% (n=4) in ANCA stimulated and 1.04±0.1% (n=4) in control IgG stimulated. This was similar to the proportion in neutrophils freshly isolated by Polymorphprep, and much less than the proportion of apoptotic neutrophils seen in positive controls. (Figure 5.20, overleaf)

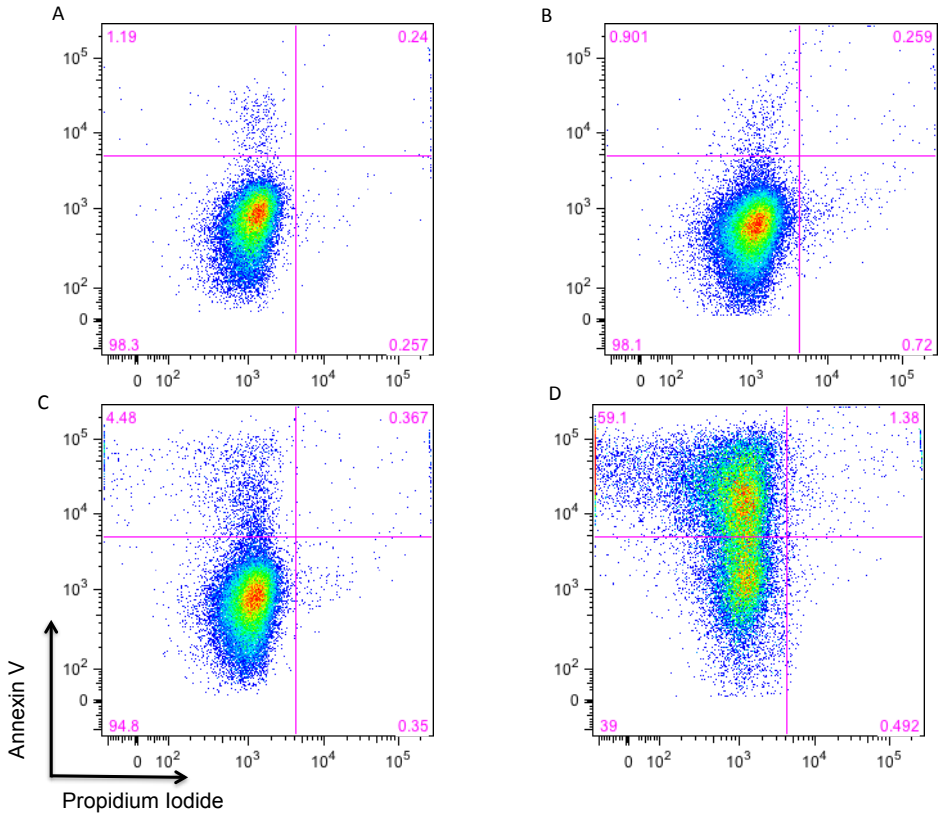

Figure 5.20 The experimental protocol does not induce significant neutrophil apoptosis as measured by Annexin V binding.

Neutrophils were purified on Polymorphprep, and sequentially incubated with MPO, ANCA and 33% NHS, before staining with Annexin V and propidium iodide(PI). (A) Representative flow cytometry plot gated on neutrophils by FS/SS after experimental protocol including ANCA stimulation showing early apoptotic (Annexin V^+ PI) and late apoptotic/necrotic (Annexin V^+PI^+) quadrants. (B) Neutrophil apoptosis assessed immediately after purification by Polymorphprep. (C) Apoptosis was assessed after 3 hours incubation in complete RPMI at 37°C, (D) Apoptosis assessed after 3 hours incubation in complete RPMI at 37°C 10ng/ml TNF and 2.5µg/ml cyclohexamide,.

ANCA induced C3 binding is not restricted to apoptotic neutrophils

As MPO expression has previously been reported on apoptotic neutrophils[54], whether ANCA induced C3 deposition was restricted to the small population of apoptotic neutrophils was determined. ANCA induced C3 deposition was assessed in parallel with annexin V binding. This demonstrated that C3 was not restricted to apoptotic (annexin V high) neutrophils (Figure 5.21).

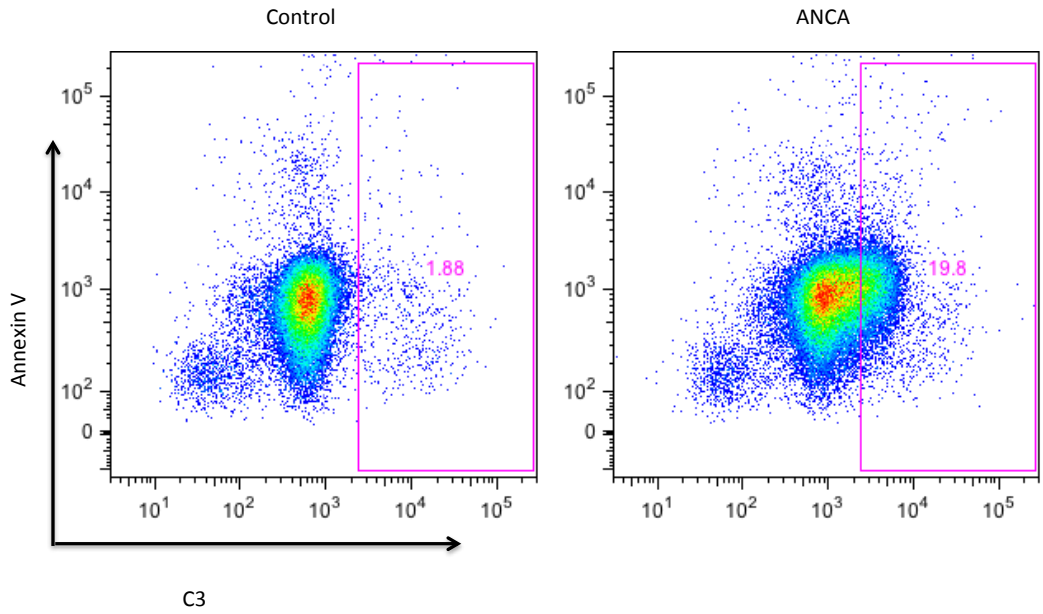

Figure 5.21 ANCA induced C3 deposition is not restricted to apoptotic neutrophils.

ANCA induced C3 deposition was assessed in parallel to annexin V binding. Representative flow cytometry plot gated on neutrophils by FS/SS showing C3 high population induced by ANCA is not apoptotic as judged by annexin V binding.

Discussion:

In this chapter the possibility that early complement activation in AAV was initiated via the classical pathway by ANCA bound to the surface of neutrophils was examined. First, development of an assay to demonstrate C1q fixing ability of ANCA in serum is described, followed by the analysis of this parameter in sera from patients with active disease. In addition, the capacity of MPO-ANCA to induce complement activation at the surface of human neutrophils was also investigated.

These data demonstrate that ANCA from a proportion of patients with active AAV, are able to fix C1q *in vitro* on single antigen beads and therefore activate the classical pathway of complement. This phenomenon was more pronounced for anti-MPO antibodies than anti-Pr3. Whilst the small number of anti-Pr3 samples tested from the Monash cohort did display C1q binding in active disease this was not replicated in the Groningen cohort. There are several possible explanations for the observed differences between MPO-ANCA and Pr3 ANCA. It is possible that patients with anti-Pr3 disease have predominance of non-complement fixing subclasses, although this is unlikely as previous studies have shown high levels of Pr3 specific IgG1 and IgG3 in this group of patients [378, 379]. Other possibilities include shielding of Pr3 in neat plasma by α-1-antirypsin, which has previously been reported to block Pr3-ANCA binding to Pr3[380]. It is also possible that an experimental error may have occurred. The Pr3 samples obtained from Groningen were analysed as a single batch and whilst it would have been desirable to re-analyse the samples this was not possible due to limited sample availability.

Many factors determine the capacity of IgG to fix C1q. These include antibody titre, affinity, subclass, antigen density, and both the number of targeted epitopes and their stereotactic arrangement. C1q fixation ability of anti-MPO serum strongly correlated with total anti-MPO IgG levels. The correlation between donor specific antibody IgG levels and C1q fixation is

described in the transplantation literature; the observed correlation in the current studies (Pearson r = 0.77) is in agreement with large studies in the transplantation literature (Pearson r ≈ 0.8)[381, 382]. Given the strength of this correlation, there is some controversy in the transplantation literature about whether measuring C1q fixation provides additive data above antigen specific IgG levels alone. This is partially related to falsely low IgG MFI levels associated with the "prozone effect" caused by high levels of complement fixing antibody binding antigen on the bead leading to complement activation and C3 split products which coat the bead leading to reduced binding of secondary IgG detection reagents[383]. This can be reduced by either adding EDTA to prevent complement activation or by serial dilution of the sample. In the data presented in this thesis, serum was diluted 1:100 to measure IgG binding, thus ensuing that no "prozone effect" will have complicated interpretation of the data.

IgG subclass is also likely to be an important determinant of C1q fixation. IgG1 and IgG3 are the dominant complement fixing subclasses[384]. In alloimmunity, the presence of IgG1 and IgG3 subclasses strongly correlates with C1q fixation although negative C1q fixation does not exclude presence of these subclasses[385]. It is therefore likely that C1q fixation represents a readout of the total complement fixing capacity of an antibody mixture incorporating subclass and titre with other factors such as geometric arrangement and antibody glycosylation.

Having established that MPO-ANCA was capable of activating the classical pathway of complement on single antigen beads, it was then investigated whether classical pathway. Even modest complement activation could potentially initiate a positive feedback loop of complement and neutrophil activation, and this could potentially be the source of the initial C5a required for neutrophil priming.

The vast majority of literature on ANCA activation of neutrophils reports experiments in which isolated neutrophils are primed with TNF +/- cytochalasin B and activation measured by ROS production. This priming induces multiple pro-inflammatory changes in neutrophils including externalisation of MPO to the neutrophil membrane. The levels of TNF used to induce neutrophil activation in the literature range from 0.1ng/ml to 10ng/ml[329, 386]. In severe sepsis, mean levels of TNF approximate 40pg/ml[387] so the higher concentrations used to prime neutrophils *in vitro* are likely to provide stimulus in significant excess of that which occurs in humans, even in severe disease states. In the current studies there was no difference in C3 deposition on neutrophils by induced by purified MPO-ANCA or control IgG when neutrophils were primed with TNF. However, increasing the concentration of TNF resulted in increased neutrophil surface C3 suggesting that in this experimental system, the effect of TNF concentration on neutrophil induced complement activation far outweighs an effect (if any) from ANCA.

A different experimental system was therefore investigated to study whether MPO-ANCA binding to the surface of neutrophils triggered complement activation. Externalisation of MPO from granules is likely to be accompanied by properdin and hence complement activation. Neutrophils have been described to acquire surface MPO from their surroundings[372, 374], so this phenomena was explored as an alternative method of allowing surface MPO to be accessible to ANCA. Having confirmed that incubation of neutrophils in MPO resulted in surface acquisition of MPO, MPO exposed neutrophils were then incubated sequentially with ANCA followed by normal serum. MPO-ANCA induced significantly more neutrophil surface C3 deposition than control IgG. The correlation of C1q fixing ability to surface C3 generation observed with the antibody preparations is also suggestive of a C1q mediated pathway. These data, in themselves are not conclusive evidence for classical pathway mediated activation, as Fc receptor mediated changes in the neutrophil that activate the alternative pathway could also be responsible. To try

and minimise this, all steps (with the exception of the serum incubation which occurred at room temperature) were performed at 4°C. This should have minimised metabolic activity of the neutrophils and complement activation mediated by neutrophil degranulation.

If the local generation of ANCA induced C3 breakdown products occurs in-vivo it has a number of potential implications. It is likely to lead to a C5 convertase, thus generating the C5a that has been shown to be important for the full degree of neutrophil activation by ANCA. The C3 breakdown products could also potentially signal into the neutrophil via the complement receptors 1 (CD25) and 3 (CD11b/18) and/or promote heterotypic aggregation. Sublytic complement attack of neutrophils has also been reported to result in the shedding of neutrophil microvesicles[388], which have been suggested to contribute to endothelial damage in AAV[389]. The C3 high population of neutrophils induced by MPO-ANCA is likely to be a result of the alternative pathway positive feedback loop inducing exponential C3 deposition on a susceptible population of neutrophils such as those with high levels of membrane MPO or low levels of complement regulatory proteins. The relatively small population of C3 high neutrophils observed is not inconsistent with clinical data. It is likely that in vivo, only a small proportion of neutrophils are activated by ANCA, as severe disease is not characterised by either neutropenia, or a dense neutrophil infiltrate in the kidney.

C4d, a specific marker of activation of the classical and lectin pathways is widely used as a biomarker of antibody mediated complement activation in human solid organ transplantation. C4d staining was therefore investigated as a more specific marker of classical pathway activation. The intensify of neutrophil C4d staining was significantly less than that of C3 breakdown products and a significant difference was not observed between MPO-ANCA and control IgG groups. Antibody induced C3 cell membrane deposition in the absence of

detectable C4d has previously been described[369] and it is possible that levels of neutrophil C4d were below the lower limit for accurate detection.

To isolate the role of the classical pathway in MPO-ANCA induced C3 deposition, a specific inhibitor, the monoclonal antibody TNT003 was used. This has previously been shown to reduce C3 deposition on antibody targeted erythrocytes[390] and complement deposition on human amniotic epithelial cells incubated with anti-HLA sera[391]. MPO-ANCA sensitised neutrophils were therefore incubated in serum containing TNT003 or isotype controls. Classical pathway inhibition resulted in large reductions in both neutrophil bound C3 and neutrophil activation markers. However, these reductions were present both in MPO-ANCA and control IgG treated neutrophils. This suggests that in this experimental system, neutrophil C3 deposition is driven by a C1s dependent mechanism. Possibilities for this include the presence of immune complexes in the IgG preparations- although significant precipitants were not seen on SDS-PAGE. A second possibility that was considered was that C3 deposition was occurring on apoptotic or necrotic neutrophils. This was excluded by assessing annexin V binding and propidum iodide permeability of neutrophils alongside C3 binding. Early apoptosis as measured by Annexin V binding was only present in around 1% of neutrophils and did not appear to be induced by the experimental protocol. In addition, the C3 high population of neutrophils were not annexin V high, suggesting that this population was not apoptotic.

In summary this work demonstrates that in the majority of patients with active disease, MPO-ANCA is able to activate the complement classical pathway in vitro on a single antigen beads. Purified MPO-ANCA also leads to C3 deposition on purified MPO incubated neutrophils. The precise mechanism of the observed C3 deposition of neutrophils and its pathophysiological relevance remain to be determined.

Further work that would bring clarity to this area includes further analysis of the IgG preparations for the presence of aggregates that could activate the classical pathway as this is the most likely source of the complement activation. It would also be useful to analyse neutrophil surface complement regulatory proteins such as CD55 and CD46 to determine whether the C3 deposition occurred in a population of neutrophils that were low for these markers. Co-measurement of C3 deposition and markers of neutrophil activation such as ROS could also determine whether the C3 deposition correlated with ANCA induced pathological changes in neutrophils.

When considering the possibility of anti-classical pathway therapy in humans, there is a theoretical concern regarding the strong predisposition to a lupus like phenotype in human with classical pathway mutations. This is due to the important role of complement in the clearance of apoptotic cells. In an attempt to address these concerns, it has been demonstrated that C1s inhibition does not significantly reduce C1q mediated phagocytosis of early apoptotic cells by macrophages[392]. Early apoptotic cells are thought to mediate the immunosuppressive effects of apoptosis and therefore, it is possible that C1s inhibition could circumvent this potential complication. However as some of the humans with C1s deficiency reported have had a lupus-like disease[393], targeting of C1s may still carry some risk.

This study set out to investigate whether ANCA induced classical pathway activation played a pathological role in ANCA induced neutrophil activation. Whilst ANCA mediated classical pathway activation occurred *in vitro* on single antigen beads, the multiple and complex interactions of complement, neutrophils and antibody made this a difficult relationship to investigate *in vitro*. Whilst ANCA induced C3 deposition on neutrophils was demonstrated, the profound effect that C1s inhibition had on both ANCA stimulated and control samples suggested that there was a significant source of classical pathway mediated complement

activation in this system and it was therefore not possible to confirm that the classical pathway activated by ANCA was responsible for inducing this deposition.

Chapter 6: Extended discussion and conclusions:

The complement system has previously been shown to be critical to disease pathogenesis in murine models of AAV. In this thesis new roles for complement beyond the priming of neutrophils by C5a for activation by ANCA are described. This chapter includes additional discussion around the role of the C5aR and C3aR in AAV- work that was presented in paper format.

C5aR1 in AAV

In chapter 2 the role that signalling through the C5aR1 plays in the generation of autoimmunity to MPO is reported, alongside experiments that show that this is mediated by the C5aR1 on DCs rather than T cells. The net result of this is that signalling through C5a exacerbates a T cell dependent model of anti-MPO glomerulonephritis. The translational implication of this finding is that C5aR1 inhibition in humans may result in a therapeutically significant reduction in autoimmunity when used as a treatment in AAV.

The effects of genetic absence or inhibition of the C5aR1 in mice immunised with MPO largely mirrored those reported in the literature. Of note the increase in Th17 generation reported in-vitro [246] was not recapitulated in vivo. This is reassuring, as the Th17 response is an important driver of autoimmune glomerular injury[125, 394]. In contrast to findings with *C5aR1*$^{-/-}$ mice, C5aR1 inhibition with PMX53 did not induce a measurable difference in Tregs. There are two possibilities for this observation. First, the degree of C5aR1 inhibition achieved by PMX53 administration may have been insufficient to impact Treg differentiation. PMX53 has a lower sensitivity for the murine C5aR1 than other species and the dose administered in the infusion pump was limited by volume and solubility constraints[395]. Whilst this dose of PMX53 has previously been reported to be effective in murine models of immune complex nephritis[320] the inhibition achieved at the immunological synapse between APCs and T cells may not have been complete. Secondly, PMX53 administration may have affected peripheral Treg generation, but measuring the proportion of T cells that were $CD25^{+}Foxp3^{+}$

was too crude a measure to detect this. If I were to revisit this I would consider alternative methods such detection of antigen specific Tregs in an antigen restimulated proliferation assay with measurement of Foxp3^{+} T cell proliferation by cell tracking dye[131].

The finding that surface activation markers on BMDCs are reduced in *C5aR1*$^{-/-}$ DCs has previously been reported[217, 244, 249], although the magnitude of difference between groups was not as substantial as observed by other investigators. This may be because the concentration of LPS used to mature DCs (1μg/ml) was sufficient to achieve robust DC activation via TLR4 activation leaving only a modest additive effect of C5a stimulation. In addition, although recombinant C5a was added to cultures, the foetal calf serum used to supplement culture medium will have contained carboxypeptidase B, the enzyme that inactivates C5a. Whilst the carboxypeptidase B enzymatic activity may have been degraded by heat treatment of the serum, it is possible that C5a breakdown meant that concentrations were insufficient for maximal stimulation throughout the incubation period.

The observation that MPO-ANCA titres were significantly reduced in *C5aR1*$^{-/-}$ supports a role for the C5aR1 in generation of humoral immunity previously observed in other models[255, 320]. As B cells do not express C5aR1[220] and transfer of *C5aR1*$^{-/-}$ DCs was associated with reduced MPO-ANCA titres it is likely that this effect is mediated via APC effect on T cells. T follicular helper (TFH) cells participate in formation of the germinal centre reaction and antibody response by B cells[396]. A detailed analysis of the how C5aR1 modulates the TFH response would help to resolve whether C5aR1 affects antibody response via TFH. Another possible avenue for investigation would be measurement of myeloid cell secreted cytokines in response to ANCA that modulate B cell development such as B-cell activating factor of the TNF family (BAFF) and TNF [397].

There are many caveats when extrapolating findings from experimental animal models to clinical practice in humans. In the studies described in this thesis autoimmunity to MPO was induced by

immunisation in Freund's complete adjuvant. This water in oil emulsion contains mycobacterial fragments with potent immune activating properties. The pattern of immune activation and break of self-tolerance is very different from the clinical situation of patients who present with established autoimmunity and active disease. It is conceivable that the propagation of autoimmunity that occurs when APCs present MPO to T cells in the context of active disease might be blunted by C5aR1 inhibition, especially considering that the APC population in the kidney highly expresses C5aR1[318]. Attempts to design an experiment to investigate this were frustrated by limitation of the autoimmune anti-MPO GN model to 4 days to preclude an autologous immune response to the anti-GBM globulin.

Whilst a T cell expressed C5aR in humans has recently been reported to be critical in the induction of Th1 immunity, in this report, the receptor is confined to the intracellular compartment. The currently available data on CCX168 do not state whether it has any action on the intracellular C5aR1[398]. If not, other strategies such as C5 or C5aR knockdown by short interfering RNA would be required to modulate this therapeutic target.

In order to confirm whether these findings in murine models presented in this thesis have translational relevance, analysis of clinical samples from the ongoing trial phase 3 trial of CCX168 in AAV could be performed. Whilst the trial design (CCX168 vs. glucocorticoids for induction of remission) makes drawing conclusions about the immunomodulatory effect of C5aR1 inhibition challenging, analysis of endpoints such as peripheral blood Th1, Th17 and FoxP3+ cells as well other serum markers of T cell activation such as soluble IL-2 receptor would be informative. Similarly, analysis of whether ANCA titres are moderated in patients receiving CCX168 would be interesting, although, since patients also receive induction therapy with rituximab or cyclophosphamide this may confound the determination of CCX168 to influence humoral anti-MPO immunity.

At present the perceived role of C5aR1 inhibition in the treatment of AAV has been confined to

induction therapy. If an immunomodulatory effect of C5aR1 inhibition is confirmed then consideration could be given to extending treatment to a maintenance therapy. The corresponding downside of any additional immunomodulatory effect would be the potential for increased infection, although based on experience with the anti-C5 monoclonal antibody eculizumab, prolonged treatment with a complement inhibitor results in only a modest increase in episodes of infection[158].

In addition to the mechanism investigated in this thesis, other reported biological effects of C5a such as the triggering of neutrophil NETosis[399], the promotion of tubulointerstitial fibrosis[326], the induction of tissue factor release[227] and the modulation of CD8 T cell response could all be the basis of future studies on additional mechanisms by which C5aR1 inhibition may effect clinical outcomes in AAV.

C3aR in AAV

In Chapter 3 the potential for a role for C3a, acting through the C3a receptor in pre-clinical models of AAV was investigated. Whilst previous studies have reported both pro and anti-inflammatory roles for C3a, it is often viewed as less potent inflammatory mediator than C5a. In studies in which mice deficient in the C3aR or C5aR1 have been investigated side-by-side, C5aR1 has usually been reported to have a more potent inflammatory effect[237, 240]. The results reported in this thesis show that deficiency of C3aR did not alter the severity of glomerulonephritis induced by either model of anti-MPO GN. This was surprising given the important roles that neutrophils play in these models and the previous description of the profound effect of C3aR deficiency on neutrophil mobilisation from the bone marrow, neutrophil NETosis and T cell mediated immunity[217, 252, 273, 287]. However, a recent report, to date only published in abstract form suggests that the distribution of the C3aR in the murine immune system characterised by a C3aR tandem-dye tomato reporter mouse is much more limited

than previously thought. In this study, C3aR expression was not detected in neutrophils, T or B cells, or splenic DCs, with expression in the immune system largely confined to macrophages[400].

Whilst the severity of glomerulonephritis was not altered in *C3ar*$^{-/-}$ mice, there were differences in other endpoints, notably the reduction in glomerular macrophages in the *C3ar*$^{-/-}$ mice in the anti-MPO IgG transfer model and the increased humoral immunity in *C3ar-/-* mice. It is possible that both these observations could represent underlying processes that impact on human pathology. The cellular infiltrate in humans with AAV in enriched for macrophages[105] and there is evidence of glomerular complement activation in active human disease. In addition, macrophages are a key promoter of fibrosis, the end result of inflammatory renal disease. The fibrotic tubulointerstitial injury associated with chronic GN is associated with adverse renal survival however, investigation of this stage of disease is not well served by current animal models of ANCA associated glomerulonephritis. C3a signalling through the C3aR has been reported to mediate pro-fibrotic responses in proteinuric and diabetic nephropathy[290, 401, 402], and it is therefore possible that C3aR could have benefits in this domain.

Whilst alterations in cellular immunity were not observed in *C3ar*$^{-/-}$ mice, the observation that signalling through the C3aR inhibited humoral immunity to MPO is consistent with some previous studies. Investigation of the B cell compartment did not reveal any differences between *C3ar*$^{-/-}$ and wild type mice. The reported lack of C3aR expression on B cells suggests that this is likely to be a B cell extrinsic process. There are several soluble factors that could induce the potentiated humoral immunity observed in *C3ar*$^{-/-}$ mice. For example, IL-1β has previously been reported to be increased in *C3ar*$^{-/-}$ mice in inflammatory conditions[286] and promotes humoral immunity to T dependent antigens[403]. A limitation of the existing murine models is that no model exists in which the generation of humoral MPO autoimmunity results in the induction of disease in the same mouse without

significant manipulation e.g. bone marrow transplant. The degree to which the observed increase in MPO-ANCA titres would result in increased pathology therefore remains undetermined.

Other potential roles for complement in AAV.

To date, the role of C3b and its breakdown products has not been investigated in experimental models of AAV. This thesis demonstrates that C3 breakdown products are present on the surface of ANCA stimulated neutrophils. Several groups have reported the presence of C3 breakdown products in the glomeruli of patients with AAV. These molecules have potent immune stimulatory properties. For instance, iC3b on either neutrophils or in the glomeruli could interact with the CR3 receptor on leucocytes. The CR3 plays diverse and context dependent roles in models of inflammation and autoimmunity[404-406]; it is therefore open to speculation whether if such an interaction occurred it would be pro or anti-inflammatory. The signalling of C5a through its second receptor the C5aR2 is another interaction which experimental data suggests may have complex effects, with both pro[189] and anti-inflammatory[407] actions reported.

Summary:

This thesis generates several original observations that are relevant to the role of complement in AAV and may inform current or future therapeutic strategies.

1) Signalling through the C5aR1 is important in generating MPO cellular and humoral autoimmunity and subsequent glomerulonephritis. This adds further evidence to support C5aR1 targeting in AAV.
2) Whilst signalling through C3aR did not appear to play a significant role in the generation of glomerular injury in murine models of anti-MPO glomerulonephritis it may play a minor role in both macrophage infiltration and the suppression of ANCA production.

3) That complement generated by the alternative pathway does not contribute to either cellular anti-MPO autoimmunity, or T cell mediated glomerular injury in a murine model of autoimmune anti-MPO glomerulonephritis.
4) The majority of ANCA from patients with active anti-MPO disease are able to fix C1q, suggesting that they are capable of activating the classical pathway. In addition, purified MPO-ANCA induces greater deposition of C3 breakdown products on MPO incubated human neutrophils than control IgG. Attempts to determine the contribution of ANCA activated classical pathway to this phenomenon were confounded by significant activation of the classical pathway in control IgG treated neutrophils.

References

1. Jennette JC, Falk RJ, Bacon PA*, et al.* 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum* 2013; **65:** 1-11.

2. Watts RA, Scott DG, Jayne DR*, et al.* Renal vasculitis in Japan and the UK--are there differences in epidemiology and clinical phenotype? *Nephrol Dial Transplant* 2008; **23:** 3928-3931.

3. Fujimoto S, Watts RA, Kobayashi S*, et al.* Comparison of the epidemiology of anti-neutrophil cytoplasmic antibody-associated vasculitis between Japan and the U.K. *Rheumatology (Oxford)* 2011; **50:** 1916-1920.

4. Xin G, Zhao MH, Wang HY. Detection rate and antigenic specificities of antineutrophil cytoplasmic antibodies in chinese patients with clinically suspected vasculitis. *Clin Diagn Lab Immunol* 2004; **11:** 559-562.

5. Flossmann O, Berden A, de Groot K*, et al.* Long-term patient survival in ANCA-associated vasculitis. *Ann Rheum Dis* 2011; **70:** 488-494.

6. Robson J, Doll H, Suppiah R*, et al.* Damage in the anca-associated vasculitides: long-term data from the European vasculitis study group (EUVAS) therapeutic trials. *Ann Rheum Dis* 2015; **74:** 177-184.

7. Lyons PA, Rayner TF, Trivedi S*, et al.* Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med* 2012; **367:** 214-223.

8. Harper L, Morgan MD, Walsh M*, et al.* Pulse versus daily oral cyclophosphamide for induction of remission in ANCA-associated vasculitis: long-term follow-up. *Ann Rheum Dis* 2012; **71:** 955-960.

9. Fauci AS, Haynes BF, Katz P*, et al.* Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years. *Ann Intern Med* 1983; **98:** 76-85.

10. De Groot K, Rasmussen N, Bacon PA*, et al.* Randomized trial of cyclophosphamide versus methotrexate for induction of remission in early systemic antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum* 2005; **52:** 2461-2469.

11. Faurschou M, Westman K, Rasmussen N*, et al.* Brief Report: long-term outcome of a randomized clinical trial comparing methotrexate to cyclophosphamide for remission induction in early systemic antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum* 2012; **64:** 3472-3477.

12. Jayne DR, Gaskin G, Rasmussen N*, et al.* Randomized trial of plasma exchange or high-dosage methylprednisolone as adjunctive therapy for severe renal vasculitis. *J Am Soc Nephrol* 2007; **18:** 2180-2188.

13. Walsh M, Casian A, Flossmann O*, et al.* Long-term follow-up of patients with severe ANCA-associated vasculitis comparing plasma exchange to intravenous methylprednisolone treatment is unclear. *Kidney Int* 2013; **84:** 397-402.

14. Stone JH, Merkel PA, Spiera R*, et al.* Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N Engl J Med* 2010; **363:** 221-232.

15. Jones RB, Tervaert JW, Hauser T*, et al.* Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N Engl J Med* 2010; **363:** 211-220.

16. Jones RB, Furuta S, Tervaert JW*, et al.* Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis: 2-year results of a randomised trial. *Ann Rheum Dis* 2015; **74:** 1178-1182.

17. Stegeman CA, Tervaert JW, de Jong PE*, et al.* Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med* 1996; **335:** 16-20.

18. Jayne D, Rasmussen N, Andrassy K*, et al.* A randomized trial of maintenance therapy for vasculitis associated with antineutrophil cytoplasmic autoantibodies. *N Engl J Med* 2003; **349:** 36-44.

19. Wegener's Granulomatosis Etanercept Trial Research G. Etanercept plus standard therapy for Wegener's granulomatosis. *N Engl J Med* 2005; **352:** 351-361.

20. Metzler C, Miehle N, Manger K*, et al.* Elevated relapse rate under oral methotrexate versus leflunomide for maintenance of remission in Wegener's granulomatosis. *Rheumatology (Oxford)* 2007; **46:** 1087-1091.

21. Pagnoux C, Mahr A, Hamidou MA*, et al.* Azathioprine or methotrexate maintenance for ANCA-associated vasculitis. *N Engl J Med* 2008; **359:** 2790-2803.

22. Guillevin L, Pagnoux C, Karras A*, et al.* Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N Engl J Med* 2014; **371:** 1771-1780.

23. Little MA, Nightingale P, Verburgh CA*, et al.* Early mortality in systemic vasculitis: relative contribution of adverse events and active vasculitis. *Ann Rheum Dis* 2010; **69:** 1036-1043.

24. Schultz J, Kaminker K. Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. *Arch Biochem Biophys* 1962; **96:** 465-467.

25. Odobasic D, Kitching AR, Holdsworth SR. Neutrophil-Mediated Regulation of Innate and Adaptive Immunity: The Role of Myeloperoxidase. *J Immunol Res* 2016; **2016:** 2349817.

26. Lanza F. Clinical manifestation of myeloperoxidase deficiency. *J Mol Med (Berl)* 1998; **76:** 676-681.

27. Odobasic D, Kitching AR, Yang Y*, et al.* Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. *Blood* 2013; **121:** 4195-4204.

28. Halbwachs-Mecarelli L, Bessou G, Lesavre P*, et al.* Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. *FEBS Lett* 1995; **374:** 29-33.

29. Csernok E, Ernst M, Schmitt W*, et al.* Activated neutrophils express proteinase 3 on their plasma membrane in vitro and in vivo. *Clin Exp Immunol* 1994; **95:** 244-250.

30. Gabay JE, Scott RW, Campanelli D*, et al.* Antibiotic proteins of human polymorphonuclear leukocytes. *Proc Natl Acad Sci U S A* 1989; **86:** 5610-5614.

31. Coeshott C, Ohnemus C, Pilyavskaya A*, et al.* Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 1999; **96:** 6261-6266.

32. Roth AJ, Ooi JD, Hess JJ*, et al.* Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis. *J Clin Invest* 2013; **123:** 1773-1783.

33. Kain R, Matsui K, Exner M*, et al.* A novel class of autoantigens of anti-neutrophil cytoplasmic antibodies in necrotizing and crescentic glomerulonephritis: the lysosomal membrane glycoprotein h-lamp-2 in neutrophil granulocytes and a related membrane protein in glomerular endothelial cells. *J Exp Med* 1995; **181:** 585-597.

34. Tan DS, Gan PY, O'Sullivan KM*, et al.* Thymic deletion and regulatory T cells prevent antimyeloperoxidase GN. *J Am Soc Nephrol* 2013; **24:** 573-585.

35. Hogan SL, Cooper GS, Savitz DA*, et al.* Association of silica exposure with anti-neutrophil cytoplasmic autoantibody small-vessel vasculitis: a population-based, case-control study. *Clin J Am Soc Nephrol* 2007; **2:** 290-299.

36. Stegeman CA, Tervaert JW, Sluiter WJ*, et al.* Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. *Ann Intern Med* 1994; **120:** 12-17.

37. Pendergraft WF, 3rd, Preston GA, Shah RR*, et al.* Autoimmunity is triggered by cPR-3(105-201), a protein complementary to human autoantigen proteinase-3. *Nat Med* 2004; **10:** 72-79.

38. Tadema H, Kallenberg CG, Stegeman CA*, et al.* Reactivity against complementary proteinase-3 is not increased in patients with PR3-ANCA-associated vasculitis. *PLoS One* 2011; **6:** e17972.

39. Kain R, Exner M, Brandes R*, et al.* Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis. *Nat Med* 2008; **14:** 1088-1096.

40. Roth AJ, Brown MC, Smith RN*, et al.* Anti-LAMP-2 antibodies are not prevalent in patients with antineutrophil cytoplasmic autoantibody glomerulonephritis. *J Am Soc Nephrol* 2012; **23:** 545-555.

41. Kessenbrock K, Krumbholz M, Schonermarck U*, et al.* Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009; **15:** 623-625.

42. Lande R, Gregorio J, Facchinetti V*, et al.* Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 2007; **449:** 564-569.

43. Sangaletti S, Tripodo C, Chiodoni C*, et al.* Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood* 2012; **120:** 3007-3018.

44. Todd SK, Pepper RJ, Draibe J*, et al.* Regulatory B cells are numerically but not functionally deficient in anti-neutrophil cytoplasm antibody-associated vasculitis. *Rheumatology (Oxford)* 2014; **53:** 1693-1703.

45. Aybar LT, McGregor JG, Hogan SL*, et al.* Reduced CD5(+) CD24(hi) CD38(hi) and interleukin-10(+) regulatory B cells in active anti-neutrophil cytoplasmic autoantibody-associated vasculitis permit increased circulating autoantibodies. *Clin Exp Immunol* 2015; **180:** 178-188.

46. Lepse N, Abdulahad WH, Rutgers A*, et al.* Altered B cell balance, but unaffected B cell capacity to limit monocyte activation in anti-neutrophil cytoplasmic antibody-associated vasculitis in remission. *Rheumatology (Oxford)* 2014; **53:** 1683-1692.

47. Reiner AP, Hartiala J, Zeller T*, et al.* Genome-wide and gene-centric analyses of circulating myeloperoxidase levels in the charge and care consortia. *Hum Mol Genet* 2013; **22:** 3381-3393.

48. Bunch DO, Mendoza CE, Aybar LT*, et al.* Gleaning relapse risk from B cell phenotype: decreased CD5+ B cells portend a shorter time to relapse after B cell depletion in patients with ANCA-associated vasculitis. *Ann Rheum Dis* 2015; **74:** 1784-1786.

49. Davies DJ, Moran JE, Niall JF*, et al.* Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology? *Br Med J (Clin Res Ed)* 1982; **285:** 606.

50. Falk RJ, Terrell RS, Charles LA*, et al.* Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci U S A* 1990; **87:** 4115-4119.

51. Brachemi S, Mambole A, Fakhouri F*, et al.* Increased membrane expression of proteinase 3 during neutrophil adhesion in the presence of anti proteinase 3 antibodies. *J Am Soc Nephrol* 2007; **18:** 2330-2339.

52. Duranton J, Bieth JG. Inhibition of proteinase 3 by [alpha]1-antitrypsin in vitro predicts very fast inhibition in vivo. *Am J Respir Cell Mol Biol* 2003; **29:** 57-61.

53. Porges AJ, Redecha PB, Kimberly WT*, et al.* Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc gamma RIIa. *J Immunol* 1994; **153:** 1271-1280.

54. Yang JJ, Tuttle RH, Hogan SL*, et al.* Target antigens for anti-neutrophil cytoplasmic autoantibodies (ANCA) are on the surface of primed and apoptotic but not unstimulated neutrophils. *Clin Exp Immunol* 2000; **121:** 165-172.

55. Kettritz R, Scheumann J, Xu Y*, et al.* TNF-alpha--accelerated apoptosis abrogates ANCA-mediated neutrophil respiratory burst by a caspase-dependent mechanism. *Kidney Int* 2002; **61:** 502-515.

56. Schreiber A, Xiao H, Jennette JC*, et al.* C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis. *J Am Soc Nephrol* 2009; **20:** 289-298.

57. Hewins P, Morgan MD, Holden N*, et al.* IL-18 is upregulated in the kidney and primes neutrophil responsiveness in ANCA-associated vasculitis. *Kidney Int* 2006; **69:** 605-615.

58. Keogan MT, Esnault VL, Green AJ*, et al.* Activation of normal neutrophils by anti-neutrophil cytoplasm antibodies. *Clin Exp Immunol* 1992; **90:** 228-234.

59. Williams JM, Ben-Smith A, Hewins P*, et al.* Activation of the G(i) heterotrimeric G protein by ANCA IgG F(ab')2 fragments is necessary but not sufficient to stimulate the recruitment of those downstream mediators used by intact ANCA IgG. *J Am Soc Nephrol* 2003; **14:** 661-669.

60. Mulder AH, Heeringa P, Brouwer E*, et al.* Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a Fc gamma RII-dependent process. *Clin Exp Immunol* 1994; **98:** 270-278.

61. Jerke U, Rolle S, Dittmar G*, et al.* Complement receptor Mac-1 is an adaptor for NB1 (CD177)-mediated PR3-ANCA neutrophil activation. *J Biol Chem* 2011; **286:** 7070-7081.

62. Kettritz R. How anti-neutrophil cytoplasmic autoantibodies activate neutrophils. *Clin Exp Immunol* 2012; **169:** 220-228.

63. Farias MG, de Lucena NP, Dal Bo S*, et al.* Neutrophil CD64 expression as an important diagnostic marker of infection and sepsis in hospital patients. *J Immunol Methods* 2014; **414:** 65-68.

64. Muller Kobold AC, Mesander G, Stegeman CA*, et al.* Are circulating neutrophils intravascularly activated in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides? *Clin Exp Immunol* 1998; **114:** 491-499.

65. Hewins P, Williams JM, Wakelam MJ*, et al.* Activation of Syk in neutrophils by antineutrophil cytoplasm antibodies occurs via Fcgamma receptors and CD18. *J Am Soc Nephrol* 2004; **15:** 796-808.

66. Veri MC, Gorlatov S, Li H*, et al.* Monoclonal antibodies capable of discriminating the human inhibitory Fcgamma-receptor IIB (CD32B) from the activating Fcgamma-receptor IIA (CD32A): biochemical, biological and functional characterization. *Immunology* 2007; **121:** 392-404.

67. Kettritz R, Jennette JC, Falk RJ. Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. *J Am Soc Nephrol* 1997; **8:** 386-394.

68. van Mirre E, Breunis WB, Geissler J*, et al.* Neutrophil responsiveness to IgG, as determined by fixed ratios of mRNA levels for activating and inhibitory FcgammaRII (CD32), is stable over time and unaffected by cytokines. *Blood* 2006; **108:** 584-590.

69. Ooi JD, Gan PY, Chen T*, et al.* FcgammaRIIB regulates T-cell autoreactivity, ANCA production, and neutrophil activation to suppress anti-myeloperoxidase glomerulonephritis. *Kidney Int* 2014; **86:** 1140-1149.

70. van der Heijden J, Breunis WB, Geissler J*, et al.* Phenotypic variation in IgG receptors by nonclassical FCGR2C alleles. *J Immunol* 2012; **188:** 1318-1324.

71. Li M, Wirthmueller U, Ravetch JV. Reconstitution of human Fc gamma RIII cell type specificity in transgenic mice. *J Exp Med* 1996; **183:** 1259-1263.

72. Kocher M, Edberg JC, Fleit HB*, et al.* Antineutrophil cytoplasmic antibodies preferentially engage Fc gammaRIIIb on human neutrophils. *J Immunol* 1998; **161:** 6909-6914.

73. Wehrli M, Cortinas-Elizondo F, Hlushchuk R*, et al.* Human IgA Fc receptor FcalphaRI (CD89) triggers different forms of neutrophil death depending on the inflammatory microenvironment. *J Immunol* 2014; **193:** 5649-5659.

74. Wu J, Ji C, Xie F*, et al.* FcalphaRI (CD89) alleles determine the proinflammatory potential of serum IgA. *J Immunol* 2007; **178:** 3973-3982.

75. Kelley JM, Monach PA, Ji C*, et al.* IgA and IgG antineutrophil cytoplasmic antibody engagement of Fc receptor genetic variants influences granulomatosis with polyangiitis. *Proc Natl Acad Sci U S A* 2011; **108:** 20736-20741.

76. Vidarsson G, Stemerding AM, Stapleton NM*, et al.* FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis. *Blood* 2006; **108:** 3573-3579.

77. Wang Y, Tian Z, Thirumalai D*, et al.* Neonatal Fc receptor (FcRn): a novel target for therapeutic antibodies and antibody engineering. *J Drug Target* 2014; **22:** 269-278.

78. Ben-Smith A, Dove SK, Martin A*, et al.* Antineutrophil cytoplasm autoantibodies from patients with systemic vasculitis activate neutrophils through distinct signaling cascades: comparison with conventional Fcgamma receptor ligation. *Blood* 2001; **98:** 1448-1455.

79. Tse WY, Nash GB, Hewins P*, et al.* ANCA-induced neutrophil F-actin polymerization: implications for microvascular inflammation. *Kidney Int* 2005; **67:** 130-139.

80. Brooks CJ, King WJ, Radford DJ*, et al.* IL-1 beta production by human polymorphonuclear leucocytes stimulated by anti-neutrophil cytoplasmic autoantibodies: relevance to systemic vasculitis. *Clin Exp Immunol* 1996; **106:** 273-279.

81. Yang JJ, Preston GA, Alcorta DA*, et al.* Expression profile of leukocyte genes activated by anti-neutrophil cytoplasmic autoantibodies (ANCA). *Kidney Int* 2002; **62:** 1638-1649.

82. Calderwood JW, Williams JM, Morgan MD*, et al.* ANCA induces beta2 integrin and CXC chemokine-dependent neutrophil-endothelial cell interactions that mimic those of highly cytokine-activated endothelium. *J Leukoc Biol* 2005; **77:** 33-43.

83. Radford DJ, Savage CO, Nash GB. Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion. *Arthritis Rheum* 2000; **43:** 1337-1345.

84. Kuligowski MP, Kwan RY, Lo C*, et al.* Antimyeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. *Blood* 2009; **113:** 6485-6494.

85. Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. *Kidney Int* 1992; **41:** 375-383.

86. Kemna MJ, Damoiseaux J, Austen J*, et al.* ANCA as a predictor of relapse: useful in patients with renal involvement but not in patients with nonrenal disease. *J Am Soc Nephrol* 2015; **26:** 537-542.

87. Fussner LA, Hummel AM, Schroeder DR*, et al.* Factors Determining the Clinical Utility of Serial Measurements of Antineutrophil Cytoplasmic Antibodies Targeting Proteinase 3. *Arthritis Rheumatol* 2016; **68:** 1700-1710.

88. Tomasson G, Grayson PC, Mahr AD*, et al.* Value of ANCA measurements during remission to predict a relapse of ANCA-associated vasculitis--a meta-analysis. *Rheumatology (Oxford)* 2012; **51:** 100-109.

89. Finkielman JD, Merkel PA, Schroeder D*, et al.* Antiproteinase 3 antineutrophil cytoplasmic antibodies and disease activity in Wegener granulomatosis. *Ann Intern Med* 2007; **147:** 611-619.

90. Quast I, Keller CW, Maurer MA*, et al.* Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. *J Clin Invest* 2015; **125:** 4160-4170.

91. Dalziel M, Crispin M, Scanlan CN*, et al.* Emerging principles for the therapeutic exploitation of glycosylation. *Science* 2014; **343:** 1235681.

92. Espy C, Morelle W, Kavian N*, et al.* Sialylation levels of anti-proteinase 3 antibodies are associated with the activity of granulomatosis with polyangiitis (Wegener's). *Arthritis Rheum* 2011; **63:** 2105-2115.

93. Pankhurst T, Nash G, Williams J*, et al.* Immunoglobulin subclass determines ability of immunoglobulin (Ig)G to capture and activate neutrophils presented as normal human IgG or disease-associated anti-neutrophil cytoplasm antibody (ANCA)-IgG. *Clin Exp Immunol* 2011; **164:** 218-226.

94. Mulder AH, Stegeman CA, Kallenberg CG. Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis: a predominant role for the IgG3 subclass of ANCA. *Clin Exp Immunol* 1995; **101:** 227-232.

95. Boomsma MM, Stegeman CA, van der Leij MJ*, et al.* Prediction of relapses in Wegener's granulomatosis by measurement of antineutrophil cytoplasmic antibody levels: a prospective study. *Arthritis Rheum* 2000; **43:** 2025-2033.

96. Nowack R, Grab I, Flores-Suarez LF*, et al.* ANCA titres, even of IgG subclasses, and soluble CD14 fail to predict relapses in patients with ANCA-associated vasculitis. *Nephrol Dial Transplant* 2001; **16:** 1631-1637.

97. Jones BE, Yang J, Muthigi A*, et al.* Gene-Specific DNA Methylation Changes Predict Remission in Patients with ANCA-Associated Vasculitis. *J Am Soc Nephrol* 2016.

98. Berden AE, Nolan SL, Morris HL*, et al.* Anti-plasminogen antibodies compromise fibrinolysis and associate with renal histology in ANCA-associated vasculitis. *J Am Soc Nephrol* 2010; **21:** 2169-2179.

99. Kain R, Tadema H, McKinney EF*, et al.* High prevalence of autoantibodies to hLAMP-2 in anti-neutrophil cytoplasmic antibody-associated vasculitis. *J Am Soc Nephrol* 2012; **23:** 556-566.

100. Suzuki K, Nagao T, Itabashi M*, et al.* A novel autoantibody against moesin in the serum of patients with MPO-ANCA-associated vasculitis. *Nephrol Dial Transplant* 2014; **29:** 1168-1177.

101. Free ME, Bunch DO, McGregor JA*, et al.* Patients with antineutrophil cytoplasmic antibody-associated vasculitis have defective Treg cell function exacerbated by the presence of a suppression-resistant effector cell population. *Arthritis Rheum* 2013; **65:** 1922-1933.

102. Ludviksson BR, Sneller MC, Chua KS*, et al.* Active Wegener's granulomatosis is associated with HLA-DR+ CD4+ T cells exhibiting an unbalanced Th1-type T cell cytokine pattern: reversal with IL-10. *J Immunol* 1998; **160:** 3602-3609.

103. Soehnlein O, Lindbom L, Weber C. Mechanisms underlying neutrophil-mediated monocyte recruitment. *Blood* 2009; **114:** 4613-4623.

104. Ooi JD, Chang J, Hickey MJ*, et al.* The immunodominant myeloperoxidase T-cell epitope induces local cell-mediated injury in antimyeloperoxidase glomerulonephritis. *Proc Natl Acad Sci U S A* 2012; **109:** E2615-2624.

105. Cunningham MA, Huang XR, Dowling JP*, et al.* Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol* 1999; **10:** 499-506.

106. Zhao L, David MZ, Hyjek E*, et al.* M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN. *Clin J Am Soc Nephrol* 2015; **10:** 54-62.

107. Muller Kobold AC, Kallenberg CG, Tervaert JW. Monocyte activation in patients with Wegener's granulomatosis. *Ann Rheum Dis* 1999; **58:** 237-245.

108. O'Reilly VP, Wong L, Kennedy C*, et al.* Urinary Soluble CD163 in Active Renal Vasculitis. *J Am Soc Nephrol* 2016; **27:** 2906-2916.

109. Weidner S, Neupert W, Goppelt-Struebe M*, et al.* Antineutrophil cytoplasmic antibodies induce human monocytes to produce oxygen radicals in vitro. *Arthritis Rheum* 2001; **44:** 1698-1706.

110. Hattar K, Bickenbach A, Csernok E*, et al.* Wegener's granulomatosis: antiproteinase 3 antibodies induce monocyte cytokine and prostanoid release-role of autocrine cell activation. *J Leukoc Biol* 2002; **71:** 996-1004.

111. Casselman BL, Kilgore KS, Miller BF*, et al.* Antibodies to neutrophil cytoplasmic antigens induce monocyte chemoattractant protein-1 secretion from human monocytes. *J Lab Clin Med* 1995; **126:** 495-502.

112. O'Brien EC, Abdulahad WH, Rutgers A*, et al.* Intermediate monocytes in ANCA vasculitis: increased surface expression of ANCA autoantigens and IL-1beta secretion in response to anti-MPO antibodies. *Sci Rep* 2015; **5:** 11888.

113. Duffield JS, Tipping PG, Kipari T*, et al.* Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. *Am J Pathol* 2005; **167:** 1207-1219.

114. Schmitt WH, Heesen C, Csernok E*, et al.* Elevated serum levels of soluble interleukin-2 receptor in patients with Wegener's granulomatosis. Association with disease activity. *Arthritis Rheum* 1992; **35:** 1088-1096.

115. Winek J, Mueller A, Csernok E*, et al.* Frequency of proteinase 3 (PR3)-specific autoreactive T cells determined by cytokine flow cytometry in Wegener's granulomatosis. *J Autoimmun* 2004; **22:** 79-85.

116. Griffith ME, Coulthart A, Pusey CD. T cell responses to myeloperoxidase (MPO) and proteinase 3 (PR3) in patients with systemic vasculitis. *Clin Exp Immunol* 1996; **103:** 253-258.

117. Ballieux BE, van der Burg SH, Hagen EC*, et al.* Cell-mediated autoimmunity in patients with Wegener's granulomatosis (WG). *Clin Exp Immunol* 1995; **100:** 186-193.

118. van der Geld YM, Huitema MG, Franssen CF*, et al.* In vitro T lymphocyte responses to proteinase 3 (PR3) and linear peptides of PR3 in patients with Wegener's granulomatosis (WG). *Clin Exp Immunol* 2000; **122:** 504-513.

119. O'Sullivan KM, Lo CY, Summers SA*, et al.* Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. *Kidney Int* 2015; **88:** 1030-1046.

120. Ruth AJ, Kitching AR, Kwan RY*, et al.* Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. *J Am Soc Nephrol* 2006; **17:** 1940-1949.

121. Summers SA, Steinmetz OM, Li M*, et al.* Th1 and Th17 cells induce proliferative glomerulonephritis. *J Am Soc Nephrol* 2009; **20:** 2518-2524.

122. Abdulahad WH, Stegeman CA, Limburg PC*, et al.* Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission. *Arthritis Rheum* 2008; **58:** 2196-2205.

123. Jovanovic DV, Di Battista JA, Martel-Pelletier J*, et al.* IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 1998; **160:** 3513-3521.

124. Hsu HC, Yang P, Wang J*, et al.* Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat Immunol* 2008; **9:** 166-175.

125. Gan PY, Steinmetz OM, Tan DS*, et al.* Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol* 2010; **21:** 925-931.

126. Long SA, Buckner JH. CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game. *J Immunol* 2011; **187:** 2061-2066.

127. Ooi JD, Snelgrove SL, Engel DR*, et al.* Endogenous foxp3(+) T-regulatory cells suppress anti-glomerular basement membrane nephritis. *Kidney Int* 2011; **79:** 977-986.

128. Gravano DM, Hoyer KK. Promotion and prevention of autoimmune disease by CD8+ T cells. *J Autoimmun* 2013; **45:** 68-79.

129. McKinney EF, Lyons PA, Carr EJ*, et al.* A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med* 2010; **16:** 586-591, 581p following 591.

130. Chang J, Eggenhuizen P, O'Sullivan KM*, et al.* CD8+ T Cells Effect Glomerular Injury in Experimental Anti-Myeloperoxidase GN. *J Am Soc Nephrol* 2016.

131. Gan PY, O'Sullivan KM, Ooi JD*, et al.* Mast Cell Stabilization Ameliorates Autoimmune Anti-Myeloperoxidase Glomerulonephritis. *J Am Soc Nephrol* 2016; **27:** 1321-1333.

132. Walker ME, Hatfield JK, Brown MA. New insights into the role of mast cells in autoimmunity: evidence for a common mechanism of action? *Biochim Biophys Acta* 2012; **1822:** 57-65.

133. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat Rev Immunol* 2008; **8:** 478-486.

134. Gan PY, Summers SA, Ooi JD*, et al.* Mast cells contribute to peripheral tolerance and attenuate autoimmune vasculitis. *J Am Soc Nephrol* 2012; **23:** 1955-1966.

135. Pfister H, Ollert M, Frohlich LF*, et al.* Antineutrophil cytoplasmic autoantibodies against the murine homolog of proteinase 3 (Wegener autoantigen) are pathogenic in vivo. *Blood* 2004; **104:** 1411-1418.

136. Schreiber A, Eulenberg-Gustavus C, Bergmann A*, et al.* Lessons from a double-transgenic neutrophil approach to induce antiproteinase 3 antibody-mediated vasculitis in mice. *J Leukoc Biol* 2016; **100:** 1443-1452.

137. Xiao H, Heeringa P, Hu P*, et al.* Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002; **110:** 955-963.

138. Huugen D, Xiao H, van Esch A*, et al.* Aggravation of anti-myeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide: role of tumor necrosis factor-alpha. *Am J Pathol* 2005; **167:** 47-58.

139. Freeley SJ, Coughlan AM, Popat RJ*, et al.* Granulocyte colony stimulating factor exacerbates antineutrophil cytoplasmic antibody vasculitis. *Ann Rheum Dis* 2013; **72:** 1053-1058.

140. Schreiber A, Xiao H, Falk RJ*, et al.* Bone marrow-derived cells are sufficient and necessary targets to mediate glomerulonephritis and vasculitis induced by anti-myeloperoxidase antibodies. *J Am Soc Nephrol* 2006; **17:** 3355-3364.

141. Little MA, Smyth L, Salama AD*, et al.* Experimental autoimmune vasculitis: an animal model of anti-neutrophil cytoplasmic autoantibody-associated systemic vasculitis. *Am J Pathol* 2009; **174:** 1212-1220.

142. Zhou W, Marsh JE, Sacks SH. Intrarenal synthesis of complement. *Kidney Int* 2001; **59:** 1227-1235.

143. Noris M, Remuzzi G. Overview of complement activation and regulation. *Semin Nephrol* 2013; **33:** 479-492.

144. de Cordoba SR, de Jorge EG. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clin Exp Immunol* 2008; **151:** 1-13.

145. Barbour TD, Ruseva MM, Pickering MC. Update on C3 glomerulopathy. *Nephrol Dial Transplant* 2016; **31:** 717-725.

146. Rawal N, Pangburn M. Formation of high-affinity C5 convertases of the alternative pathway of complement. *J Immunol* 2001; **166:** 2635-2642.

147. Harris CL, Heurich M, Rodriguez de Cordoba S*, et al.* The complotype: dictating risk for inflammation and infection. *Trends Immunol* 2012; **33:** 513-521.

148. Barbour T, Johnson S, Cohney S*, et al.* Thrombotic microangiopathy and associated renal disorders. *Nephrol Dial Transplant* 2012; **27:** 2673-2685.

149. Fremeaux-Bacchi V, Fakhouri F, Garnier A*, et al.* Genetics and outcome of atypical hemolytic uremic syndrome: a nationwide French series comparing children and adults. *Clin J Am Soc Nephrol* 2013; **8:** 554-562.

150. Legendre CM, Licht C, Muus P*, et al.* Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med* 2013; **368:** 2169-2181.

151. Pickering MC, D'Agati VD, Nester CM*, et al.* C3 glomerulopathy: consensus report. *Kidney Int* 2013; **84:** 1079-1089.

152. Cook HT, Pickering MC. Histopathology of MPGN and C3 glomerulopathies. *Nat Rev Nephrol* 2015; **11:** 14-22.

153. Zhang Y, Meyer NC, Wang K*, et al.* Causes of alternative pathway dysregulation in dense deposit disease. *Clin J Am Soc Nephrol* 2012; **7:** 265-274.

154. Sethi S, Fervenza FC, Zhang Y*, et al.* C3 glomerulonephritis: clinicopathological findings, complement abnormalities, glomerular proteomic profile, treatment, and follow-up. *Kidney Int* 2012; **82:** 465-473.

155. Gale DP, de Jorge EG, Cook HT*, et al.* Identification of a mutation in complement factor H-related protein 5 in patients of Cypriot origin with glomerulonephritis. *Lancet* 2010; **376:** 794-801.

156. Zand L, Kattah A, Fervenza FC*, et al.* C3 glomerulonephritis associated with monoclonal gammopathy: a case series. *Am J Kidney Dis* 2013; **62:** 506-514.

157. Brodsky RA. Paroxysmal nocturnal hemoglobinuria. *Blood* 2014; **124:** 2804-2811.

158. Hillmen P, Muus P, Roth A*, et al.* Long-term safety and efficacy of sustained eculizumab treatment in patients with paroxysmal nocturnal haemoglobinuria. *Br J Haematol* 2013; **162:** 62-73.

159. Sethi S, Quint PS, O'Seaghdha CM*, et al.* C4 Glomerulopathy: A Disease Entity Associated With C4d Deposition. *Am J Kidney Dis* 2016; **67:** 949-953.

160. Sethi S, Sullivan A, Smith RJ. C4 dense-deposit disease. *N Engl J Med* 2014; **370:** 784-786.

161. Ali A, Schlanger L, Nasr SH*, et al.* Proliferative C4 Dense Deposit Disease, Acute Thrombotic Microangiopathy, a Monoclonal Gammopathy, and Acute Kidney Failure. *Am J Kidney Dis* 2016; **67:** 479-482.

162. Maillard N, Wyatt RJ, Julian BA*, et al.* Current Understanding of the Role of Complement in IgA Nephropathy. *J Am Soc Nephrol* 2015; **26:** 1503-1512.

163. Espinosa M, Ortega R, Sanchez M*, et al.* Association of C4d deposition with clinical outcomes in IgA nephropathy. *Clin J Am Soc Nephrol* 2014; **9:** 897-904.

164. Kim SJ, Koo HM, Lim BJ*, et al.* Decreased circulating C3 levels and mesangial C3 deposition predict renal outcome in patients with IgA nephropathy. *PLoS One* 2012; **7:** e40495.

165. Gharavi AG, Kiryluk K, Choi M*, et al.* Genome-wide association study identifies susceptibility loci for IgA nephropathy. *Nat Genet* 2011; **43:** 321-327.

166. Zhu L, Zhai YL, Wang FM*, et al.* Variants in Complement Factor H and Complement Factor H-Related Protein Genes, CFHR3 and CFHR1, Affect Complement Activation in IgA Nephropathy. *J Am Soc Nephrol* 2015; **26:** 1195-1204.

167. Xie J, Kiryluk K, Li Y*, et al.* Fine Mapping Implicates a Deletion of CFHR1 and CFHR3 in Protection from IgA Nephropathy in Han Chinese. *J Am Soc Nephrol* 2016.

168. Rosenblad T, Rebetz J, Johansson M*, et al.* Eculizumab treatment for rescue of renal function in IgA nephropathy. *Pediatr Nephrol* 2014; **29:** 2225-2228.

169. Ring T, Pedersen BB, Salkus G*, et al.* Use of eculizumab in crescentic IgA nephropathy: proof of principle and conundrum? *Clin Kidney J* 2015; **8:** 489-491.

170. Pickering MC, Walport MJ. Links between complement abnormalities and systemic lupus erythematosus. *Rheumatology (Oxford)* 2000; **39:** 133-141.

171. Eggleton P, Ukoumunne OC, Cottrell I*, et al.* Autoantibodies against C1q as a Diagnostic Measure of Lupus Nephritis: Systematic Review and Meta-analysis. *J Clin Cell Immunol* 2014; **5:** 210.

172. Trouw LA, Groeneveld TW, Seelen MA*, et al.* Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes. *J Clin Invest* 2004; **114:** 679-688.

173. Birmingham DJ, Bitter JE, Ndukwe EG*, et al.* Relationship of Circulating Anti-C3b and Anti-C1q IgG to Lupus Nephritis and Its Flare. *Clin J Am Soc Nephrol* 2016; **11:** 47-53.

174. Pickering MC, Ismajli M, Condon MB*, et al.* Eculizumab as rescue therapy in severe resistant lupus nephritis. *Rheumatology (Oxford)* 2015; **54:** 2286-2288.

175. Coppo R, Peruzzi L, Amore A*, et al.* Dramatic effects of eculizumab in a child with diffuse proliferative lupus nephritis resistant to conventional therapy. *Pediatr Nephrol* 2015; **30:** 167-172.

176. Barilla-Labarca ML, Toder K, Furie R. Targeting the complement system in systemic lupus erythematosus and other diseases. *Clin Immunol* 2013; **148:** 313-321.

177. Beck LH, Jr., Bonegio RG, Lambeau G*, et al.* M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *N Engl J Med* 2009; **361:** 11-21.

178. Tomas NM, Beck LH, Jr., Meyer-Schwesinger C*, et al.* Thrombospondin type-1 domain-containing 7A in idiopathic membranous nephropathy. *N Engl J Med* 2014; **371:** 2277-2287.

179. Jennette JC, Iskandar SS, Dalldorf FG. Pathologic differentiation between lupus and nonlupus membranous glomerulopathy. *Kidney Int* 1983; **24:** 377-385.

180. Cybulsky AV, Rennke HG, Feintzeig ID*, et al.* Complement-induced glomerular epithelial cell injury. Role of the membrane attack complex in rat membranous nephropathy. *J Clin Invest* 1986; **77:** 1096-1107.

181. Saran AM, Yuan H, Takeuchi E*, et al.* Complement mediates nephrin redistribution and actin dissociation in experimental membranous nephropathy. *Kidney Int* 2003; **64:** 2072-2078.

182. Schulze M, Donadio JV, Jr., Pruchno CJ*, et al.* Elevated urinary excretion of the C5b-9 complex in membranous nephropathy. *Kidney Int* 1991; **40:** 533-538.

183. Lhotta K, Wurzner R, Konig P. Glomerular deposition of mannose-binding lectin in human glomerulonephritis. *Nephrol Dial Transplant* 1999; **14:** 881-886.

184. Bally S, Debiec H, Ponard D*, et al.* Phospholipase A2 Receptor-Related Membranous Nephropathy and Mannan-Binding Lectin Deficiency. *J Am Soc Nephrol* 2016.

185. Xiao H, Schreiber A, Heeringa P*, et al.* Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol* 2007; **170:** 52-64.

186. Schwaeble WJ, Lynch NJ, Clark JE*, et al.* Targeting of mannan-binding lectin-associated serine protease-2 confers protection from myocardial and gastrointestinal ischemia/reperfusion injury. *Proc Natl Acad Sci U S A* 2011; **108:** 7523-7528.

187. Freeley SJ, Popat RJ, Parmar K*, et al.* Experimentally-induced anti-myeloperoxidase vasculitis does not require properdin, MASP-2 or bone marrow derived C5. *J Pathol* 2016.

188. Huugen D, van Esch A, Xiao H*, et al.* Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int* 2007; **71:** 646-654.

189. Xiao H, Dairaghi DJ, Powers JP*, et al.* C5a receptor (CD88) blockade protects against MPO-ANCA GN. *J Am Soc Nephrol* 2014; **25:** 225-231.

190. Kimura Y, Miwa T, Zhou L*, et al.* Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood* 2008; **111:** 732-740.

191. Gou SJ, Yuan J, Chen M*, et al.* Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. *Kidney Int* 2013; **83:** 129-137.

192. Gou SJ, Yuan J, Wang C*, et al.* Alternative complement pathway activation products in urine and kidneys of patients with ANCA-associated GN. *Clin J Am Soc Nephrol* 2013; **8:** 1884-1891.

193. Manenti L, Vaglio A, Gnappi E*, et al.* Association of Serum C3 Concentration and Histologic Signs of Thrombotic Microangiopathy with Outcomes among Patients with ANCA-Associated Renal Vasculitis. *Clin J Am Soc Nephrol* 2015.

194. Augusto JF, Langs V, Demiselle J*, et al.* Low Serum Complement C3 Levels at Diagnosis of Renal ANCA-Associated Vasculitis Is Associated with Poor Prognosis. *PLoS One* 2016; **11:** e0158871.

195. Villacorta J, Diaz-Crespo F, Acevedo M*, et al.* Circulating C3 levels predict renal and global outcome in patients with renal vasculitis. *Clin Rheumatol* 2016.

196. Chen SF, Wang H, Huang YM*, et al.* Clinicopathologic characteristics and outcomes of renal thrombotic microangiopathy in anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis. *Clin J Am Soc Nephrol* 2015; **10:** 750-758.

197. Xing GQ, Chen M, Liu G*, et al.* Complement activation is involved in renal damage in human antineutrophil cytoplasmic autoantibody associated pauci-immune vasculitis. *J Clin Immunol* 2009; **29:** 282-291.

198. Chen M, Xing GQ, Yu F*, et al.* Complement deposition in renal histopathology of patients with ANCA-associated pauci-immune glomerulonephritis. *Nephrol Dial Transplant* 2009; **24:** 1247-1252.

199. Haas M, Eustace JA. Immune complex deposits in ANCA-associated crescentic glomerulonephritis: a study of 126 cases. *Kidney Int* 2004; **65:** 2145-2152.

200. Hilhorst M, van Paassen P, van Rie H*, et al.* Complement in ANCA-associated glomerulonephritis. *Nephrol Dial Transplant* 2015.

201. Zwirner J, Felber E, Herzog V*, et al.* Classical pathway of complement activation in normal and diseased human glomeruli. *Kidney Int* 1989; **36:** 1069-1077.

202. Regele H, Exner M, Watschinger B*, et al.* Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. *Nephrol Dial Transplant* 2001; **16:** 2058-2066.

203. Persson U, Gullstrand B, Pettersson A*, et al.* A Candidate Gene Approach to ANCA-Associated Vasculitis Reveals Links to the C3 and CTLA-4 Genes but not to the IL1-Ra And Fc gamma-RIIa Genes. *Kidney & Blood Pressure Research* 2013; **37:** 641-648.

204. Patel H, Smith RA, Sacks SH*, et al.* Therapeutic strategy with a membrane-localizing complement regulator to increase the number of usable donor organs after prolonged cold storage. *J Am Soc Nephrol* 2006; **17:** 1102-1111.

205. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002; **8:** 582-587.

206. Hao J, Chen M, Zhao MH. Involvement of protein kinase C in C5a-primed neutrophils for ANCA-mediated activation. *Mol Immunol* 2013; **54:** 68-73.

207. Jayne DR, Bruchfeld AN, Harper L*, et al.* Randomized Trial of C5a Receptor Inhibitor Avacopan in ANCA-Associated Vasculitis. *J Am Soc Nephrol* 2017.

208. Matthews KW, Mueller-Ortiz SL, Wetsel RA. Carboxypeptidase N: a pleiotropic regulator of inflammation. *Mol Immunol* 2004; **40:** 785-793.

209. Klos A, Tenner AJ, Johswich KO*, et al.* The role of the anaphylatoxins in health and disease. *Mol Immunol* 2009; **46:** 2753-2766.

210. Gerard NP, Lu B, Liu P*, et al.* An anti-inflammatory function for the complement anaphylatoxin C5a-binding protein, C5L2. *J Biol Chem* 2005; **280:** 39677-39680.

211. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Mol Immunol* 2011; **48:** 1631-1642.

212. Chenoweth DE, Hugli TE. Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. *Proc Natl Acad Sci U S A* 1978; **75:** 3943-3947.

213. Fureder W, Agis H, Willheim M*, et al.* Differential expression of complement receptors on human basophils and mast cells. Evidence for mast cell heterogeneity and CD88/C5aR expression on skin mast cells. *J Immunol* 1995; **155:** 3152-3160.

214. Zwirner J, Gotze O, Begemann G*, et al.* Evaluation of C3a receptor expression on human leucocytes by the use of novel monoclonal antibodies. *Immunology* 1999; **97:** 166-172.

215. Patzelt J, Mueller KA, Breuning S*, et al.* Expression of anaphylatoxin receptors on platelets in patients with coronary heart disease. *Atherosclerosis* 2015; **238:** 289-295.

216. Morelli A, Larregina A, Chuluyan I*, et al.* Expression and modulation of C5a receptor (CD88) on skin dendritic cells. Chemotactic effect of C5a on skin migratory dendritic cells. *Immunology* 1996; **89:** 126-134.

217. Strainic MG, Liu J, Huang D*, et al.* Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 2008; **28:** 425-435.

218. Lalli PN, Strainic MG, Yang M*, et al.* Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. *Blood* 2008; **112:** 1759-1766.

219. Dunkelberger J, Zhou L, Miwa T*, et al.* C5aR expression in a novel GFP reporter gene knockin mouse: implications for the mechanism of action of C5aR signaling in T cell immunity. *J Immunol* 2012; **188:** 4032-4042.

220. Karsten CM, Laumonnier Y, Eurich B*, et al.* Monitoring and Cell-Specific Deletion of C5aR1 Using a Novel Floxed GFP-C5aR1 Reporter Knock-in Mouse. *J Immunol* 2015; **194:** 1841-1855.

221. van Werkhoven MB, Damman J, Daha MR*, et al.* Novel insights in localization and expression levels of C5aR and C5L2 under native and post-transplant conditions in the kidney. *Mol Immunol* 2013; **53:** 237-245.

222. Gueler F, Rong S, Gwinner W*, et al.* Complement 5a receptor inhibition improves renal allograft survival. *J Am Soc Nephrol* 2008; **19:** 2302-2312.

223. el-Lati SG, Dahinden CA, Church MK. Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells. *J Invest Dermatol* 1994; **102:** 803-806.

224. Ehrengruber MU, Geiser T, Deranleau DA. Activation of human neutrophils by C3a and C5A. Comparison of the effects on shape changes, chemotaxis, secretion, and respiratory burst. *FEBS Lett* 1994; **346:** 181-184.

225. Schindler R, Gelfand JA, Dinarello CA. Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide or IL-1 itself. *Blood* 1990; **76:** 1631-1638.

226. Monsinjon T, Gasque P, Chan P*, et al.* Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. *FASEB J* 2003; **17:** 1003-1014.

227. Ikeda K, Nagasawa K, Horiuchi T*, et al.* C5a induces tissue factor activity on endothelial cells. *Thromb Haemost* 1997; **77:** 394-398.

228. Foreman KE, Vaporciyan AA, Bonish BK*, et al.* C5a-induced expression of P-selectin in endothelial cells. *J Clin Invest* 1994; **94:** 1147-1155.

229. Karsten CM, Kohl J. The immunoglobulin, IgG Fc receptor and complement triangle in autoimmune diseases. *Immunobiology* 2012; **217:** 1067-1079.

230. Shushakova N, Skokowa J, Schulman J*, et al.* C5a anaphylatoxin is a major regulator of activating versus inhibitory FcgammaRs in immune complex-induced lung disease. *J Clin Invest* 2002; **110:** 1823-1830.

231. Tsuboi N, Ernandez T, Li X*, et al.* Regulation of human neutrophil Fcgamma receptor IIa by C5a receptor promotes inflammatory arthritis in mice. *Arthritis Rheum* 2011; **63:** 467-478.

232. Karsten CM, Pandey MK, Figge J*, et al.* Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcgammaRIIB and dectin-1. *Nat Med* 2012; **18:** 1401-1406.

233. Kolev M, Le Friec G, Kemper C. Complement--tapping into new sites and effector systems. *Nat Rev Immunol* 2014; **14:** 811-820.

234. Czermak BJ, Sarma V, Pierson CL*, et al.* Protective effects of C5a blockade in sepsis. *Nat Med* 1999; **5:** 788-792.

235. Hollmann TJ, Mueller-Ortiz SL, Braun MC*, et al.* Disruption of the C5a receptor gene increases resistance to acute Gram-negative bacteremia and endotoxic shock: opposing roles of C3a and C5a. *Mol Immunol* 2008; **45:** 1907-1915.

236. Pan H, Shen Z, Mukhopadhyay P*, et al.* Anaphylatoxin C5a contributes to the pathogenesis of cisplatin-induced nephrotoxicity. *Am J Physiol Renal Physiol* 2009; **296:** F496-504.

237. Peng Q, Li K, Smyth LA*, et al.* C3a and C5a promote renal ischemia-reperfusion injury. *J Am Soc Nephrol* 2012; **23:** 1474-1485.

238. Khameneh HJ, Ho AW, Laudisi F*, et al.* C5a Regulates IL-1beta Production and Leukocyte Recruitment in a Murine Model of Monosodium Urate Crystal-Induced Peritonitis. *Front Pharmacol* 2017; **8:** 10.

239. Lee H, Zahra D, Vogelzang A*, et al.* Human C5aR knock-in mice facilitate the production and assessment of anti-inflammatory monoclonal antibodies. *Nat Biotechnol* 2006; **24:** 1279-1284.

240. Banda NK, Hyatt S, Antonioli AH*, et al.* Role of C3a receptors, C5a receptors, and complement protein C6 deficiency in collagen antibody-induced arthritis in mice. *J Immunol* 2012; **188:** 1469-1478.

241. Andersson C, Wenander CS, Usher PA*, et al.* Rapid-onset clinical and mechanistic effects of anti-C5aR treatment in the mouse collagen-induced arthritis model. *Clin Exp Immunol* 2014; **177:** 219-233.

242. Heimbach L, Li Z, Berkowitz P*, et al.* The C5a receptor on mast cells is critical for the autoimmune skin-blistering disease bullous pemphigoid. *J Biol Chem* 2011; **286:** 15003-15009.

243. Li K, Fazekasova H, Wang N*, et al.* Expression of complement components, receptors and regulators by human dendritic cells. *Mol Immunol* 2011; **48:** 1121-1127.

244. Peng Q, Li K, Wang N*, et al.* Dendritic cell function in allostimulation is modulated by C5aR signaling. *J Immunol* 2009; **183:** 6058-6068.

245. Liu T, Xu G, Guo B*, et al.* An essential role for C5aR signaling in the optimal induction of a malaria-specific CD4+ T cell response by a whole-killed blood-stage vaccine. *J Immunol* 2013; **191:** 178-186.

246. Weaver DJ, Jr., Reis ES, Pandey MK*, et al.* C5a receptor-deficient dendritic cells promote induction of Treg and Th17 cells. *Eur J Immunol* 2010; **40:** 710-721.

247. Schmudde I, Strover HA, Vollbrandt T*, et al.* C5a receptor signalling in dendritic cells controls the development of maladaptive Th2 and Th17 immunity in experimental allergic asthma. *Mucosal Immunol* 2013; **6:** 807-825.

248. Hashimoto M, Hirota K, Yoshitomi H*, et al.* Complement drives Th17 cell differentiation and triggers autoimmune arthritis. *J Exp Med* 2010; **207:** 1135-1143.

249. Li K, Fazekasova H, Wang N*, et al.* Functional modulation of human monocytes derived DCs by anaphylatoxins C3a and C5a. *Immunobiology* 2012; **217:** 65-73.

250. Kwan WH, Hashimoto D, Paz-Artal E*, et al.* Antigen-presenting cell-derived complement modulates graft-versus-host disease. *J Clin Invest* 2012; **122:** 2234-2238.

251. Liu J, Lin F, Strainic MG*, et al.* IFN-gamma and IL-17 production in experimental autoimmune encephalomyelitis depends on local APC-T cell complement production. *J Immunol* 2008; **180:** 5882-5889.

252. Strainic MG, Shevach EM, An FQ*, et al.* Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta 1 signaling and induction of Foxp3(+) regulatory T cells. *Nature Immunology* 2013; **14:** 162-171.

253. van der Touw W, Cravedi P, Kwan WH*, et al.* Cutting edge: Receptors for C3a and C5a modulate stability of alloantigen-reactive induced regulatory T cells. *J Immunol* 2013; **190:** 5921-5925.

254. Arbore G, West EE, Spolski R*, et al.* T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4(+) T cells. *Science* 2016; **352:** aad1210.

255. Wenderfer SE, Ke B, Hollmann TJ*, et al.* C5a receptor deficiency attenuates T cell function and renal disease in MRLlpr mice. *J Am Soc Nephrol* 2005; **16:** 3572-3582.

256. Li Q, Peng Q, Xing G*, et al.* Deficiency of C5aR prolongs renal allograft survival. *J Am Soc Nephrol* 2010; **21:** 1344-1353.

257. Wilken HC, Gotze O, Werfel T*, et al.* C3a(desArg) does not bind to and signal through the human C3a receptor. *Immunol Lett* 1999; **67:** 141-145.

258. Maslowska M, Wang HW, Cianflone K. Novel roles for acylation stimulating protein/C3adesArg: a review of recent in vitro and in vivo evidence. *Vitam Horm* 2005; **70:** 309-332.

259. Ames RS, Li Y, Sarau HM*, et al.* Molecular cloning and characterization of the human anaphylatoxin C3a receptor. *J Biol Chem* 1996; **271:** 20231-20234.

260. Crass T, Ames RS, Sarau HM*, et al.* Chimeric receptors of the human C3a receptor and C5a receptor (CD88). *J Biol Chem* 1999; **274:** 8367-8370.

261. Legler DF, Loetscher M, Jones SA*, et al.* Expression of high- and low-affinity receptors for C3a on the human mast cell line, HMC-1. *Eur J Immunol* 1996; **26:** 753-758.

262. Ruan BH, Li X, Winkler AR*, et al.* Complement C3a, CpG oligos, and DNA/C3a complex stimulate IFN-alpha production in a receptor for advanced glycation end product-dependent manner. *J Immunol* 2010; **185:** 4213-4222.

263. Martin U, Bock D, Arseniev L*, et al.* The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes. *J Exp Med* 1997; **186:** 199-207.

264. Kirchhoff K, Weinmann O, Zwirner J*, et al.* Detection of anaphylatoxin receptors on CD83+ dendritic cells derived from human skin. *Immunology* 2001; **103:** 210-217.

265. Kretzschmar T, Jeromin A, Gietz C*, et al.* Chronic myelogenous leukemia-derived basophilic granulocytes express a functional active receptor for the anaphylatoxin C3a. *Eur J Immunol* 1993; **23:** 558-561.

266. Fischer WH, Hugli TE. Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response. *J Immunol* 1997; **159:** 4279-4286.

267. Werfel T, Kirchhoff K, Wittmann M*, et al.* Activated human T lymphocytes express a functional C3a receptor. *J Immunol* 2000; **165:** 6599-6605.

268. Liszewski MK, Kolev M, Le Friec G*, et al.* Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity* 2013; **39:** 1143-1157.

269. Ghannam A, Fauquert JL, Thomas C*, et al.* Human complement C3 deficiency: Th1 induction requires T cell-derived complement C3a and CD46 activation. *Mol Immunol* 2014; **58:** 98-107.

270. Braun MC, Reins RY, Li TB*, et al.* Renal expression of the C3a receptor and functional responses of primary human proximal tubular epithelial cells. *J Immunol* 2004; **173:** 4190-4196.

271. Asgari E, Le Friec G, Yamamoto H*, et al.* C3a modulates IL-1beta secretion in human monocytes by regulating ATP efflux and subsequent NLRP3 inflammasome activation. *Blood* 2013; **122:** 3473-3481.

272. Takabayashi T, Vannier E, Clark BD*, et al.* A new biologic role for C3a and C3a desArg: regulation of TNF-alpha and IL-1 beta synthesis. *J Immunol* 1996; **156:** 3455-3460.

273. Guglietta S, Chiavelli A, Zagato E*, et al.* Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. *Nat Commun* 2016; **7:** 11037.

274. Zwirner J, Werfel T, Wilken HC*, et al.* Anaphylatoxin C3a but not C3a(desArg) is a chemotaxin for the mouse macrophage cell line J774. *Eur J Immunol* 1998; **28:** 1570-1577.

275. Peng Q, Li K, Anderson K*, et al.* Local production and activation of complement up-regulates the allostimulatory function of dendritic cells through C3a-C3aR interaction. *Blood* 2008; **111:** 2452-2461.

276. Li K, Anderson KJ, Peng Q*, et al.* Cyclic AMP plays a critical role in C3a-receptor-mediated regulation of dendritic cells in antigen uptake and T-cell stimulation. *Blood* 2008; **112:** 5084-5094.

277. Lajoie S, Lewkowich IP, Suzuki Y*, et al.* Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol* 2010; **11:** 928-935.

278. Drouin SM, Corry DB, Hollman TJ*, et al.* Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J Immunol* 2002; **169:** 5926-5933.

279. Ghannam A, Pernollet M, Fauquert JL*, et al.* Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells. *J Immunol* 2008; **181:** 5158-5166.

280. Le Friec G, Sheppard D, Whiteman P*, et al.* The CD46-Jagged1 interaction is critical for human TH1 immunity. *Nat Immunol* 2012; **13:** 1213-1221.

281. Lim H, Kim YU, Drouin SM*, et al.* Negative regulation of pulmonary Th17 responses by C3a anaphylatoxin during allergic inflammation in mice. *PLoS One* 2012; **7:** e52666.

282. Kwan WH, van der Touw W, Paz-Artal E*, et al.* Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. *J Exp Med* 2013; **210:** 257-268.

283. Pepys MB. Role of complement in induction of antibody production in vivo. Effect of cobra factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. *J Exp Med* 1974; **140:** 126-145.

284. Dempsey PW, Allison ME, Akkaraju S*, et al.* C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 1996; **271:** 348-350.

285. Kawamoto S, Yalcindag A, Laouini D*, et al.* The anaphylatoxin C3a downregulates the Th2 response to epicutaneously introduced antigen. *J Clin Invest* 2004; **114:** 399-407.

286. Kildsgaard J, Hollmann TJ, Matthews KW*, et al.* Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. *J Immunol* 2000; **165:** 5406-5409.

287. Wu MC, Brennan FH, Lynch JP*, et al.* The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization. *Proc Natl Acad Sci U S A* 2013; **110:** 9439-9444.

288. Wende E, Laudeley R, Bleich A*, et al.* The complement anaphylatoxin C3a receptor (C3aR) contributes to the inflammatory response in dextran sulfate sodium (DSS)-induced colitis in mice. *PLoS One* 2013; **8:** e62257.

289. Boos L, Campbell IL, Ames R*, et al.* Deletion of the complement anaphylatoxin C3a receptor attenuates, whereas ectopic expression of C3a in the brain exacerbates, experimental autoimmune encephalomyelitis. *J Immunol* 2004; **173:** 4708-4714.

290. Tang Z, Lu B, Hatch E*, et al.* C3a mediates epithelial-to-mesenchymal transition in proteinuric nephropathy. *J Am Soc Nephrol* 2009; **20:** 593-603.

291. Bao L, Wang Y, Haas M*, et al.* Distinct roles for C3a and C5a in complement-induced tubulointerstitial injury. *Kidney Int* 2011; **80:** 524-534.

292. Bao L, Wang Y, Chang A*, et al.* Unrestricted C3 activation occurs in Crry-deficient kidneys and rapidly leads to chronic renal failure. *J Am Soc Nephrol* 2007; **18:** 811-822.

293. Wenderfer SE, Wang H, Ke B*, et al.* C3a receptor deficiency accelerates the onset of renal injury in the MRL/lpr mouse. *Mol Immunol* 2009; **46:** 1397-1404.

294. Bao L, Osawe I, Haas M*, et al.* Signaling through up-regulated C3a receptor is key to the development of experimental lupus nephritis. *J Immunol* 2005; **175:** 1947-1955.

295. Mathieu MC, Sawyer N, Greig GM*, et al.* The C3a receptor antagonist SB 290157 has agonist activity. *Immunol Lett* 2005; **100:** 139-145.

296. Martinelli S, Urosevic M, Daryadel A*, et al.* Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. *J Biol Chem* 2004; **279:** 44123-44132.

297. Bamberg CE, Mackay CR, Lee H*, et al.* The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. *J Biol Chem* 2010; **285:** 7633-7644.

298. Klickstein LB, Bartow TJ, Miletic V*, et al.* Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. *J Exp Med* 1988; **168:** 1699-1717.

299. Witko-Sarsat V, Rieu P, Descamps-Latscha B*, et al.* Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 2000; **80:** 617-653.

300. Ruiz S, Henschen-Edman AH, Tenner AJ. Localization of the site on the complement component C1q required for the stimulation of neutrophil superoxide production. *J Biol Chem* 1995; **270:** 30627-30634.

301. Wirthmueller U, Dewald B, Thelen M*, et al.* Properdin, a positive regulator of complement activation, is released from secondary granules of stimulated peripheral blood neutrophils. *J Immunol* 1997; **158:** 4444-4451.

302. Camous L, Roumenina L, Bigot S*, et al.* Complement alternative pathway acts as a positive feedback amplification of neutrophil activation. *Blood* 2011; **117:** 1340-1349.

303. Shingu M, Nonaka S, Nishimukai H*, et al.* Activation of complement in normal serum by hydrogen peroxide and hydrogen peroxide-related oxygen radicals produced by activated neutrophils. *Clin Exp Immunol* 1992; **90:** 72-78.

304. Harboe M, Johnson C, Nymo S*, et al.* Properdin binding to complement activating surfaces depends on initial C3b deposition. *Proc Natl Acad Sci U S A* 2017; **114:** E534-E539.

305. Gasser O, Schifferli JA. Microparticles released by human neutrophils adhere to erythrocytes in the presence of complement. *Exp Cell Res* 2005; **307:** 381-387.

306. Yuen J, Pluthero FG, Douda DN*, et al.* NETosing Neutrophils Activate Complement Both on Their Own NETs and Bacteria via Alternative and Non-alternative Pathways. *Front Immunol* 2016; **7:** 137.

307. Amara U, Kalbitz M, Perl M*, et al.* Early expression changes of complement regulatory proteins and C5A receptor (CD88) on leukocytes after multiple injury in humans. *Shock* 2010; **33:** 568-575.

308. Berger M, Medof ME. Increased expression of complement decay-accelerating factor during activation of human neutrophils. *J Clin Invest* 1987; **79:** 214-220.

309. Jones J, Morgan BP. Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation. *Immunology* 1995; **86:** 651-660.

310. Gan PY, Holdsworth SR, Kitching AR*, et al.* Myeloperoxidase (MPO)-specific CD4+ T cells contribute to MPO-anti-neutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis. *Cell Immunol* 2013; **282:** 21-27.

311. Morgan M, Bulmer AC, Woodruff TM*, et al.* Pharmacokinetics of a C5a receptor antagonist in the rat after different sites of enteral administration. *Eur J Pharm Sci* 2008; **33:** 390-398.

312. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci U S A* 2005; **102:** 5126-5131.

313. Hebert MJ, Takano T, Papayianni A*, et al.* Acute nephrotoxic serum nephritis in complement knockout mice: relative roles of the classical and alternate pathways in neutrophil recruitment and proteinuria. *Nephrol Dial Transplant* 1998; **13:** 2799-2803.

314. Devi S, Li A, Westhorpe CL*, et al.* Multiphoton imaging reveals a new leukocyte recruitment paradigm in the glomerulus. *Nat Med* 2013; **19:** 107-112.

315. Yoshida M, Iwahori T, Nakabayashi I*, et al.* In vitro production of myeloperoxidase anti-neutrophil cytoplasmic antibody and establishment of Th1-type T cell lines from peripheral blood lymphocytes of patients. *Clin Exp Rheumatol* 2005; **23:** 227-230.

316. Odobasic D, Gan PY, Summers SA*, et al.* Interleukin-17A promotes early but attenuates established disease in crescentic glomerulonephritis in mice. *Am J Pathol* 2011; **179:** 1188-1198.

317. Fang C, Miwa T, Song WC. Decay-accelerating factor regulates T-cell immunity in the context of inflammation by influencing costimulatory molecule expression on antigen-presenting cells. *Blood* 2011; **118:** 1008-1014.

318. Weiss S, Rosendahl A, Czesla D*, et al.* The complement receptor C5aR1 contributes to renal damage but protects the heart in angiotensin II-induced hypertension. *Am J Physiol Renal Physiol* 2016; **310:** F1356-1365.

319. Bao L, Osawe I, Puri T*, et al.* C5a promotes development of experimental lupus nephritis which can be blocked with a specific receptor antagonist. *Eur J Immunol* 2005; **35:** 2496-2506.

320. Alexander JJ, Chaves LD, Chang A*, et al.* Abrogation of immune complex glomerulonephritis by native carboxypeptidase and pharmacological antagonism of the C5a receptor. *Cell Mol Immunol* 2015.

321. Hung CY, Hurtgen BJ, Bellecourt M*, et al.* An agonist of human complement fragment C5a enhances vaccine immunity against Coccidioides infection. *Vaccine* 2012; **30:** 4681-4690.

322. Hao J, Meng LQ, Xu PC*, et al.* p38MAPK, ERK and PI3K signaling pathways are involved in C5a-primed neutrophils for ANCA-mediated activation. *PLoS One* 2012; **7:** e38317.

323. Miyabe Y, Miyabe C, Murooka TT*, et al.* Complement C5a Receptor is the Key Initiator of Neutrophil Adhesion Igniting Immune Complex-induced Arthritis. *Sci Immunol* 2017; **2**.

324. Konrad S, Engling L, Schmidt RE*, et al.* Characterization of the murine IgG Fc receptor III and IIB gene promoters: a single two-nucleotide difference determines their inverse responsiveness to C5a. *J Biol Chem* 2007; **282:** 37906-37912.

325. Miyabe Y, Miyabe C, Murooka TT*, et al.* Complement C5a receptor is the key initiator of neutrophil adhesion igniting immune complex–induced arthritis. *Science Immunology* 2017; **2:** eaaj2195.

326. Boor P, Konieczny A, Villa L*, et al.* Complement C5 mediates experimental tubulointerstitial fibrosis. *J Am Soc Nephrol* 2007; **18:** 1508-1515.

327. Quintana LF, Perez NS, De Sousa E*, et al.* ANCA serotype and histopathological classification for the prediction of renal outcome in ANCA-associated glomerulonephritis. *Nephrol Dial Transplant* 2014; **29:** 1764-1769.

328. Apostolopoulos J, Ooi JD, Odobasic D*, et al.* The isolation and purification of biologically active recombinant and native autoantigens for the study of autoimmune disease. *J Immunol Methods* 2006; **308:** 167-178.

329. Charles LA, Caldas ML, Falk RJ*, et al.* Antibodies against granule proteins activate neutrophils in vitro. *J Leukoc Biol* 1991; **50:** 539-546.

330. Daffern PJ, Pfeifer PH, Ember JA*, et al.* C3a is a chemotaxin for human eosinophils but not for neutrophils. I. C3a stimulation of neutrophils is secondary to eosinophil activation. *J Exp Med* 1995; **181:** 2119-2127.

331. Clarke EV, Tenner AJ. Complement modulation of T cell immune responses during homeostasis and disease. *J Leukoc Biol* 2014; **96:** 745-756.

332. Sanders JS, Huitma MG, Kallenberg CG*, et al.* Plasma levels of soluble interleukin 2 receptor, soluble CD30, interleukin 10 and B cell activator of the tumour necrosis factor family during follow-up in vasculitis associated with proteinase 3-antineutrophil cytoplasmic antibodies: associations with disease activity and relapse. *Ann Rheum Dis* 2006; **65:** 1484-1489.

333. Huang XR, Tipping PG, Shuo L*, et al.* Th1 responsiveness to nephritogenic antigens determines susceptibility to crescentic glomerulonephritis in mice. *Kidney Int* 1997; **51:** 94-103.

334. O'Flynn J, Dixon KO, Faber Krol MC*, et al.* Myeloperoxidase directs properdin-mediated complement activation. *J Innate Immun* 2014; **6:** 417-425.

335. Ruth AJ, Kitching AR, Semple TJ*, et al.* Intrinsic renal cell expression of CD40 directs Th1 effectors inducing experimental crescentic glomerulonephritis. *J Am Soc Nephrol* 2003; **14:** 2813-2822.

336. Kitching AR, Holdsworth SR, Tipping PG. IFN-gamma mediates crescent formation and cell-mediated immune injury in murine glomerulonephritis. *J Am Soc Nephrol* 1999; **10:** 752-759.

337. Morgan EL, Weigle WO, Hugli TE. Anaphylatoxin-mediated regulation of the immune response. I. C3a-mediated suppression of human and murine humoral immune responses. *J Exp Med* 1982; **155:** 1412-1426.

338. Mueller-Ortiz SL, Hollmann TJ, Haviland DL*, et al.* Ablation of the complement C3a anaphylatoxin receptor causes enhanced killing of Pseudomonas aeruginosa in a mouse model of pneumonia. *Am J Physiol Lung Cell Mol Physiol* 2006; **291:** L157-165.

339. Dutow P, Fehlhaber B, Bode J*, et al.* The complement C3a receptor is critical in defense against Chlamydia psittaci in mouse lung infection and required for antibody and optimal T cell response. *J Infect Dis* 2014; **209:** 1269-1278.

340. Quell KM, Karsten CM, Kordowski A*, et al.* Monitoring C3aR Expression Using a Floxed tdTomato-C3aR Reporter Knock-in Mouse. *J Immunol* 2017.

341. Laumonnier Y, Karsten CM, Kohl J. Novel insights into the expression pattern of anaphylatoxin receptors in mice and men. *Mol Immunol* 2017.

342. Klos A, Bank S, Gietz C*, et al.* C3a receptor on dibutyryl-cAMP-differentiated U937 cells and human neutrophils: the human C3a receptor characterized by functional responses and 125I-C3a binding. *Biochemistry* 1992; **31:** 11274-11282.

343. Huang XR, Holdsworth SR, Tipping PG. Evidence for delayed-type hypersensitivity mechanisms in glomerular crescent formation. *Kidney Int* 1994; **46:** 69-78.

344. Harboe M, Ulvund G, Vien L*, et al.* The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin Exp Immunol* 2004; **138:** 439-446.

345. Banda NK, Takahashi K, Wood AK*, et al.* Pathogenic complement activation in collagen antibody-induced arthritis in mice requires amplification by the alternative pathway. *J Immunol* 2007; **179:** 4101-4109.

346. Hietala MA, Jonsson IM, Tarkowski A*, et al.* Complement deficiency ameliorates collagen-induced arthritis in mice. *J Immunol* 2002; **169:** 454-459.

347. Nataf S, Carroll SL, Wetsel RA*, et al.* Attenuation of experimental autoimmune demyelination in complement-deficient mice. *J Immunol* 2000; **165:** 5867-5873.

348. Nelson KC, Zhao M, Schroeder PR*, et al.* Role of different pathways of the complement cascade in experimental bullous pemphigoid. *J Clin Invest* 2006; **116:** 2892-2900.

349. Watanabe H, Garnier G, Circolo A*, et al.* Modulation of renal disease in MRL/lpr mice genetically deficient in the alternative complement pathway factor B. *J Immunol* 2000; **164:** 786-794.

350. Elliott MK, Jarmi T, Ruiz P*, et al.* Effects of complement factor D deficiency on the renal disease of MRL/lpr mice. *Kidney Int* 2004; **65:** 129-138.

351. Robson MG, Cook HT, Pusey CD*, et al.* Antibody-mediated glomerulonephritis in mice: the role of endotoxin, complement and genetic background. *Clin Exp Immunol* 2003; **133:** 326-333.

352. Thurman JM, Tchepeleva SN, Haas M*, et al.* Complement alternative pathway activation in the autologous phase of nephrotoxic serum nephritis. *Am J Physiol Renal Physiol* 2012; **302:** F1529-1536.

353. Stover CM, Luckett JC, Echtenacher B*, et al.* Properdin plays a protective role in polymicrobial septic peritonitis. *J Immunol* 2008; **180:** 3313-3318.

354. Matsumoto M, Fukuda W, Circolo A*, et al.* Abrogation of the alternative complement pathway by targeted deletion of murine factor B. *Proc Natl Acad Sci U S A* 1997; **94:** 8720-8725.

355. Liu J, Miwa T, Hilliard B*, et al.* The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. *J Exp Med* 2005; **201:** 567-577.

356. Ambrus JL, Jr., Peters MG, Fauci AS*, et al.* The Ba fragment of complement factor B inhibits human B lymphocyte proliferation. *J Immunol* 1990; **144:** 1549-1553.

357. Cardone J, Le Friec G, Vantourout P*, et al.* Complement regulator CD46 temporally regulates cytokine production by conventional and unconventional T cells. *Nat Immunol* 2010; **11:** 862-871.

358. Wagner C, Ochmann C, Schoels M*, et al.* The complement receptor 1, CR1 (CD35), mediates inhibitory signals in human T-lymphocytes. *Mol Immunol* 2006; **43:** 643-651.

359. Jozsi M, Prechl J, Bajtay Z*, et al.* Complement receptor type 1 (CD35) mediates inhibitory signals in human B lymphocytes. *J Immunol* 2002; **168:** 2782-2788.

360. Ong GL, Mattes MJ. Mouse strains with typical mammalian levels of complement activity. *J Immunol Methods* 1989; **125:** 147-158.

361. Bergman I, Basse PH, Barmada MA*, et al.* Comparison of in vitro antibody-targeted cytotoxicity using mouse, rat and human effectors. *Cancer Immunol Immunother* 2000; **49:** 259-266.

362. Ratelade J, Verkman AS. Inhibitor(s) of the classical complement pathway in mouse serum limit the utility of mice as experimental models of neuromyelitis optica. *Mol Immunol* 2014; **62:** 104-113.

363. Campbell AK, Morgan BP. Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. *Nature* 1985; **317:** 164-166.

364. Mellbye OJ, Mollnes TE, Steen LS. IgG subclass distribution and complement activation ability of autoantibodies to neutrophil cytoplasmic antigens (ANCA). *Clin Immunol Immunopathol* 1994; **70:** 32-39.

365. Chen G, Sequeira F, Tyan DB. Novel C1q assay reveals a clinically relevant subset of human leukocyte antigen antibodies independent of immunoglobulin G strength on single antigen beads. *Human Immunology* 2011; **72:** 849-858.

366. Pfeifer PH, Kawahara MS, Hugli TE. Possible mechanism for in vitro complement activation in blood and plasma samples: futhan/EDTA controls in vitro complement activation. *Clin Chem* 1999; **45:** 1190-1199.

367. Inglis JE, Radziwon KA, Maniero GD. The serum complement system: a simplified laboratory exercise to measure the activity of an important component of the immune system. *Adv Physiol Educ* 2008; **32:** 317-321.

368. Ollert MW, Kadlec JV, David K*, et al.* Antibody-mediated complement activation on nucleated cells. A quantitative analysis of the individual reaction steps. *J Immunol* 1994; **153:** 2213-2221.

369. AlMahri A, Holgersson J, Alheim M. Detection of complement-fixing and non-fixing antibodies specific for endothelial precursor cells and lymphocytes using flow cytometry. *Tissue Antigens* 2012; **80:** 404-415.

370. Tosic L, Sutherland WM, Kurek J*, et al.* Preparation of monoclonal antibodies to C3b by immunization with C3b(i)-sepharose. *J Immunol Methods* 1989; **120:** 241-249.

371. Noronha IL, Kruger C, Andrassy K*, et al.* In situ production of TNF-alpha, IL-1 beta and IL-2R in ANCA-positive glomerulonephritis. *Kidney Int* 1993; **43:** 682-692.

372. Lau D, Mollnau H, Eiserich JP*, et al.* Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc Natl Acad Sci U S A* 2005; **102:** 431-436.

373. Kothari N, Keshari RS, Bogra J*, et al.* Increased myeloperoxidase enzyme activity in plasma is an indicator of inflammation and onset of sepsis. *J Crit Care* 2011; **26:** 435 e431-437.

374. Hess C, Sadallah S, Schifferli JA. Induction of neutrophil responsiveness to myeloperoxidase antibodies by their exposure to supernatant of degranulated autologous neutrophils. *Blood* 2000; **96:** 2822-2827.

375. Haller H, Eichhorn J, Pieper K*, et al.* Circulating leukocyte integrin expression in Wegener's granulomatosis. *J Am Soc Nephrol* 1996; **7:** 40-48.

376. Paidassi H, Tacnet-Delorme P, Garlatti V*, et al.* C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. *J Immunol* 2008; **180:** 2329-2338.

377. Shive MS, Salloum ML, Anderson JM. Shear stress-induced apoptosis of adherent neutrophils: a mechanism for persistence of cardiovascular device infections. *Proc Natl Acad Sci U S A* 2000; **97:** 6710-6715.

378. Nowack R, Grab I, Flores-Suarez LF*, et al.* ANCA titres, even of IgG subclasses, and soluble CD14 fail to predict relapses in patients with ANCA-associated vasculitis. *Nephrology Dialysis Transplantation* 2001; **16:** 1631-1637.

379. Holland M, Hewins P, Goodall M*, et al.* Anti-neutrophil cytoplasm antibody IgG subclasses in Wegener's granulomatosis: a possible pathogenic role for the IgG4 subclass. *Clin Exp Immunol* 2004; **138:** 183-192.

380. Rooney CP, Taggart C, Coakley R*, et al.* Anti-proteinase 3 antibody activation of neutrophils can be inhibited by alpha1-antitrypsin. *Am J Respir Cell Mol Biol* 2001; **24:** 747-754.

381. Tambur AR, Herrera ND, Haarberg KM*, et al.* Assessing Antibody Strength: Comparison of MFI, C1q, and Titer Information. *Am J Transplant* 2015; **15:** 2421-2430.

382. Taylor CJ, Kosmoliaptsis V, Martin J*, et al.* Technical Limitations of the C1q Single-Antigen Bead Assay to Detect Complement Binding HLA-Specific Antibodies. *Transplantation* 2016.

383. Schwaiger E, Wahrmann M, Bond G*, et al.* Complement component C3 activation: the leading cause of the prozone phenomenon affecting HLA antibody detection on single-antigen beads. *Transplantation* 2014; **97:** 1279-1285.

384. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 2014; **5:** 520.

385. Schaub S, Honger G, Koller MT*, et al.* Determinants of C1q binding in the single antigen bead assay. *Transplantation* 2014; **98:** 387-393.

386. Franssen CF, Huitema MG, Muller Kobold AC*, et al.* In vitro neutrophil activation by antibodies to proteinase 3 and myeloperoxidase from patients with crescentic glomerulonephritis. *J Am Soc Nephrol* 1999; **10:** 1506-1515.

387. Gordon AC, Lagan AL, Aganna E*, et al.* TNF and TNFR polymorphisms in severe sepsis and septic shock: a prospective multicentre study. *Genes Immun* 2004; **5:** 631-640.

388. Stein JM, Luzio JP. Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. *Biochem J* 1991; **274 (Pt 2):** 381-386.

389. Hong Y, Eleftheriou D, Hussain AA*, et al.* Anti-neutrophil cytoplasmic antibodies stimulate release of neutrophil microparticles. *J Am Soc Nephrol* 2012; **23:** 49-62.

390. Shi J, Rose EL, Singh A*, et al.* TNT003, an inhibitor of the serine protease C1s, prevents complement activation induced by cold agglutinins. *Blood* 2014; **123:** 4015-4022.

391. Thomas KA, Valenzuela NM, Gjertson D*, et al.* An Anti-C1s Monoclonal, TNT003, Inhibits Complement Activation Induced by Antibodies Against HLA. *Am J Transplant* 2015; **15:** 2037-2049.

392. Colonna L, Parry GC, Panicker S*, et al.* Uncoupling complement C1s activation from C1q binding in apoptotic cell phagocytosis and immunosuppressive capacity. *Clin Immunol* 2016; **163:** 84-90.

393. Bienaime F, Quartier P, Dragon-Durey MA*, et al.* Lupus nephritis associated with complete C1s deficiency efficiently treated with rituximab: a case report. *Arthritis Care Res (Hoboken)* 2010; **62:** 1346-1350.

394. Steinmetz OM, Summers SA, Gan PY*, et al.* The Th17-defining transcription factor RORgammat promotes glomerulonephritis. *J Am Soc Nephrol* 2011; **22:** 472-483.

395. Woodruff TM, Strachan AJ, Sanderson SD*, et al.* Species dependence for binding of small molecule agonist and antagonists to the C5a receptor on polymorphonuclear leukocytes. *Inflammation* 2001; **25:** 171-177.

396. Qi H. T follicular helper cells in space-time. *Nat Rev Immunol* 2016; **16:** 612-625.

397. Cain D, Kondo M, Chen H*, et al.* Effects of acute and chronic inflammation on B-cell development and differentiation. *J Invest Dermatol* 2009; **129:** 266-277.

398. Bekker P, Dairaghi D, Seitz L*, et al.* Characterization of Pharmacologic and Pharmacokinetic Properties of CCX168, a Potent and Selective Orally Administered Complement 5a Receptor Inhibitor, Based on Preclinical Evaluation and Randomized Phase 1 Clinical Study. *PLoS One* 2016; **11:** e0164646.

399. Yousefi S, Mihalache C, Kozlowski E*, et al.* Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* 2009; **16:** 1438-1444.

400. Laumonnier Y, Karsten CM, Czabanska A*, et al.* Defining C3aR expression in myeloid and lymphoid cells using a novel Td-Tomato-C3aR reporter knockin mouse. *Immunobiology* 2016; **221:** 1180.

401. Li L, Yin Q, Tang X*, et al.* C3a receptor antagonist ameliorates inflammatory and fibrotic signals in type 2 diabetic nephropathy by suppressing the activation of TGF-beta/smad3 and IKBalpha pathway. *PLoS One* 2014; **9:** e113639.

402. Morigi M, Locatelli M, Rota C*, et al.* A previously unrecognized role of C3a in proteinuric progressive nephropathy. *Sci Rep* 2016; **6:** 28445.

403. Nakae S, Asano M, Horai R*, et al.* Interleukin-1 beta, but not interleukin-1 alpha, is required for T-cell-dependent antibody production. *Immunology* 2001; **104:** 402-409.

404. Barbour TD, Ling GS, Ruseva MM*, et al.* Complement receptor 3 mediates renal protection in experimental C3 glomerulopathy. *Kidney Int* 2016; **89:** 823-832.

405. Bullard DC, Hu X, Schoeb TR*, et al.* Critical requirement of CD11b (Mac-1) on T cells and accessory cells for development of experimental autoimmune encephalomyelitis. *J Immunol* 2005; **175:** 6327-6333.

406. Tang T, Rosenkranz A, Assmann KJ*, et al.* A role for Mac-1 (CDIIb/CD18) in immune complex-stimulated neutrophil function in vivo: Mac-1 deficiency abrogates sustained Fcgamma receptor-dependent neutrophil adhesion and complement-dependent proteinuria in acute glomerulonephritis. *J Exp Med* 1997; **186:** 1853-1863.

407. Hao J, Wang C, Yuan J*, et al.* A pro-inflammatory role of C5L2 in C5a-primed neutrophils for ANCA-induced activation. *PLoS One* 2013; **8:** e66305.