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The Pathogenesis of Antineutrophil Cytoplasmic Antibody Renal Vasculitis

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<http://dx.doi.org/10.5772/54637>

1. Introduction

The vasculitides comprise a heterogeneous group of diseases characterized by inflammation and destruction of blood vessels. Vessels of any size can be involved which explains the diverse spectrum of clinical diseases attributed to vasculitis. While the immunological basis of disease for vasculitis was recognized over thirty years ago,[1] a standardized classification system was only adopted nearly twenty years later. The initial classification system proposed by the American College of Rheumatology attempted to classify vasculitis according to standardized criteria.[2] The subsequent system described by the Chapel Hill Conference on the Nomenclature of Systemic Vasculitis[3] introduced a system which coupled contemporary commonly used disease names and the size of vessel(s) involved.

1.1. Small vessel vasculitis

Necrotizing arteritis is common to many forms of vasculitis, but involvement of vessels smaller than arteries is unique to small vessel vasculitis.[4] A clinical report of 'Vasculitis' originated from the mid-nineteenth century[5] and clinical descriptions of these diseases were published in the 1930s,[6] however it was not until the 1950s that Wegener's Granulomatosis, Churg Strauss Syndrome and Microscopic polyangiitis were identified as unique clinical entities.[7] In the 1980s it was appreciated that the small vessel vasculitides represented a clinically distinct form of disease.[8] These small vessel vasculitides will be the primary focus of this chapter.

Classification	Disease Name
Large Vessel Vasculitis	Giant Cell (Temporal) Arteritis
	Takayasu's Arteritis
Medium Sized Vessel Vasculitis	Polyarteritis Nodosa
	Kawasaki's disease
Small Vessel Vasculitis	Wegener's Granulomatosis*
	Churg Strauss Syndrome*
	Microscopic Polyangiitis*
	Henoch Schonlein Purpura
	Essential Cryoglobulinaemic Vasculitis
	Cutaneous Leukocytoclastic Angiitis

*These diseases have subsequently been renamed.

Table 1. The Chapel Hill Conference on the Nomenclature of Systemic Vasculitis

2. Antineutrophil cytoplasmic antibody associated vasculitis

2.1. Background and chapter overview

Glomerulonephritis is a common cause of renal failure both worldwide and in Australia. Rapidly progressive or crescentic glomerulonephritis represents the most severe form of the disease and antineutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) accounts for >50% and more likely up to 80% of all cases of rapidly progressive glomerulonephritis. The AAVs are considered a heterogenous group of systemic autoimmune conditions characterised by necrotising inflammation of small to medium sized arteries, capillaries and venules. The disease is diagnosed by detecting ANCA in the serum which characteristically is directed against myeloperoxidase (MPO) or proteinase 3 (PR3). The two most severe clinical manifestations of disease are rapidly progressive glomerulonephritis and pulmonary haemorrhage due to pulmonary capillaritis. These syndromes are associated with significant morbidity and untreated have a mortality that approaches 100%. Renal vasculitis occurs in more than 50% of patients at presentation but in 70-85% of patients with AAV during the course of their disease [9]. While current treatments for active ANCA vasculitis are often life-saving they are toxic and more than 1 in 3 patients will suffer a significant treatment related adverse event.[10] A better understanding of the *critical molecular events* which underlie the disease process will help identify more specific targeted therapies.

In the early 1980s two Australian groups based in Melbourne, from St Vincent's Hospital[11] and the Austin Hospital[12] described the association of antibodies directed against the neutrophil cytoplasm in patients with rapidly progressive glomerulonephritis. These reports represented key advances in our understanding of the pathogenesis of au-

toimmune small vessel vasculitis. Subsequent work by a Dutch group helped establish the correlation between ANCAs and the three clinical syndromes; Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome.[13] More recently these syndromes have been renamed to generate nomenclature free from the use of eponyms. [14-16] The new nomenclature proposed and adopted into the literature and clinical practice in 2011 is as follows; Microscopic Polyangiitis (MPA), Granulomatosis with polyangiitis, (GPA), formally known as Wegener's, Allergic Granulomatosis and Angiitis (AGA) formally known as Churg Strauss Disease and Renal Limited Vasculitis (RLV).[14] This new terminology will be adopted for the remainder of this chapter.

In this chapter, we will concentrate on renal injury resulting from AAV which has formed the basis for clinical and experimental studies. For both MPA and GPA target autoantigens have been identified which are constituents of neutrophils. For MPA, myeloperoxidase (MPO) is usually the target autoantigen, while antibodies to proteinase 3 (PR3) are usually detectable in patients with clinical features of GPA. In both clinical and experimental AAV (GPA or MPA) two separate key steps are required for the development of glomerulonephritis and renal injury. The first critical step involves the development of systemic autoimmunity to the target antigen, MPO or PR3. The second step involves antigen specific nephritogenic immune responses driving glomerular injury and renal disease.

2.2. The development of systemic autoimmunity in MPA and GPA

The development of autoimmunity is a complex process, multifactorial in origin, which involves the loss of tolerance and enhanced cellular and humoral activity.[17] In AAV, disease is defined and characterized by antibodies detected against MPO or PR3. While antibodies form the diagnostic hallmark of disease, cellular immunity is critical and is required for the development of humoral immunity and the subsequent generation of B cells and production of ANCAs. A role for cellular immunity has been defined in both clinical and experimental ANCA vasculitis. In addition to adaptive immune cells, innate immune cells contribute to the generation of autoimmunity with evidence for involvement of different cell types in this disease process.

2.3. The initiation and progression of rapidly progressive glomerulonephritis and renal injury in AAV

Enhanced cellular autoimmunity and innate cells stimulate B cells resulting in the production of antigen specific ANCAs. These auto-antibodies bind to and activate circulating neutrophils. These activated neutrophils are recruited to glomerular capillaries,[18] where they degranulate and initiate renal injury. Degranulating neutrophils release their noxious constituents and also deposit MPO [19] and probably PR3 in the glomerulus. Later, CD4+ T cells recognise the autoantigen (MPO/PR3) in the glomerulus and attract additional immune effector cells; this results in severe renal injury. In both clinical and experimental settings cellular nephritogenic immunity, humoral immunity and innate immune cells are critical for the development of rapidly progressive glomerulonephritis.[20-24] Our current treatment regimes were designed to target these cells, or combinations of them.

In this chapter we will focus on the pathogenesis of the ANCA associated vasculitides, focussing on AAV attributable to MPA and GPA. We will pay attention to the development of autoimmunity and concentrate on end organ injury in the kidney, a critical target of the small vessel vasculitides. Interestingly, while GPA and MPA share many diagnostic and clinical features and patients with these diseases have been grouped together in many clinical trials, more recent evidence including a landmark genetic study, suggests that GPA and MPA represent two different diseases. While we will discuss GPA and MPA separately, there is stronger experimental evidence linking MPO with disease. This includes several small animal studies which have confirmed pathogenic roles for cellular and humoral autoimmunity, directed against MPO, which closely resemble human disease. Our discussion will focus on the disease pathogenesis of AAV and attempt to define future directions for study which ultimately may lead to therapeutic interventions. Information has been made available from human studies assessing mechanisms of disease as well as experimental studies, utilizing rodent models of vasculitis. Further insights into disease pathogenesis can be gained from clinical trials, including those with negative results.

3. Genetic and epigenetic basis of disease in ANCA vasculitis

Consistent with improved mechanistic studies the last decade has witnessed significant advances in our understanding of the role of both the genetic and epigenetic factors driving AAV. While a detailed description and discussion of these factors is beyond the scope of this chapter it would be remiss not to discuss several recent key studies. It is important to note that all results discussed in this section are from clinical studies. It should also be noted that while the varying genetic background of commonly used laboratory rodents may contribute to a particular pattern and severity of disease in experimental AAV, the relevance and correlation of this to human disease is less clear.

A genetic basis for AAV has long been suspected, however this was recently confirmed by a publication which demonstrated a relative risk of 1:56 for first degree relatives of patients with GPA.[25] This rate is similar to that seen in other autoimmune diseases with an established genetic component which contributes to injury. This study followed on from previous studies which had suggested a genetic link into AAV. Many of the candidate genes identified as being over represented in vasculitis patients are associated with genes which encode proteins involved in the immune system. These include several genes encoded in the human leukocyte antigen (HLA) as well as genes encoding protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), cytotoxic T-lymphocyte antigen 4 (*CTLA4*), Interleukin (*IL*)-2, *PRTN3* which encodes PR3, α 1 anti-trypsin (*AAT*), complement related genes, *CD18*, *IL-10*, *CD226* as well as the Fc gamma receptors; *FCGR2A*, *FCGR3B* (for both copy number high and copy number low). For a detailed review of the individual genes linked with clinical disease, the authors recommend the review by Willcocks and colleagues, whose work with Ken Smith has been instrumental in advancing knowledge in this field.[26] It is important to acknowledge that while genetic variation of these genes has been associated with an increased incidence of AAV,

many of these genes display aberrant expression in several autoimmune diseases. This is not surprising considering several of these genes encode proteins critical for maintenance of the immune system, including the function of innate immune cells, T lymphocytes, B lymphocytes and regulatory cells. There are several limitations to these studies. Some studies which linked aberrant gene expression with AAV included patients with only one form of the disease (i.e. GPA, MPA, RLV or AGA), while other studies were less specific and included all patients who had detectable ANCA levels. Furthermore several of these associations were not confirmed when assessing disease in different population groups and hence results from these early studies suggested that there was, at best, a modest link between genetic background and disease.[26-27]

In a genome wide association study with over 10 000 patients (including controls), not only was a genetic component confirmed but the antigenic specificity for AAV, i.e. for MPO or PR3 was found to have distinct genetic associations. For patients with ANCA directed against PR3, there was a strong genetic association with *HLA-DP* and genes encoding $\alpha 1$ -AT-SERPINA1 and PTN3. Conversely patients with antibodies directed against MPO showed a strong association with *HLA-DQ*.[28] The observation that there were different genetic associations for MPO-ANCA and PR3-ANCA strengthens the proposal that these diseases represented two different clinical entities. Furthermore the stronger genetic component to PR3 related disease identified in earlier studies was substantiated.

An epigenetic basis for disease has also been proposed. Neutrophil levels of the chromatin modification protein complex, H3K27me3, required for gene silencing were decreased in patients with AAV, at both the MPO and PR3 loci. This phenomenon was dependent on the transcription factor encoding gene, RUNX3. Interestingly RUNX3 message was found to be decreased in patients with AAV compared to healthy controls. These studies provided the first evidence that epigenetic modifications present in AAV patients could impair gene silencing and result in aberrant expression of the target auto-antigens, MPO and PR3.[29] These recently published genetic and epigenetic studies have added considerably to our understanding of AAV.

4. Environmental factors driving disease in ANCA vasculitis

In addition to genetic factors, environmental factors contribute to the loss of tolerance, the development of autoimmunity (to MPO or PR3) and subsequent organ injury. Environmental triggers that have been implicated in disease pathogenesis include environmental toxins, pharmacological therapies and infections, for which there is the strongest evidence.

Epidemiological studies have demonstrated increased incidence of ANCA vasculitis, and more specifically MPA, is increased in patients exposed to a variety of environmental toxins,[30] in particular silica.[31] This is thought to result from environmental toxins serving as adjuvants to the immune system.[32] The development of ANCAs, in particular those reactive to MPO, is not uncommon after treatment with propylthiouracil,[33] although systemic disease following treatment is uncommon. Overt MPA with focal ne-

crotising glomerulonephritis has been described in patients treated with penicillamine[34] and hydralazine.[35] The rarity of these phenomena has prevented us from learning more about disease pathogenesis.

Links between infection and ANCA vasculitis have been suggested for some time, with seasonal variation in disease presentation suggesting a correlation with microbial infection.[36] Moreover results from several studies suggested that infection(s) may predate disease initiation and/or relapse in GPA, MPA and pulmonary vasculitis.[37-40] It must be noted that these results are contentious and other studies have not confirmed them.[30] However, nasal colonization with *Staphylococcus Aureus* is significantly increased in patients with GPA and increases the relative risk of relapse over 7 fold.[37] In a key study, published more than 15 years ago, it was shown that prophylactic antibiotic therapy (co-trimoxazole) successfully decreased disease relapses in ANCA vasculitis. This effect was presumed to result from decreased nasal carriage of *Staphylococcus Aureus*. [41] Interestingly, despite this finding long-term maintenance therapy with co-trimoxazole is not the standard of care in many centres, which may reflect concerns about the long-term safety of the drug. Consistent with an infective trigger to the development of AAV; features of vasculitis have been described in patients with bacterial endocarditis.[42-43] Despite the strong evidence linking infection with the development of autoimmunity (MPO/ PR3) and the ensuing organ injury few mechanistic links have been provided, until recently.

Several mechanisms have been proposed to link infection with the development of AAV, including the use of complementary proteins, molecular mimicry and the ligation of Toll like receptors (TLRs) which heighten innate and adaptive immune responses as well as activating resident kidney cells. A series of clinical and experimental studies have supported each of these concepts, however it is likely that these mechanisms act, at least partially, in combination.

Molecular mimicry refers to the development of antibodies to host proteins after (repeated) exposure to foreign antigens, this occurs due to structural similarities between host and foreign proteins. Molecular mimicry has been proposed as a reason for the loss of tolerance to self and the subsequent development of autoimmunity.[44] In a series of elegant experiments it was demonstrated that antibodies to the lysosomal associated membrane protein-2 (LAMP-2) were highly prevalent in patients with ANCA vasculitis. Furthermore LAMP-2 was pathogenic and administration of polyclonal LAMP-2 to rodents resulted in a characteristic pattern of AAV, with focal necrotising glomerulonephritis, similar to that observed in human renal vasculitis. We will discuss LAMP-2 in more detail later in this chapter. There is homology between the immunodominant LAMP-2 epitope and the peptide of FimH, which is a component of the fimbriae of Gram negative bacteria. It is hypothesized that certain patients infected with Gram negative bacteria would generate antibodies to LAMP-2 and develop vasculitis, through the process of molecular mimicry.[45] This highly plausible theory provides one explanation for the clinical association between infection and the development of ANCAs or LAMP-2 antibodies.

An earlier study reported that a form of molecular mimicry could link *Staphylococcus Aureus* infection with the development of AAV. This process was more complex and involved the use of complementary proteins. The authors observed that patients who were PR3-ANCA positive

also had antibodies to a complementary PR3. Complementary PR3 is the protein sequence resulting from transcription of the antisense DNA strand of the PR3 gene. Subsequently it was found that mice immunized with complementary PR3 also developed PR3-ANCA, suggesting a form of molecular mimicry. Pendergraft et al proposed that loss of tolerance, with the development of autoantibodies, could develop as a consequence of immune responses directed against a complementary protein to the autoantigen.[46]

Both of these studies utilized human samples and elegant rodent models to propose infections as initiators of autoimmunity and renal vasculitis. Further work in this field is required to facilitate a better understanding of how molecular mimicry functions in humans and what organisms could be involved.

Infections activate and ligate Toll-like receptors (TLRs). These receptors are innate pattern and danger recognition receptors, ubiquitously expressed on immune cells, and resident tissue cells, which heighten innate and adaptive immune responses in response to infection or danger signals. Ligation of TLRs after infection can stimulate host immune responses, promoting auto-inflammatory and auto-immune responses. Furthermore TLR ligation can stimulate endothelial cells and other resident kidney cells to generate a cytokine milieu conducive to the recruitment of inflammatory leukocytes.

5. The role of adaptive immunity in the development of ANCA autoimmunity and glomerulonephritis

5.1. The role of humoral immunity in AAV pathogenesis

Since their description in the 1980s antibodies directed against MPO and PR3 have formed the diagnostic hallmark of AAV. While not entirely specific there is a strong association between MPO-ANCA and MPA, while PR3 is commonly associated with GPA. Clinical and experimental studies have supported the notion that ANCA are pathogenic. Furthermore therapies targeting (humoral immunity and) ANCAs, including plasma exchange[47] and the anti-CD20 monoclonal antibody Rituximab,[48-49] have been successful in clinical practice. Most of the experimental evidence has supported a role for MPO in disease, but more recently an animal model of PR3-associated vasculitis has also been developed. This represents a significant advance and it is anticipated that this model will facilitate an improved understanding of the pathogenesis of PR3-AAV. In this section, we will also discuss other roles for B cells including their function as antigen presenting cells (APCs) and as potential regulators of disease.

Are ANCAs pathogenic? There has been increasing evidence supporting a pathogenic role for ANCAs. Results from *in vitro* studies demonstrate that ANCAs activate primed neutrophils which degranulate and deposit autoantigens in glomeruli. Similarly results from *in vivo* studies, including an expanding number of animal models, have confirmed a pathogenic role for ANCAs. *In vitro* studies have consistently demonstrated that neutrophils from patients with AAV express increased amounts of the target antigens (MPO/PR3) on their cell surface. [50] These auto-antigens are targets for ANCA binding. Furthermore, several cytokines

including tumor necrosis factor (TNF), IL-18 and granulocyte macrophage colony stimulating factor can prime neutrophils in AAV, increasing auto-antigen expression which facilitates ANCA binding.[51-53] Binding of ANCA to the neutrophil is associated with increased adherence to the endothelium, superoxide generation and cytokine production.[51, 54] The effect of neutrophils and their interaction with the endothelium will be discussed in greater detail later in this chapter.

Animal studies have demonstrated a pathogenic role for ANCAs. The model described by Xiao et al was one of the first murine models of AAV, which produced severe renal injury. The observed renal injury bore considerable resemblance to that seen in human rapidly progressive glomerulonephritis. In this model MPO deficient mice were immunized with MPO. Subsequently the spleens of these MPO deficient mice were transferred into recombinant activation gene knockout (RAG2^{-/-}) mice, which lack adaptive immunity. After transfer of splenocytes (from MPO immunized MPO^{-/-} mice) RAG2^{-/-} mice developed humoral autoimmunity with the production of MPO-ANCAs. Kidneys from these mice displayed the hallmarks of severe crescentic glomerulonephritis. The authors also performed a passive transfer experiment, administering MPO-ANCAs to RAG2^{-/-} mice. The passive transfer of MPO-ANCA to RAG2^{-/-} mice resulted in a milder form of glomerular injury compared to that seen after splenocyte transfer.[55] These experiments highlighted the pathogenic role for MPO-ANCAs, however, it should be noted that the severe injury occurring after the transfer of splenocytes could reflect cellular immunity contributing to renal injury. None the less, the passive transfer of ANCAs to mice has consistently resulted in a degree of renal injury, which is neutrophil,[56] lipopolysaccharide[57], TLR4[58] and complement [59] dependent.

Additional evidence for a pathogenic role for MPO in driving AAV and renal injury was demonstrated in Wistar-Kyoto rats. Rats developed focal necrotizing glomerulonephritis and pulmonary vasculitis after immunization with purified human MPO. Furthermore a pathogenic role for the chemokine CXCL1 (the rodent homolog of human IL-8) in neutrophil-endothelial interactions was demonstrated, by analysis of neutrophil migration in the capillary beds.[60] Recently Little has described a model of vasculitis, dependent on PR3-ANCA, which develops in mice with a humanised immune system. This model was generated by treating irradiated NOD-scid-IL-2R γ ^{-/-} mice with human haematopoietic cells. In NOD-scid-IL-2R γ ^{-/-} mice there are multiple deficiencies in the function of both innate and adaptive immune cells. These chimeric mice were then treated with human immunoglobulin from patients with PR3-ANCA vasculitis or control serum. In control treated mice no glomerular injury was observed, however mice treated with PR3-ANCA demonstrated (at least mild) glomerulonephritis, while more severe injury was observed in 17% of PR3-ANCA treated mice.[61] While further work is required to confirm that this murine model is robust, it is anticipated that it will provide a good basis to explore the pathogenic nature of PR3-ANCA in clinical practice.

Another potential antigenic target is LAMP-2. Antibodies to LAMP-2 were reliably detected in more than 90% of patients with active ANCA associated necrotising crescentic glomerulonephritis. LAMP-2 antibodies were detected even when MPO-ANCA and PR3-ANCA could not be detected, suggesting this test may have improved diagnostic sensitivity and could possibly be useful for serological diagnosis in patients with renal limited vasculitis, who

traditionally are found to be ANCA negative. Antibodies to LAMP-2 were also pathogenic and administration of human LAMP-2 antibodies to Wistar Kyoto rats resulted in pauci-immune focal necrotizing glomerulonephritis.[45] Subsequently, the authors working with several collaborative groups, have verified the prevalence of antibodies to LAMP-2 in cohorts of ANCA patients from a range of European countries. Three different techniques; enzyme linked immunosorbent assay; western blotting and an indirect immunofluorescence assay were all readily able to detect antibodies. Interestingly antibodies were undetectable shortly after treatment, although they were detectable during clinical relapse, highlighting the potential usefulness of these antibodies in clinical practice.[62] However studies from the United States could not confirm these findings, where the sensitivity of detecting LAMP-2 antibodies was much lower than that seen within the European studies.[63] The divergence of results is interesting and suggests that further work is required to facilitate assays which could result in the development of better diagnostic tools.

Most studies examining the pathogenic role of B cells in AAV have focussed on their role as effector cells, however B cells have a more diverse range of functions than autoantibody production alone. In other scenarios B cells are considered antigen presenting cells, while they possibly influence T cell responses.[64]

The B cell activating factor (BAFF) has also been shown to be elevated in patients with AAV,[65] which is exciting considering the therapeutic promise shown with BAFF inhibitors in systemic lupus erythematosus (SLE).[66] B cells may also contribute to disease in other ways and a detailed analysis of renal biopsies from patients with AAV demonstrated significant B cell infiltration, including organized B cell clusters.[67] In addition to pro-inflammatory responses B cell also display regulatory function and produce IL-10, a regulatory cytokine. Interestingly in patients with SLE regulatory B cells (Bregs) are impaired and are unable to suppress effector T cells.[68] While this has not been explored to date in vasculitis, it remains possible that heightened humoral and cellular immunity occurs as a consequence of impaired Bregs.

In concluding, B cells form the diagnostic hallmarks of ANCA vasculitis and are pathogenic. The success observed in clinical practice with therapies which chiefly target B cells has not been fully elucidated and may extend beyond autoantibody inhibition. Interestingly, Rituximab was shown to treat the clinical symptoms of GPA, even when ANCAs were not detectable.[69] An in-depth understanding of the role of humoral immunity is awaited and may help direct future therapies.

5.2. The role of cellular immunity in AAV pathogenesis

While ANCAs are diagnostic and pathogenic in AAV, cellular immunity is an essential requirement for the initiation and continued production of auto-reactive B cell responses and for driving effector cell responses in the kidney. Evidence for a key role for cellular autoimmunity in AAV comes from several lines of evidence, including observational studies in humans, reports of refractory disease responding to treatments targeting T cells and extensive murine studies showing a pathogenic role for T cells in the development of autoimmunity. Vasculitis involving the glomerular capillary bed has little or no antibody deposition, but

rather demonstrates delayed type hypersensitivity responses, including fibrin deposition. This is most likely to be a consequence of auto-reactive CD4⁺ effector cells recognizing MPO, which is present in glomeruli in both human and experimental ANCA vasculitis [70-72]. In addition to enhancing inflammation, regulatory T cells (Tregs) are likely to have an important role in modulating immune responses and glomerular injury.

T cells are active participants in the loss of tolerance and the development of autoimmunity in AAV. Firstly we know that ANCAs are class switched high affinity antibodies which are (therefore) dependent on T cells for their generation.[73] Secondly, in proliferation studies, it has been demonstrated that auto-reactive T cells from patients with AAV respond to MPO and PR3,[74] while markers of T cell activity are increased in parallel with disease activity.[75-76] Furthermore, in renal biopsy samples from patients with AAV, the number of infiltrating T cells correlates with the severity of injury. Additional evidence supporting a pathogenic role for T cells was provided when 15 patients with refractory vasculitis, resistant to other therapies, were successfully treated with anti-thymocyte globulin, which targets T cells.[77]

Early studies supported a role for T helper (Th) 1 (and possibly Th2) cells in the pathogenesis of AAV. Peripheral blood lymphocytes from patients with MPO-ANCA were shown to produce IFN γ when stimulated.[78] The more recently defined Th17 cells represent a distinct lineage of CD4⁺ T cells, which are characterized by the production of IL-17A.[79] Two key human studies supported a role for Th17 cells in ANCA vasculitis. Firstly it was demonstrated that when peripheral blood from GPA patients was stimulated with PR3, there was an increased percentage of IL-17A producing CD4⁺ T cells (Th17). After stimulation no difference in IFN γ production was seen, suggesting that Th1 cells were not involved. The authors proposed that this skewed Th17 response supported a role for Th17 cells in disease.[80] A subsequent study demonstrated that sera from patients with active AAV consistently displayed a Th17 phenotype. Cytokines associated with Th17 cells, including IL-17A and IL-23, were increased in patients with acute AAV, while levels of IFN γ were unchanged. Interestingly immunosuppressive therapy did not consistently decrease IL-23 or IL-17 production.[23] In a study of human ANCA biopsies it has been shown that IL-17A producing CD4⁺ T cells constitute part of the inflammatory infiltrate and correspond with disease severity.[81] In addition, murine models have provided strong evidence for a pathogenic role for CD4⁺ T cells in glomerulonephritis.

An MPO-dependent murine model which demonstrates considerable homology to human ANCA vasculitis, where mice develop autoimmunity to MPO and focal necrotising glomerulonephritis was described. Immunization of C57BL/6 wild type mice with MPO results in cellular and humoral autoimmunity to MPO. A small dose of sheep anti-mouse glomerular basement membrane serum is subsequently administered. Treatment of chicken ovalbumin (OVA) immunized mice with this dose of sheep anti-mouse glomerular basement membrane serum does not result in significant renal injury. However in mice immunized with MPO and then sheep anti-mouse glomerular basement membrane serum significant renal injury is seen. Depletion of CD4⁺ effector cells significantly attenuated glomerular injury in this model, while experiments performed in B cell-deficient mice did not show renal protection.[72] These results provide strong evidence for a pathogenic role for CD4⁺ effector cells contributing to rapidly

progressive glomerulonephritis in MPO-ANCA vasculitis. Subsequent work from this group has supported a role for both Th1 and Th17 cells in disease. Firstly, using IL-17A^{-/-} mice it was shown that the development of cellular autoimmunity and necrotizing glomerulonephritis was IL-17A dependent. Secondly in the absence of IL-17A there was a decrease in glomerular neutrophil and macrophage recruitment and renal injury was attenuated. These results highlight the potential therapeutic benefits of IL-17A blockade in AAV.[24] This group has also elucidated that both IL-17A and IFN γ can drive nephritogenic autoimmunity and renal injury in AAV. Interestingly ligation of different TLRs dictated the pattern of cytokine production, TLR2 ligation promoted the development of Th17 autoimmunity, while TLR9 ligation drove Th1 autoimmunity. Mice which developed Th17 induced renal injury were successfully treated with anti-IL-17A monoclonal antibody (mAb). Conversely in mice that developed predominant Th1 driven injury, administration of anti-IFN γ mAb attenuated renal injury.[82] Work from Richard Kitching's group has further refined our understanding of the role of CD4⁺ T cells in the pathogenesis of AAV. Using 20 amino acid sequence peptides they identified the immunodominant MPO CD4⁺ T cell epitope. Subsequently they produced T cell clones which were specific for this immunodominant MPO epitope, which were then injected into mice. Using three different techniques it was demonstrated that when the MPO peptide (or whole MPO) was deposited in glomeruli focal necrotising glomerulonephritis was driven by antigen specific CD4⁺ T cells.[83] These key studies have helped define how effector T cells drive glomerular injury.

6. The role of Th17 cells in autoimmunity and glomerulonephritis

The original description of Th1, IFN γ producing and Th2, IL-4 producing, T helper cells by Mosmann and Coffman [84] has been expanded to include a new subset of Th cells, the IL-17A producing Th17 cells.[79, 85-86] While the prototypic cytokine produced by Th17 cells is IL-17A, these cells produce numerous other cytokines, including the ubiquitous IL-6, TNF and IL-1 β . [85] Two transcription factors are critical for the development of Th17 cells; STAT3 and Ror γ t.[87-88] For the induction and maintenance Th17 cells, several cytokines are required, these include; IL-23,[89] IL-6, TGF- β , [90-93] while IL-21 is required for amplification of Th17 cells.[94-96]

Prior to the discovery of Th17 cells, autoimmunity was believed to be predominantly a Th1-mediated phenomenon. There were inconsistencies, however, in this paradigm, for example IFN γ ^{-/-} mice developed exaggerated organ inflammation and injury in experimental autoimmune models.[97-98] Subsequently it was demonstrated that organ injury (in the most common autoimmune model, experimental autoimmune encephalomyelitis [EAE]) was unchanged in IL-12p35^{-/-} mice (functionally Th1 deficient), while injury was significantly attenuated in IL-12p40^{-/-} (functionally Th1 and Th17 deficient) and IL-12p19^{-/-} (functionally Th17 deficient) mice.[99] Similarly IL-17A^{-/-} mice were protected from EAE, [100] while increased IL-17 expression was seen in patients with multiple sclerosis, [101] a common autoimmune disease seen in clinical practice, which is the human equivalent of EAE. Further studies have implicated Th17 cells in several autoimmune diseases including rheumatoid

arthritis,[102] consistent with this finding IL-17A^{-/-} mice are protected from murine experimental arthritis.[103-104] IL-17A has been implicated in inflammatory bowel disease, both experimental[105] and clinical[106] as well as human inflammatory skin conditions.[107-108]

7. Th17 cells in the kidney

Early studies performed in gene deficient mice supported a role for Th17 related cytokines in the development of experimental autoimmune glomerulonephritis[109] and sheep anti-mouse glomerular basement membrane disease.[110] A pathogenic role for ROR γ t, the key IL-17A transcription factor, was also demonstrated in a murine model of crescentic glomerulonephritis.[111] A direct role for Th17 cells acting as effectors was subsequently published. The antigen, ovalbumin (OVA), was planted in the kidneys of RAG1^{-/-} mice, after the conjugation of OVA to a non-nephritogenic antibody specific for the glomerular basement membrane. This was followed by the administration of Th-17 polarized ovalbumin specific CD4⁺ T cells, which resulted in neutrophil mediated proliferative glomerulonephritis.[112] Detailed reviews of the role of Th17 cells in kidney disease have recently been published.[113-114]

Th17 cells are a distinct line of CD4⁺ T helper cells with unique transcription factors and effector cytokines. These cells are active participants in the development of autoimmunity but are also involved as effector cells in autoimmune conditions including rapidly progressive glomerulonephritis.

In addition to CD4⁺ effector T cells other T cells are likely to contribute to AAV. Several years ago it was demonstrated that CD4⁺ effector memory cells (Tem) were increased in the blood of GPA patients in remission, compared to those with active disease.[115] While Tem were decreased in the blood, they were increased in the urine of patients with active disease - suggesting that these cells may influence renal injury during active disease.[116] Further *in vitro* studies suggested that in GPA patients these cells could mediate endothelial injury and thus play a role in driving glomerular injury.[117] Fewer studies have assessed potential pathogenic roles of CD8⁺ T cells in AAV, however it would seem likely that these cells are involved. A study assessing gene expression and outcome in AAV and SLE patients suggested that CD8⁺ T cell signatures and increased CD8⁺ T cell memory populations were associated with poorer outcomes.[118] It was hoped that results from these studies would facilitate more individualised treatments. It would seem important that we further explore the role of CD8⁺ T cells in AAV.

Regulatory T cells (Tregs) represent a subset of CD4⁺ CD25⁺ T cells which perform a key role in regulating inflammation and tissue injury. These cells are identified through the expression of FoxP3, which is considered a master regulator of Tregs. In several autoimmune diseases, including Goodpasture's disease, Tregs are required for the maintenance of tolerance and loss of Treg function can result in the development of autoimmunity and organ injury.[119] In GPA clinical studies have shown that although circulating FoxP3-expressing Tregs vary in number their suppressive capacity is reduced.[120] In MPA patients (and experimentally) FoxP3-expressing Tregs display diminished capacity to suppress antigen specific MPO responses an

effect mediated through tryptophan.[121] Our current understanding of the role of Tregs in AAV is limited and further studies are required to improve our knowledge of their role in disease pathogenesis in order to facilitate treatments aimed at optimizing their therapeutic potential. It is well known that Th17 cells and Tregs require many of the same cytokines for growth and development and it has been postulated that they have an inverse relationship. Whilst this explanation may be simplistic it is attractive to hypothesise that both the initiation of disease and flares seen in AAV could be attributed to an imbalance in the Th17: Treg ratio; with Th17 overactivity promoting disease. This imbalance could be targeted in future treatment protocols.

8. Innate immune responses in ANCA associated vasculitis

8.1. Neutrophils, key effector cells, in ANCA associated vasculitis

Neutrophils play a critical role in the pathogenesis of ANCA vasculitis. Not only are neutrophils the primary effector cells in the kidney but neutrophils also contain the target autoantigens, MPO, PR3 (and LAMP-2) and hence are directly involved in the auto-immune process. We will discuss three different aspects of neutrophil involvement in disease, (a) The role of the Neutrophil in the development of Autoimmunity, (b) Neutrophil Activation by ANCAs and (c) Neutrophil Endothelial Interactions, which initiate glomerular injury.

8.1.1. *The role of the neutrophil in the development of autoimmunity*

It is well established that ANCAs bind to the autoantigens, MPO or PR3, located on the cell surface of the neutrophil. How and why these autoantigens translocate to the cell surface is poorly understood. We know that neutrophils die through apoptosis or necrosis and data suggests that neutrophil death through apoptosis can promote the loss of tolerance to MPO or PR3. After cell death neutrophils release granule constituents, including MPO and PR3, which translocate to the cell surface[122-123] where they serve as antigenic targets. This phenomenon was thought to occur exclusively after neutrophil death through apoptosis, which is possibly related to a slower mechanism of cell death, although the operational mechanisms of this system require further clarification.

An additional pathway linking neutrophil cell death and autoimmunity has recently been proposed, involving a distinct method of neutrophil death involving neutrophil extracellular traps (NETs). Neutrophils extrude NETs which consist of chromatin structures and include anti-microbial peptides such as; MPO, PR3 elastin, cathepsin, and lactoferrin.[124] Dying neutrophils extrude NETs to kill invading pathogens in a process recently named NETosis. It is understood that neutrophils, through NETosis, contribute to the development of autoimmunity, a concept well established in SLE. In SLE, in response to chronic autoantibody stimulation neutrophils and their NETs activate plasmacytoid dendritic cells which secrete IFN α . [125-127] NETosis has been linked with glomerular injury in AAV, through the enhancement of endothelial-leukocyte interaction,[71] however only recently have NETs been implicated in the development of ANCA autoimmunity. NETotic neutrophils interacted with

myeloid dendritic cells (mDC). This interaction was not observed when neutrophils died by necrosis or apoptosis. This process was dependent on both TNF and IFN γ and in their absence NETosis did not occur. The interaction between the NETotic neutrophil and the mDC resulted in the transfer of MPO and PR3 to the mDC, which potentially could induce and promote adaptive immune responses. This process was confirmed to be pathogenic *in vivo*. Mice were immunized with mDCs co-cultured with NETotic neutrophils (6 times intraperitoneally) and three months later they developed ANCAs and showed evidence of renal injury. The mice also displayed features consistent with systemic auto-immune disease. A similar process was thought to be present in human AAV. Assessing skin lesions from patients with MPO-ANCA vasculitis revealed an interaction between mDCs and neutrophils, with uploading of the auto-antigens.[128] While this process is not yet completely understood, NETosis potentially explains how autoantigens are recognized by antigen presenting cells, activating cellular and humoral autoimmunity in AAV.

While neutrophil apoptosis and NETosis provide some insight into the role of the neutrophil in the development of AAV, there remain several 'gaps' in our knowledge. Why AAV patients develop autoimmunity to MPO/PR3, with an associated clinical syndrome and yet they do not develop autoantibodies to other neutrophil constituents which are released after cell death is unclear. The driving factors behind apoptosis and NETosis have not been well established. Further studies in this area are required before definitive conclusions can be reached. In addition to promoting autoimmunity the ANCA-neutrophil interaction is a key to two other mechanisms of injury, ANCA binding to neutrophils leading to neutrophil activation and an oxidative burst, and the recruitment of ANCA bound neutrophils to the glomerulus where they initiate renal injury.

8.1.2. Neutrophil activation by ANCAs

The first paper suggesting a role for ANCA in activating neutrophils was published over 20 years ago. In this landmark paper it was shown that both MPO-ANCA and PR3-ANCA could bind to primed neutrophils. After neutrophil priming by tumour necrosis factor (TNF) MPO and PR3 were translocated to the cell surface, providing an autoantibody antigenic target. Neutrophil binding by ANCAs produced an oxidative burst and resulted in degranulation. [115] Other authors have confirmed this process and shown that it is also Fc γ RII-dependent.[129] Cytokine priming of neutrophils is important for ANCA binding as it increases surface expression of the autoantigens and mobilizes the NADPH oxidase complex, further increasing ANCA binding.[27, 53] In addition to TNF, IL-18 and granulocyte macrophage colony stimulating factor can prime neutrophils and enhance ANCA binding.[52]

The pathogenic role of TNF in AAV has attracted significant interest both experimentally and clinically. In a passive transfer model of MPO-ANCA vasculitis pre-treatment with lipopolysaccharide (LPS) increased systemic inflammation and glomerular injury. The *in vitro* oxidative burst induced by MPO-ANCA required TNF and anti-TNF mAb treatment attenuated renal injury *in vivo*. [57] Similar results were found by other authors who demonstrated that mAb directed against TNF successfully attenuated established glomerulonephritis in a rat model of AAV. Despite intact humoral responses there was a decrease in functional and histological

injury in rats receiving the treatment.[130] The anti-TNF mAb, Infliximab, has been used with some success in patients with AAV. While Infliximab therapy was useful in treating patients with refractory disease,[131-132] disappointingly the addition of TNF blockade (Infliximab[133] or Adalimumab[134]) to standard treatment regimes did not result in an improvement in clinical outcomes and Etanercept, (a fusion protein TNF inhibitor) could not decrease relapse rates in GPA.[115] Despite the lack of efficacy of TNF blockade in these trials, it would seem that TNF has a central role in the pathogenesis and in selected patients the use of TNF blockade may be associated with clinical improvement. In addition to TNF other cytokines and chemokines are likely to be important in AAV. Interestingly it was first appreciated over 15 years ago that neutrophils stimulated by ANCA produce IL-1 β . [135] Few studies have further investigated this observation. Recently the Inflammasome, which is a pattern recognition receptor which is characterized by the production of IL-1 β , has been shown to promote auto-inflammatory and auto-immune disease. It is possible that the Inflammasome is involved in vasculitis. This is clinically relevant because diseases resulting from overstimulation of the Inflammasome have been successfully treated with monoclonal antibody therapy.[136] This warrants further investigation.

The intracellular signalling pathways activated by ANCA neutrophil binding are multiple, although several pathways are shared. Whereas the Fc portion of ANCA IgG activates tyrosine kinase pathways,[137] the F(ab')₂ portion activates a G protein pathway.[138] Despite initiating two separate pathways, these pathways converge on the p21^{ras} GTPase, which is essential for many neutrophil functions.[139] The identification of these pathways, combined with the development of antibodies directed against specific components of these pathways, predominantly used in preclinical models, has increased expectations of their potential therapeutic use in autoimmune diseases including renal vasculitis.[140-141]

8.1.3. Neutrophil- endothelial interactions

The interaction between neutrophils and endothelial cells is important in the initiation of glomerular lesions, including fibrinoid necrosis, which is frequently observed in patients with renal vasculitis. Under normal physiological conditions neutrophils do not interact with the endothelium, however when the endothelium is activated resulting in increased expression of adhesion molecules and chemokines (and neutrophils are activated) neutrophil recruitment, binding and transmigration is increased. Our understanding of this complex dynamic has been improved through the use of in vitro systems, which include flow chambers mimicking blood flow in human capillaries. For these studies neutrophils from healthy controls and patients with AAV have been compared. Further information has been gleaned from experimental models using live imaging of the kidney, including intravital microscopy.

It is likely that TNF production and complement activation in AAV patients results in a persistent low grade activation of neutrophils.[142] Results from in vitro studies have shown that neutrophils exposed to ANCA bind to human umbilical vein endothelial cells (HUVECs), [143-144] with up-regulation of CD11b, an adhesion molecule.[145-146] In a flow system set up to mirror blood flow in human capillaries, ANCA treated neutrophils demonstrated increased adhesion and transmigration which was β 2 integrin and CXCR2 (neutrophil cell

surface receptors) dependent.[146] This is likely to resemble what happens in human AAV, where expression of both $\beta 1$ and $\beta 2$ integrins are increased in circulating neutrophils,[147] and the adhesion molecules ICAM and VCAM are expressed on glomerular endothelial cells.[148] Additional results from human studies have implicated IL-8 in leukocyte recruitment which was also shown to correlate with glomerular injury.[51] Neutrophil degranulation with the accompanying release of reactive oxygen species, proteases[149-150] and an oxidative burst[151-152] directly leads to endothelial injury. Evidence for enhanced endothelial injury includes increased levels of endothelial cell microparticles in active disease, which subsequently reduce when the disease remits.[153-154] This is in direct contrast to the restorative endothelial progenitor cells which are decreased when disease is active.[155-156] It has been suggested that the pro-angiogenic protein, angiopoietin-2, may act locally to promote inflammation and endothelial cell injury.[154] It is likely that several mechanisms combine to result in endothelial injury. We know that neutrophil degranulation also results in deposition of the neutrophil constituents, MPO and PR3 in the glomerular bed,[157] and these deposited autoantigens provide targets for antigen specific T and B cells, which recruit additional effector cells, promoting a vicious cycle of injury.

Many of the original studies assessing neutrophil recruitment to the capillaries used intravital imaging of mesenteric and cremasteric vessels. These vessels are more accessible and provide some parallels with leukocyte recruitment seen in renal and lung vasculitis. More recently Michael Hickey's group have pioneered new methods for assessing neutrophil physiology in the inflamed glomerulus, which has considerably improved our understanding of leukocyte behaviour in glomerulonephritis.[158-159].

In vitro studies performed in a flow chamber have shown that human neutrophils treated with ANCA display altered patterns of rolling, adhesion and transmigration.[146, 160] Using intravital microscopy to visualise mesenteric postcapillary venules Little et al found that administration of MPO-ANCA induced neutrophil adhesion and transmigration. Similarly studies using intravital microscopy to visualize murine cremasteric postcapillary venules demonstrated increased neutrophil adhesion and transmigration after the passive transfer of MPO-ANCA. Neutrophil recruitment was both Fc γ receptor and $\beta 2$ integrin dependent.[161] While these studies provided valuable insight into neutrophil recruitment and transmigration in inflamed tissues in AAV, it remained unclear if the observations seen in the postcapillary venules could be replicated in the glomerulus. The use of live imaging of the murine kidney has facilitated the study of leukocyte behaviour in models of glomerular injury. Differences in neutrophil behaviour in the inflamed glomerulus have been noted. In the heterologous phase of renal injury induced after administration of sheep anti-mouse GBM serum, neutrophil recruitment occurred via rapid arrest and occurred in the absence of rolling.[158] Relevant to AAV, in mice treated with LPS and MPO-ANCA glomerular neutrophil recruitment occurred in a lymphocyte function-associated antigen (LFA-1)(a leukocyte integrin) dependent manner. However if an increased dose of MPO-ANCA was used (without LPS priming), neutrophil recruitment was $\alpha 4$ -integrin dependent, but $\beta 2$ -integrin independent.[18] These studies highlight how MPO-ANCA can induce glomerular neutrophil recruitment through many different pathways and furthermore demonstrate that the glomerulus is

a unique organ in which neutrophil migration differs from other postcapillary venules. While it is likely that injury in humans with renal vasculitis is a consequence of several mechanisms (discussed above) acting in tandem, direct visualization of the kidney appears to be the best technique to assess glomerulonephritis. In addition to the mechanisms detailed above there are likely to be several other factors which contribute to pathogenic neutrophil-endothelial interaction and the ensuing rapidly progressive glomerulonephritis, several of these are discussed later in this chapter.

8.1.3.1. The role of NETs in neutrophil-endothelial interactions and glomerulonephritis in AAV

The role of neutrophil extracellular traps (NETs) in the development of autoimmunity to MPO and PR3 has been discussed earlier. A further role for NETs in driving effector responses in renal vasculitis was described in an innovative paper published in 2009. In this manuscript, Kessenbrock et al, found that primed neutrophils cultured with ANCAs resulted in the development of NETs and these chromatin fibres contained the auto-antigens MPO and PR3. When neutrophils were recruited to inflamed glomeruli, degranulation of the neutrophil and NETs resulted in the deposition of these autoantigens in the glomerulus. Furthermore they demonstrated that in human kidney biopsies, from patients with AAV, NET formation was associated with areas of high neutrophil influx and acute injury.[71] It is likely that deposition of MPO and PR3 could directly result in glomerular injury, however these autoantigens could also serve as targets for auto-reactive CD4+ T cells and B cells further increasing the influx of inflammatory cells and exacerbating glomerular injury.

8.1.3.2. A pathogenic role for neutrophil microparticles in AAV

Microparticles in neutrophils contain an abundance of adhesion molecules and proteases which include the ANCA auto-antigens PR3 and MPO.[162] Recent data has shown ANCAs can induce the release of neutrophil microparticles from primed neutrophils. These microparticles bind to endothelial cells through an up-regulation of adhesion molecules and result in increased endothelial reactive oxygen species and released pro-inflammatory cytokines including, IL-6 and IL-8. The clinical relevance of this was supported by data which demonstrated that neutrophil microparticles were more readily detected in patients (children) with active AAV, while levels were suppressed in healthy controls and patients with inactive disease.[163] Several other mechanisms of neutrophil microparticle release have been described, including those triggered by the complement system, which is also active in renal vasculitis. These studies further highlight the complex nature of neutrophil induced glomerular injury in renal vasculitis. It is likely that the synchrony of many innate immune cells and adaptive immunity result in the severe injury observed in rapidly progressive glomerulonephritis and acute kidney injury.

8.2. The role of Toll Like Receptors (TLRs) in ANCA associated vasculitis

The innate pattern recognition receptors, TLRs, recognise molecular patterns commonly found in bacterial and viral organisms.[164] In response to invading microbes, TLR ligation results in a 'hard-wired' activation of the innate immune system and heightened adaptive immune

responses. While TLRs are required for protection from invading microbes, inappropriate stimulation can result in the development of autoimmunity and organ injury,[165] including renal disease.[166] In several experimental models of kidney disease, including acute kidney injury,[167] lupus nephritis[168] and crescentic glomerulonephritis,[169] we and others have demonstrated pathogenic roles for TLRs. However their role in AAV is likely to be dual. Firstly ligation of TLRs heightens innate and adaptive immune response, which in turn leads to the loss of tolerance and the development of autoimmunity. Secondly, TLRs activate both effector cells and resident kidney cells, increasing glomerular inflammation and renal injury. This is important as infections are known to promote injury in AAV, with TLRs providing a link between infection and the development of AAV and disease relapses. While many TLRs have been implicated in autoimmunity studies in AAV have largely concentrated on the surface receptors, TLR2 and TLR4, as well as the intracellular TLR9.

There is clinical evidence implicating TLRs in the loss of tolerance in AAV. Stimulation of peripheral blood mononuclear cells (PBMCs) from GPA patients with a TLR9 ligand resulted in increased ANCA production.[170] Moreover in patients with AAV in remission TLR9 expression is increased on B lymphocytes and when these B lymphocytes were cultured with a TLR9 ligand they produced ANCA.[171] These studies support a role for infection (through ligation of TLR9) promoting humoral autoimmunity. Expression of TLR2, TLR4 and TLR9 on B lymphocytes, T lymphocytes, natural killer (NK) cells, monocytes and granulocytes from AAV patients (and controls) was assessed. Amongst AAV patient's monocytes and NK cells had increased TLR expression.[172] We have provided supporting evidence for a pathogenic role for TLRs using experimental models of AAV. Immunization of WT mice with a TLR ligand and MPO resulted in the loss of tolerance with the development of cellular and humoral autoimmune responses and later necrotising glomerulonephritis. Interestingly immunization with a TLR9 ligand and MPO resulted in T-bet dependent IFN γ production and macrophage mediated renal injury. Conversely autoimmunity induced by a TLR2 ligand and MPO resulted in ROR- γ dependent Th17 autoimmunity and neutrophil mediated renal injury.[82]

However, TLRs are also likely to be involved in effector responses. While TLRs are expressed at low and often undetectable levels in normal kidney biopsies, increased expression has been seen in glomerulonephritis. In lupus nephritis glomerular and tubular TLR9 expression was shown to be increased in both children and adults.[173-174] In studies assessing patterns of TLR2, TLR4 and TLR9 in glomerulonephritis, strong TLR2 and TLR4 staining was seen in the inflammatory infiltrates of patients with AAV.[175] We have stained renal biopsies from patients with AAV and found that TLR9 staining is positive in the glomeruli (unpublished data), as illustrated.

Further studies have supported an interaction between AAV and TLRs, when epithelial cells, from kidney and lung, primed with PR3-ANCA serum produced exaggerated cytokine levels after TLR stimulation.[176] While early studies suggested that lipopolysaccharide (LPS) enhanced effector responses in AAV,[57] our understanding of this process has increased. We demonstrated that highly purified LPS, a pure TLR4 ligand, increased neutrophil recruitment and glomerular injury after the passive transfer of MPO-ANCA, in a TLR4 dependent manner.

We used bone marrow chimeras to define the relative contributions of bone marrow cell TLR4 and intrinsic renal cell TLR4 to the disease process. We found that both bone marrow and resident kidney cell TLR4 were required for maximal neutrophil recruitment and renal injury. [58] These studies highlighted the importance of TLR4 (and potentially other TLRs) in driving effector responses in ANCA vasculitis.

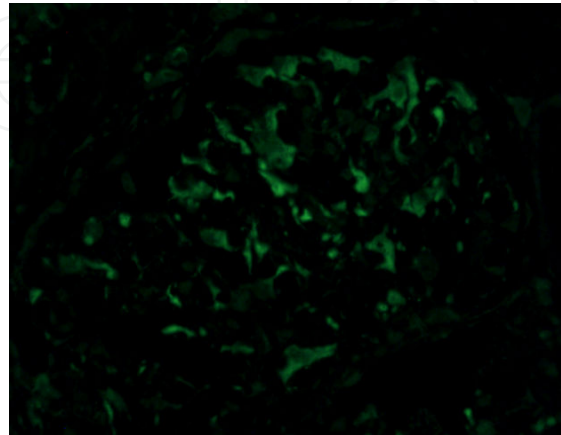


Figure 1. TLR9 staining in a kidney biopsy from a patient with PR3-ANCA vasculitis. While TLR9 staining was not detectable in normal kidney samples stained with a mAb directed against TLR9 and visualized under immunofluorescent light, TLR9 staining was readily detectable in patients with crescentic glomerulonephritis and PR3-ANCA vasculitis.

8.3. The role of complement in ANCA associated vasculitis

The complement system is recognized as one of the phylogenetically oldest components of human immune defence. This highly regulated system of proteins (together with their regulatory inhibitors) compromise an important part of host defence. In response to either innate or adaptive stimuli activation of the complement system results in a cascade of amplification and cleavage steps with the generation of anaphylatoxins (C5a and C3a) and a terminal attack complex capable of lysing cells.[177] Three complement pathways are well described, namely, the classical pathway, the alternate pathway which is initiated by recognition of foreign surfaces and the mannose binding lectin pathway.[178] More recently a pathway which is initiated by coagulation and fibrinolytic proteins has been described.[179] In addition to its role in host defence, activation of the complement cascade can result in tissue injury and has been implicated in many forms of glomerulonephritis and kidney injury. Traditionally complement was not considered critical to the pathogenesis of AAV as renal injury was considered 'pauci immune' in nature and hence free from complement (and immune complex) deposition. Interestingly complement is frequently observed in renal and skin biopsies from patients with AAV,[180-182] while in vitro studies have demonstrated a role for complement in ANCA-neutrophil interactions.

From historical data we know that when neutrophils are activated by ANCA, the complement cascade is triggered and C3a is produced.[183] We also know that priming neutrophils with C5a enhances ANCA-neutrophil interactions,[184] an effect mediated by p38 mitogen-

activated protein kinase, extracellular signal-regulated kinase and phosphoinositol 3-kinase. [185] Results from clinical studies have shown that serum and urine levels of C5a are elevated in patients with active disease strongly supporting the notion that the complement cascade is activated in active AAV. [186] Strong support for a pathogenic role for complement has also been provided from experimental studies. In an extensive set of experiments, the North Carolina group robustly demonstrated that complement depletion (achieved through the use of cobra venom serum) abrogated disease, an effect mediated through C5 and Factor B. [183] Factor B is critical for alternative pathway activation. Similarly inhibition of C5 using a mAb successfully attenuated experimental anti-MPO induced glomerulonephritis. [59] These studies detailing a pathogenic role for the alternative pathway in ANCA induced glomerulonephritis have helped improve our understanding of the disease. More recently a mAb directed against C5, Eculizumab (also known as Soliris and manufactured by Alexion Pharmaceuticals) has been licensed for the treatment of several complement mediated diseases, including paroxysmal nocturnal hemoglobinuria. There is growing interest that C5 inhibition could be used for the treatment of glomerulonephritis and organ injury induced by AAV and this has formed the basis of a clinical trial currently underway in the United States.

8.4. Dendritic cells as antigen presenting cells and effector cells in AAV

Evidence from experimental models has supported a role for dendritic cells (DCs) in initiating and promoting immune responses in autoimmune diseases. [187] These specialised antigen presenting cells (APCs) recognise antigens through pattern-recognition receptors and co-ordinate the initiation and maintenance of the immune response. [188] Little is currently known about antigen presentation and the subsequent development of autoimmunity in AAV. It is likely that DCs are involved in two processes, firstly in the development of autoimmunity through interaction with dying neutrophils and also, acting locally, promoting kidney injury where their presence in renal biopsy samples positively correlates with injury.

A pathogenic role for DCs in human AAV was recognised several years ago. When immature DCs were isolated from GPA patients and cultured with PR3, markers of DC activation, CD80 and CD86 were increased. These antigen primed DCs were able to produce IFN γ , consistent with a Th1 phenotype. [189] In an experimental model of MPO induced ANCA vasculitis, we have shown that pulsing DCs with MPO is an effective means of inducing cellular and humoral autoimmunity directed against MPO. Furthermore, using our murine model of focal necrotising glomerulonephritis, these mice developed severe functional and histological renal injury (unpublished data). It is likely that up-regulation of DCs is (at least partially) TLR mediated and in AAV this could result from infection. After immunizing WT mice with a TLR2 or TLR9 ligand and MPO we found an increase in DC maturation (assessed as an increase in CD86 expression), compared to mice immunized with MPO alone. [82] In additional unpublished work, pilot studies have shown that stimulation of DCs with a TLR9 ligand and MPO results in increased CD40, CD80 and CD86 expression, compared to DCs stimulated with MPO alone, this is demonstrated below. These clinical and experimental studies implicate DCs in the loss of tolerance to MPO.

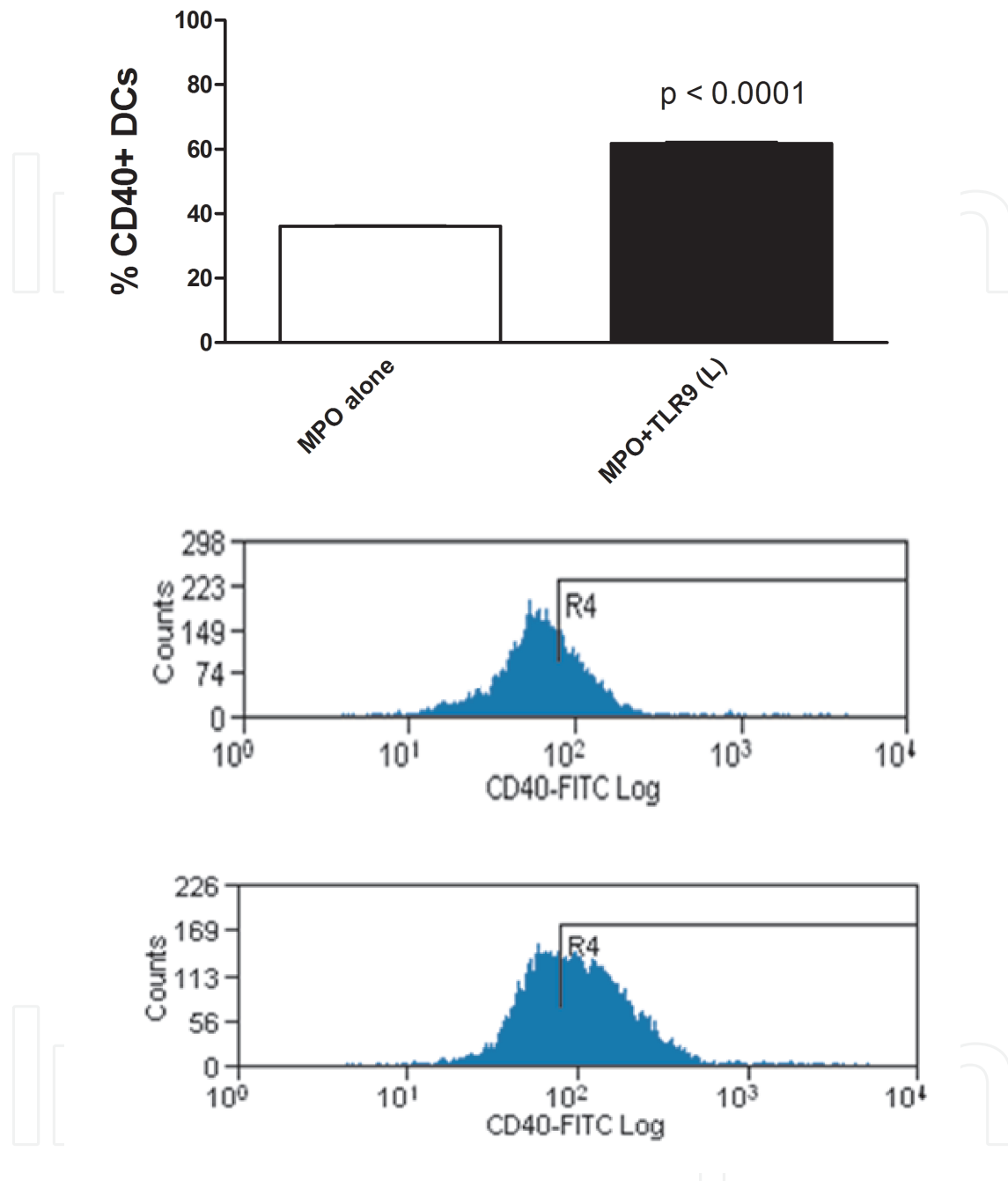


Figure 2. Dendritic cell (DC) activation after culturing DCs with MPO and a TLR9 ligand. After culturing DCs with MPO and control or a TLR 9 ligand, there was an increase on CD40+ DCs shown schematically in the top figure. In the middle figure we see representative CD40 expression from DCs treated with control and MPO, while the bottom figure shows the increase in CD40 expression after treatment with MPO and a TLR9 ligand.

A limitation of both the clinical and experimental studies is that these studies have largely focussed on myeloid DCs and not plasmacytoid DCs. In other diseases, including systemic lupus erythematosus, plasmacytoid DCs have been shown to be potent inducers of Type 1 IFNs and drive the development of autoimmunity.[190] In addition to their role in the

development of autoimmune responses DCs represent a component of the characteristic inflammatory infiltrate seen on renal biopsy samples from patients with AAV. Increased numbers of immature (CD209+) and mature (CD208+) DCs were found in renal biopsies from patients with AAV.[191] While these studies do not prove that DCs are pathogenic in renal vasculitis, their association with the inflammatory infiltrate suggests that they may be involved in the promotion of renal inflammation and injury.

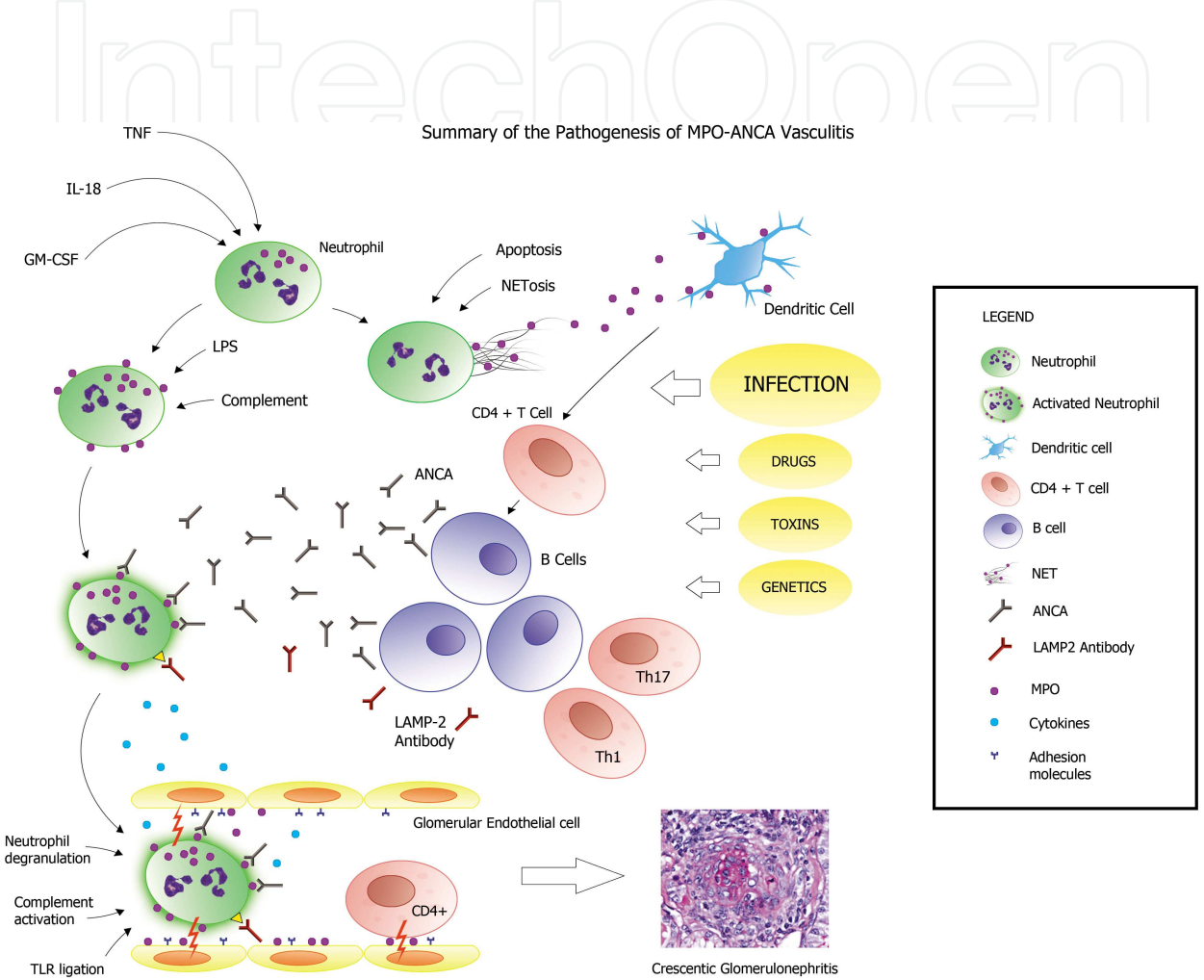


Figure 3. Summary of the Pathogenesis of MPO-ANCA Vasculitis. A summary of the events which contribute to the loss of tolerance to MPO, with the development of autoimmunity resulting in rapidly progressive crescentic glomerulonephritis.

9. The role of resident kidney cells in ANCA associated renal vasculitis

Results from many of the studies discussed above have demonstrated that the kidney is not an 'innocent bystander' in the disease process. Rather the kidney provides an anatomic and physiological milieu which is well suited to recruit inflammatory cells. In our studies we have found that TLR ligation increased ANCA induced glomerular neutrophil recruitment and injury, which required contributions from both bone marrow and resident kidney cells.[58]

Our studies strongly supported a role for glomerular endothelial cells in this disease process and the role of the endothelium in promoting inflammation and injury is well known. It is likely that other glomerular cell types contribute to injury and immunofluorescent staining of kidney biopsies from patients with AAV has demonstrated that podocytes and tubulointerstitial cells are major producers of IL-18, which is involved in neutrophil recruitment.[52] Similarly after staining human biopsies from AAV patients with crescentic glomerulonephritis the pathogenic isoform of the stress response protein kinase p38MAPK was detected in the podocyte, further implicating the role of this specialised cell in driving glomerular injury.[192] In addition to the glomerular injury observed in ANCA associated renal vasculitis tubular lesions, most notably peritubular inflammatory capillaritis, are common and are associated with a poor prognosis.[193] The interstitium is a prime target for inflammatory cells as many of the tubular epithelial cells express MHCII, TLRs and complement receptors, with which they can interact.[194] Furthermore peritubular capillaries display physiological characteristics similar to postcapillary venules which further increases the recruitment of inflammatory cells commonly observed in crescentic glomerulonephritis.[195-196] In conclusion it is apparent that the kidney harbours a particular environment which facilitates the recruitment of inflammatory cells and subsequent renal injury making it the key target for injury in AAV.

10. Conclusions

As new concepts of autoimmunity and cellular functions are elucidated in both innate and humoral immunity our scope of understanding of this complex disease entity continues to expand. Whilst an appreciation of the involvement of the adaptive immune dysfunction that contributes to AAV is well established new and varied innate immune system mechanisms of pathogenesis are emerging. Recent work investigating neutrophil functions and life cycle including the newly identified and described NETosis, along with imaging modalities allowing accurate characterisation of neutrophil trafficking and interactions with endothelial cells of the vessel wall provide us with a better understanding of the important role these cells have to play in this multifactorial disease process. The huge range of new biologic agents and advancing therapeutic technologies bring with them the possibilities of more effective, targeted, less toxic therapies for our patients.

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