ANCA-associated vasculitis: pathogenesis, models and pre-clinical testing

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Abstract

Our understanding of ANCA-associated vasculitis (AAV) has developed greatly since the discovery of anti-neutrophil cytoplasmic antibodies (ANCA), directed against neutrophil components, in 1982. Observations in human disease, and increasingly sophisticated studies *in vitro* and in rodent models *in vivo* have allowed a nuanced understanding of many aspects of the immunopathogenesis of disease, including the significance of ANCA as a diagnostic and monitoring tool as well as a mediator of microvascular injury. The mechanisms of leukocyte recruitment and tissue injury, and the role of T cells, are increasingly understood. Unexpected findings, such as the role of complement, have also been uncovered through experimental studies and human observations. This review focusses on the pathogenesis of AAV, highlighting the challenges in finding new, less toxic treatments and potential therapeutic targets in this disease. The current suite of rodent models is reviewed, and future directions in the study of this complex and fascinating disease are suggested.

Introduction

The anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV) are small vessel vasculitides characterized by autoimmunity to and autoantibodies specific for neutrophil granule components, predominantly proteinase 3 (PR3) and myeloperoxidase (MPO). While AAV can affect a variety of vascular beds, these diseases typically involve the kidney and respiratory tracts, together with features of systemic inflammation. Untreated, AAV is fatal with a median survival of 5 months.[1] While current therapies, many of which were introduced more than 30 years ago, have improved survival, AAV is still associated with a substantial increase in morbidity and mortality, not only due to the disease but also to the side effects of immunosuppression.[2]

For a significant period, from its initial characterization in the 1930s, our understanding of AAV was based only on observations of syndromic clinical presentations and patterns of pathology and histopathology. Since the discovery of ANCA in 1982,[3] we have made impressive progress in understanding the pathogenesis of this disease. Major autoantigens have been defined, the actions of ANCA in activating neutrophils have been elucidated, and we are coming to the realization that the pattern of effector responses in these conditions are unique to AAV. Serum ANCA measurements have become valuable diagnostic tools. These insights have come largely from careful observations of human disease, combined with the development and use of *in vitro* and *in vivo* disease models. To further unravel the pathogenesis of these complex diseases, in order to produce targeted therapies, researchers require robust and reproducible in vitro and in vivo disease models. This review focus on granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA). It will highlight some of the key features of the pathogenesis of AAV, discuss *in vitro* and *in vivo* model systems, highlight some of the strengths and weaknesses or the current suite of available models, and suggest future directions. It will not include discussion of eosinophilic granulomatosis with polyangiitis (EGPA; Churg-Strauss), a small to medium vessel vasculitis in which approximately 40% of patients are ANCA positive. EGPA is less well characterized than GPA or MPA, disease models have not been established and the role of ANCA in its pathogenesis is not clear.

Historical Perspective

Our understanding of the pathogenesis of AAV has evolved over time, often with surprising results. The discovery and characterization of ANCA and their autoantigens was the first real

evidence that GPA (then known as Wegener's granulomatosis) and MPA were autoimmune diseases. Prior to the discovery of ANCA, Stillmant et al[4] made a seminal observation – that the majority of cases of rapidly progressive necrotizing and crescentic glomerulonephritis (GN) had little or no evidence of glomerular complement or immunoglobulin deposition- i.e., they were 'pauci-immune.' Until this time, it was thought that all cases were associated with immune complex deposition or due to anti-glomerular basement membrane (GBM) disease. It was soon recognized that these pauci-immune cases of GN also had clinical evidence of systemic small vessel vasculitis and were strongly associated with circulating ANCA. Subsequently, the major targeted autoantigens were defined to be neutrophil lysosomal enzymes MPO and PR3.

The diagnostic importance of serum ANCA estimation and the development of *in vitro* systems where human ANCA were shown to bind to and activate human neutrophils,[5] (and in some studies, monocytes),[6] placed the focus squarely on a potential pathological role for ANCA in this disease. Key murine *in vivo* studies performed by transferring (anti-MPO) ANCA demonstrated that ANCA could induce GN,[7] while later, complement was identified as being critical in neutrophil activation and disease.[8] A further pathway to tissue injury, that of antigen specific T cells (initially CD4+ cells, now also CD8+ cells)[9-11] has been described in experimental anti-MPO GN where autoreactive effector T cells can recognize MPO, planted in glomeruli by ANCA-activated neutrophils and further promote injury. The discovery of a process whereby neutrophils extrude extracellular traps (NETs),[12] containing histones decorated with both MPO and PR3 led to their discovery in AAV[13] and their potential relevance in effector responses, neutrophil activation and loss of tolerance and promotion of autoimmunity.[13-15] Increasingly, data show differences in clinical features and genetic associations between anti-MPO and anti-PR3 autoimmunity, suggesting that MPO-AAV and PR3-AAV are two different but overlapping diseases. The emerging importance of these autoantigens has led to suggestions that a more accurate nomenclature for cases of AAV may be to define the disease according to autoantigen involved (i.e. MPO-AAV or PR3-AAV) and then add the relevant clinical or syndromic manifestations, usually using the terms GPA or MPA (reviewed in Hilhorst, 2015[16]). While this nomenclature has yet to be formally adopted, *in vitro* and *in vivo* models of ANCA-associated glomerulonephritis and of AAV have, for the most part, been founded on active or passive immunity either to MPO or to PR3. Therefore, in this review, where the focus is on models of disease, we will define the diseases according to their target autoantigen (e.g. MPO-AAV or PR3-AAV).

The Pathogenesis of AAV

<u>Epidemiology, genetics and risk factors</u>

AAV are rare diseases, with an variable but increasing incidence recently reported at 20 cases per million/year.[17] There is a slightly increased prevalence in males, which is more pronounced in PR3-AAV than in MPO-AAV.[16] MPO-AAV is more common in Asia, Oceania, and Southern Europe, whereas PR3-AAV predominates in the northern parts of Europe and North America.[16] Two large genome wide association studies (GWAS) have shown an association with polymorphisms in MHC class II genes and AAV; the association was strongest between HLA-DP (DP4, from other studies) and PR3-AAV, with a weaker association between HLA-DQ and MPO-AAV.[18, 19] PR3-AAV is associated with the gene encoding the PR3 antigen itself, PRTN3 as well as SERPINA, the gene for α1-antitrypsin.[18] Interestingly, there were distinct and different genetic associations with autoimmunity to PR3 and MPO, which were stronger than any genetic associations with GPA or MPA clinical phenotype.[18] This, and the fact that polymorphisms in the gene encoding for PR3 were associated with PR3-AAV contribute to the growing evidence that autoimmunity to PR3 is pathogenic and not an epiphenomenon. Increased expression of MPO and PR3 by neutrophils is also associated with disease and subject to genetic influence. A study by Ciavatta et al[20] showed that epigenetic modifications associated with silencing of MPO and PR3 genes are disrupted in patients with vasculitis, potentially contributing to inappropriate overexpression of ANCA antigens on neutrophils and setting the stage for disease to occur.

Both laboratory and clinical studies indicate that infection has an important association with AAV. Nasal carriage of Staphylococcus aureus in PR3-AAV patients is associated with higher risks of disease relapse.[21] A prospective trial showed that antibiotic prophylaxis was associated with a reduction in disease relapse rates in patients with predominantly PR3 vasculitis.[21] Molecular mimicry involving a sequence homology between complementary PR3 and parts of the *S. aureus* genome has been proposed to explain this association, although *in vivo* evidence of this is lacking.[16] Laboratory data that support the role of infection in disease pathogenesis include the discovery of LAMP-2 autoantibodies, which have sequence homology to bacterial adhesion FimH, in patients with AAV,[22] and the contribution of bacterial components such as LPS[23] and innate immune receptors such as toll like receptors (TLRs)[24, 25] to disease. Silica exposure is also associated with the development

of MPO-AAV,[26] although the mechanism for this is not yet clear. Drugs such as propothiouracil, hydralazine and levamisole-contaminated cocaine have also been associated with the development of vasculitis. Dual PR3 and MPO positivity is more common in vasculitis induced by some drugs, suggesting that these drugs may provoke a generalized loss of tolerance.[27]

Figure 1 shows key steps in AAV pathogenesis. The development of AAV firstly requires autoimmunity to the target antigen, MPO or PR3. Loss of tolerance to ANCA antigens and development of autoimmunity is a complex and multifactorial process. Data show that central T cell tolerance, at least to MPO, is mediated by AIRE (autoimmune regulator)-induced thymic MPO expression.[28] However, it is likely that in AAV, as in other autoimmune diseases, that central tolerance is incomplete, and healthy humans have low titer "natural" ANCA.[29] Whilst the mechanisms that underpin loss of tolerance to MPO and PR3 are unclear, innate immune responses, stimulated by bacterial peptides or components released from dying or damaged cells, produce powerful signals through pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that induce dysregulation of adaptive immune responses and may result in autoimmunity.[25, 30] NETs are also implicated, and experimental data show that dendritic cells (DCs) loaded with NETs can induce loss of tolerance to both MPO and PR3.[14] A recent study by Millet et al indicates that PR3 on the surface of apoptotic neutrophils facilitates a loss of tolerance by interfering with the anti-inflammatory responses that normally follow phagocytosis, instead provoking the secretion of inflammatory cytokines and a Th17 response.[31] Some studies implicate autoantigen complementarity in PR3-AAV, where tolerance may be lost via reactivity to complementary PR3 (expressed by the antisense PR3 strand) with T cell reactivity and antibodies that then induce anti-idiotype PR3-ANCA.[32] Functional abnormalities in regulatory T cells (Tregs), cells critical for the maintenance of tolerance in the periphery have been observed in humans with AAV.[33] Whether similar abnormalities exit in regulatory B cells is less clear.

Clinical and experimental data indicate that T cell help is required for the activation of autoreactive B cells and the production of ANCA. The exact conditions that cause B cells to start producing ANCA are unclear. Following the loss of tolerance, an antigen specific effector response develops to initiate and direct tissue injury. ANCA induce neutrophils to adhere to glomerular capillary endothelium. [34] For this to occur, circulating neutrophils must

be activated by cytokines, danger associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) and complement. Activated neutrophils localize to vulnerable microvascular beds, including glomerular capillaries, where they degranulate and release NETs, causing endothelial injury. This process also deposits ANCA antigens (including both MPO and PR3) in glomeruli. At least in the case of MPO, MPO can then be recognized by antigen-specific effector T cells, contributing to injury and potentially also to the development of further cell mediated autoimmunity.

ANCA: pathogenic, present and absent

Since they were first described in the 1980s, ANCA directed against MPO and PR3 have been used to diagnose AAV clinically, and have provided important insights into disease pathogenesis. Inconsistencies between the presence of ANCA and clinically evident disease caused debate about whether the ANCA themselves were pathogenic, or merely bystanders in the disease process. While evidence supports a pathogenic role of ANCA, there is a complex relationship between autoantibodies and disease. Levels of ANCA do not necessarily correlate with disease severity, remission induction or relapse.[35] ANCA have been described in healthy individuals,[29] and patients who phenotypically present with GPA or MPA can test ANCA negative by conventionally used assays.[36] Recent work by Roth et al showed that ceruloplasmin fragments within plasma may act to mask pathogenic anti-MPO autoantibodies in "ANCA negative" patients, preventing their detection by conventional serologic assays.[37] It is possible that there is another pathway, not involving conventional ANCA antigens, that is responsible for the phenomenon of "ANCA negative" ANCA-associated vasculitis. While MPO and PR3 are the best known and currently recognized ANCA antigens in AAV, other antigens can be responsible for an ANCA pattern of staining in indirect immunofluorescence in humans, and experimental studies have in some situations implicated other antigens in disease pathogenesis. Although ANCA recognizing lactoferrin, cathepsin and elastase are not known to be pathogenic, ANCA recognizing other antigens such as moesin[38] or LAMP-2[22] may be.

Falk et al first demonstrated that ANCA can bind to and activate neutrophils, which then degranulate and produce reactive oxygen species (ROS).[5] It has since been shown that ANCA binding to neutrophils also activates intracellular signaling pathways to alter cytoskeletal proteins, increase cytokine production, alter adhesion molecule expression and conformation, with adherence to the endothelium.[39] ANCA also induces primed neutrophils to form NETs,

histone rich complexes containing the autoantigens MPO and PR3.[13] These ANCA-induced NETs can activate dendritic cells and autoreactive B cells.[13] Compared to neutrophils from controls, neutrophils from patients with AAV are primed, with increased expression of PR3 and MPO on their surface, and show increased ROS production in the presence of ANCA.[40] In AAV, some macrophages can also express surface proteins that are known ANCA targets,[15] and ANCA can also bind to cells of the monocyte/macrophage lineage.[6, 41] Although the relationship between the presence and titer of ANCA and AAV is not clear-cut, clinical evidence supports a pathogenic role of ANCA. This evidence includes the efficacy of antibody depletion therapy such as plasma exchange,[42] and a case report of placental transfer of MPO-ANCA from a mother with active vasculitis to the fetus, who developed pulmonary hemorrhage and microscopic hematuria as a neonate.[43] The B cell-depleting anti-CD20 monoclonal antibody rituximab is efficacious in AAV.[44, 45] Although its actions have been attributed to direct effects on humoral immunity and ANCA production, B cells have other roles in autoimmune diseases and B cell depletion may affect adaptive immunity by several mechanisms. *In vivo* models of AAV have proven, by passive transfer of anti-MPO antibodies, that these antibodies and neutrophils mediate disease. They have also led to an understanding of the role of complement in ANCA associated neutrophil activation and have provided the pre-clinical data that has led to clinical trials using new inhibitors of C5a.

Determinants of pathogenicity

The persistence of ANCA in remission, and the re-appearance of ANCA without disease, both suggest that not all ANCA are pathogenic. There are a number of characteristics of IgG ANCA that might influence their pathogenicity, including titer, subclass, epitope and affinity. 'Natural' anti-MPO antibodies, sometimes present in healthy individuals, are of a lower titer and avidity than those from patients with AAV.[46] In both humans with MPO-AAV and in mouse models, the MPO-ANCA response involves all IgG subclasses and is directed against a variety of epitopes on MPO.[37] Fcγ receptor ligation by ANCAs must occur for full activation of leukocytes; in humans and in rodents, different IgG subclasses have different affinity for leukocyte Fcγ receptors. *In vitro* studies suggested that IgG3-ANCA have more capacity to activate neutrophils compared to other IgG subclasses, which may explain their tendency to cause renal injury.[47] Compared to ANCA of other subclasses, IgG3 ANCA are better at capturing rolling neutrophils, converting them to a static, adhesive state.[46] However, IgG1 ANCA can also induce neutrophil ROS production.[46] Although several patient studies

have examined associations between ANCA subclass and disease, no firm conclusions have been reached.[48, 49]

Epitope specificity of ANCA

Epitope mapping of both MPO[37] and PR3[50] have been performed; in particular, a study looking at MPO-ANCA has added to our understanding of disease significantly. MPO-ANCA are directed against multiple epitopes, mostly clustered on the carboxy terminus of the heavy chain. Roth et al,[37] via a high sensitivity, epitope excision and mass spectrometry approach studied MPO-ANCA epitopes from patients with active disease, and remission, as well as 'natural' antibodies from healthy controls, and found that one linear epitope, corresponding to amino acids 447-459 on MPO, was exclusive to patients with active disease. Titers of antibodies to this epitope declined in remission. Antibodies to $MPO_{447-459}$ induced ROS from neutrophils, whereas antibodies to natural epitopes were poor inducers of ROS. Intriguingly, pathogenic epitopes on MPO are located close to natural epitopes, and one could speculate that antibodies first develop against natural epitopes, and then subsequently spread to disease-causing epitopes.

Complement and AAV

Given the paucity of complement in AAV lesions, it had been thought that complement played little role in disease. However, over the last 10 years, complement has been defined as playing an important role, to the point where phase II human trials using anti-C5a therapies have been conducted. This interest has been fortunate to coincide with a renaissance in complement biology, with significant advances in understanding the system's complex biology, its pivotal role in some diseases, and in new treatments targeting the complement system. Stimulated human neutrophils can release properdin, which has an autocrine role in activating the alternate pathway. Patients with AAV have evidence of alternate pathway serum complement activation.[51] Studies in mice injected with anti-MPO antibodies showed roles for C5, C5a and C5aR, and the alternative pathway, but not C4.[8, 52] In a passive transfer model, pretreatment with an anti-C5 monoclonal antibody abrogated renal injury; treatment one day after antibody transfer also resulted in significantly decreased renal injury.[8] Recent studies in mice with a human C5a receptor showed disease could be limited by CCX168, a small molecule C5a inhibitor now in human trials in AAV.[52] C5a is present both on leukocytes and on endothelial cells and both seem to be required for disease.[53]

T cell autoimmunity and AAV

Evidence for a role of T cells in AAV comes from their presence in glomeruli, periglomerular and tubulointerstitial regions of renal biopsies, [54] as well as the efficacy of T cell depleting therapy in a small group of patients with refractory GPA.[55] Experimental work by Ruth et al in the active autoimmunity to MPO with anti-GBM globulin trigger model (described later in this article) suggested a potential role for CD4+ T cells after CD4+ depletion significantly reduced glomerular injury.[9] The immunodominant CD4+ T cell epitope, $MPO_{409-428}$, has been defined in mice,[10] and a T cell transfer model using this epitope induces GN,[56] confirming that CD4+ T cells contribute to injury in experimental AAV.

Upon activation, $CD4^+$ T cells tend to differentiate into distinct $CD4^+$ T effector subsets (Th1, Th2, Th17, Tfh and regulatory T cells [Tregs]) with typical patterns of cytokine secretion which engage effectors in different ways. IFN-γ secreting Th1 cells are important in some forms of glomerular disease and MPO-specific Th1 cells have been shown to mediate AAV in a cell transfer model.[10] IL-17A and IL-23 (the key Th17 cytokine and the upstream cytokine necessary for maintenance of Th17 cells) are elevated in the serum of patients with active vasculitis compared to healthy controls or those with quiescent disease,[33] indicating a role for Th17 cells which has also been borne out in *in vitro* and *in vivo* studies, showing ANCA induces IL-17A secretion from activated neutrophils,[57] and a murine model showing that IL-17A deficient mice were protected from anti-MPO GN.[58] The actions of CD4+ effector cells can be suppressed by Tregs. A number of lines of evidence suggest that the suppressive function of Tregs cells is impaired in AAV, either through lower frequency of these cells, or their defective function.[59] Induction of anti-MPO specific Tregs has been shown to modulate GN in mice with established anti-MPO autoimmunity.[60]

Leukocyte recruitment/tissue specificity

The development of *in vitro* models of neutrophil activation and the subsequent use of flow chamber systems showed that ANCA could upregulate and/or change the conformation of adhesion molecules to induce adhesion and recruitment. In particular, flow chamber studies implicate selectins, β2 integrins (especially CD11b) and neutrophil chemokines in mediating ANCA induced neutrophil recruitment to the activated endothelium in vitro.[61, 62] Several studies have examined neutrophil-endothelial interactions in vivo using intravital microscopy. Little et al[63] used both active disease and passive MPO-ANCA transfer in the WKY rat to

demonstrate neutrophil adhesion, migration and injury to the gut mesentery. When treated with a rat IL-8 homologue, there was significant neutrophil recruitment – which was further enhanced in the presence of ANCA. Nolan et al[64] also used a conventional vascular bed, the mouse cremaster, and found that anti-MPO antibodies augmented cytokine or chemokine induced leukocyte recruitment in an FcγR and β2 integrin dependent manner. Kuligowski et al[34] used intravital microscopy of the glomerulus to show that i) anti-MPO antibodies bound to and altered the phenotype of mouse neutrophils in vivo with and without LPS; ii) anti-MPO antibodies could induce neutrophil recruitment to glomeruli without adhesion in cremasteric post capillary venules, iii) different adhesion molecules mediated recruitment with and without priming: β2 integrins with LPS and anti-MPO antibodies, and α4 integrin, a molecule expressed on more activated neutrophils, with higher dose anti-MPO antibodies. Despite these and other studies, as yet there is no clear picture of why some microvascular beds are more vulnerable than others.

The biology of severity and prognosis in AAV

A current treatment conundrum in AAV is how to predict patients who will relapse, so that treatment intensity and duration (both a significant burden in AAV) can be individualized, or at least patients can be stratified into "low risk" and "high risk". Notwithstanding that relapse is more common in PR3-AAV, there is no current reliable way to predict relapse in AAV patient who have remitted. However, human and experimental data indicate that CD8+ T cells may also participate in AAV.[11, 65] "T cell exhaustion" is a phenomenon initially described in chronic infection or cancer, where CD8+ T cell dysfunction occurs in the setting of persistent exposure to antigen, characterized by impaired effector function, expression of inhibitory receptors and a relative lack of CD4+ positive co-stimulatory signals.[66] Recent evidence shows that in AAV and in other autoimmune diseases a "non-exhausted" T cell phenotype is a sign of poor prognosis, with a higher chance of AVV relapse.[65] These findings may pave the way for a more rational and individualized approaches to immunosuppression in AAV.

Management of AAV

Current management of AAV is centered on the induction of remission using corticosteroids and more potent immunosuppression such as cyclophosphamide or rituximab. This induction phase of intense immunosuppression, is followed, after remission is attained, by a

maintenance phase, using one of several possible drugs, including azathioprine, methotrexate or mycophenolate. While survival rates in AAV have improved over the years, morbidity and mortality, and the burden of toxicity from corticosteroids and from other immunosuppression remains high. It has been estimated that of the current mortality in the first year after diagnosis AAV, approximately 50% is related to the activity and consequences of the condition itself and the other 50% is a consequence of treatment.[2] Nonetheless, due to well-designed trials conducted by collaborative groups such as the European Vasculitis Study Group (EUVAS), we have a clearer picture of what agents are optimal for remission induction and maintenance. Cyclophosphamide has long been regarded as standard of care. However, RCT have shown in remission induction that rituximab (a monoclonal antibody against CD20 on B cells) is essentially equally efficacious, but has a similar rate of adverse events.[44, 45] High doses of glucocorticoids were used in both the rituximab and cyclophosphamide arms of these RCTs and could explain some of the adverse events seen in both groups. However, whether rituximab would retain its efficacy with a lower side effect rate when used in conjunction with low dose or in steroid free regimens is uncertain. Newer treatments are being evaluated in clinical trials, including anti-C5a therapy (the small molecule C5a inhibitor (CCX168) and anti-BAFF antibodies.[67] Many challenges remain, including finding better biomarkers to indicate and predict disease activity, remission and relapse, employing more targeted therapies and managing co-morbidities and the side effects of therapies more effectively.

Aims of better treatment for AAV

To help treat AAV more effectively key issues the need to be addressed include:

1) The development and use of therapies that induce higher rates of remission.

2) Using therapies and therapeutic regimens that are steroid sparing and less toxic than current immunosuppressive agents.

3) The development of more specific therapies. Antigen-specific therapies that target only ANCA antigen specific T or B cells would be ideal, as these treatments would leave protective immunity intact. Defining critical molecular disease pathways may also enhance the development of more specific immunosuppression

4) Testing of currently available anti-inflammatory cytokine monoclonal antibodies and receptor blockers in AAV. These therapies, including anti-TNF, anti-IL-1, anti-IL-6 and anti-IL-17A therapies have been shown the be effective in autoimmune inflammatory

diseases such as psoriasis, ankylosing spondylitis and rheumatoid arthritis.[67] However, despite the fact that AAV has many similar features to these conditions, suggesting possible responsiveness to blocking these cytokines, many of these therapies, with the exception of an unsuccessful randomized clinical trial of anti-TNF treatment, have not been tested in AAV.[67]

5) Better stratification of patients, both in terms of severity and tissue involvement, but also in terms of autoantigen. Are MPO-AAV and PR3-AAV separate diseases that need subtly different treatment approaches?

6) More reliable assessment of remission status in individual patients.

7) More effectively identifying those patients that are less likely and those that are more likely to relapse, via gene expression profiling or biomarkers, either singly or in combination.

8) Systematically using a more patient centered approach, with multidisciplinary involvement to improve patient well-being during and after treatment, as an addition to overall outcomes.

9) As with other forms or renal disease, targeting the prevention and slowing the progression of fibrosis after the effective treatment of the inflammatory component of AAV.

Potential areas for future research in AAV are summarized in Table 1.

Why model disease?

While much of our understanding of the pathogenesis of AAV has come from the many careful observations of human disease, testing hypotheses derived from these observations in relevant *in vitro* and *in vivo* models of AAV has also been critical to our advances in understanding. Understanding disease pathogenesis is in itself essential as a rational approach to identify drugable pathways and therapeutic targets. Even with the advent of big science, big data and data science and experiments that are designed to be less focused observations, animal models still have a key role. For example, genetic variants identified by GWAS or WES/WGS (with subsequent more focused sequencing) that are potentially pathogenic can now, at least in concept, be modelled using CRISPR/Cas9 based technology *in vitro* and *in vivo*, potentially allowing more rapid identification of abnormalities in specific pathways and confirmation of their pathogenic potential. Similarly, the capacity to genetically delete (or conditionally delete) specific candidate therapeutic target molecules can better define whether these molecules are critical in disease development. Insertion of human genes into rodents

that do not normally express these genes can allow for testing of specific therapies. An example of this is the insertion of the gene for human C5a receptor into mice, humanizing the mice with respect to complement function. This was used to show that CCX168 a small molecule human C5a receptor antagonist was efficacious in a model of AAV[52] and paved the way for human trials with this molecule. Such discoveries may in themselves lead to better (and potentially more personalized) therapies, and may trigger investigations of potentially damaging pathways that can be targeted by existing or new therapies. *In vitro* models, usually where ANCA are added to normal neutrophils, allow control over most elements of the system (including cell type, as well as the dose of ANCA, priming agent and therapy) and limit the complexity in biological systems. They have roles both in understanding pathogenesis at a molecular and cellular level, and potentially in screening molecules and drugs relatively rapidly. *In vivo* animal studies, while more complex than *in vitro* work, remain a relatively reductionist approach to the study of AAV, the use of inbred rodent strains allowing researchers to develop tractable models of autoimmunity and GN. Different models, for example passive transfer of immune reactants, versus disease induced by active autoimmunity inform us in different ways about AAV pathogenesis.

Current models of ANCA-associated vasculitis

In vitro models

Most evidence for the pathogenicity of ANCA has arisen from in a number of vitro experiments showing that ANCA bind to neutrophils in an antigen-specific and Fc dependent manner.[68, 69] In contrast to *in vivo* experiments in AAV, PR3 models predominate in *in vitro* studies. Low dose TNF is commonly used for priming of neutrophils; this occurs via a p38-MAPK pathway, which, when inhibited, prevents ANCA-mediated neutrophil activation.[70] Other inflammatory stimuli, including IL-18 and TLR9 ligands have also been used as priming stimuli *in vitro* (*in vivo* LPS is typically used). A more detailed understanding of PR3 expression, which occurs on only a subset of neutrophils, was afforded by the identification of NB1 (CD177), a PR3 presenting membrane receptor. Jerke et al showed that NB1 and PR3 co-localize with the β_2 integrin, MAC-1 (CD11b) in cholesterol enriched parts of the plasma membrane. An interaction between the extracellular domains of NB1 and Mac-1 then allows signal transduction, facilitating PR3-ANCA mediated neutrophil degranulation and ROS production.[71]

ANCA-activated leukocytes release pro-inflammatory cytokines which upregulate endothelial cell adhesion molecules and result in leukocyte adhesion, ultimately leading to vessel wall necrosis. Using intravital microscopy, Kuligowski et al showed that α_4 and β_2 integrins on leukocytes are key to this process in glomeurli.[34] Although most *in vitro* studies have focused on characterizing the series of events that follow ANCA binding to neutrophils, monocytes also express MPO and PR3 and react to ANCA IgG. ANCA IgG binding to monocytes can trigger ROS production and upregulation of CD14 and CD18.[6] An anti-PR3 monoclonal antibody and PR-3 ANCA, but not an anti-MPO monoclonal antibody or ANCA, induce sFlt-1, an inhibitor of vascular endothelial growth factor released from monocytes, which may have a role in preventing endothelial repair in AAV.[72]

Rodent models in unravelling the pathogenesis of AAV

A number of different rodent models of AAV have been used to explore disease pathogenesis, each with its own limitations and complexities. Models of AAV are summarized in Table 2. Models of MPO-AAV and their impact on understanding of disease will be outlined first, followed by a discussion of anti-PR3 disease, and then disease using other antigens or susceptible mice. Table 3 outlines some of the potential therapeutic targets studied in these animal models.

Passive transfer models

Passive transfer of anti-MPO antibodies

In 2002, Xiao and colleagues established that transfer of anti-MPO antibodies, induced by immunizing *Mpo*^{-/-} mice with native mouse MPO in Freund's adjuvant, induces focal necrotizing crescentic GN and in some cases, pulmonary capillaritis.[7] Both *Rag2*^{-/-} mice, which lack functional T and B cells, and C57BL/6 mice were used in these experiments, with milder disease occurring in the C57BL/6 mice compared to the *Rag2*^{-/-} mice, over the course of 6 to 13 days. Since this successful model, a large amount of work in experimental AAV has been performed using the passive transfer approach.[7, 73]

The protection from renal injury that occurred in mice treated with a neutrophil depleting antibody in this model subsequently uncovered the key role of these cells in disease.[74] Accumulation of neutrophils in glomeruli occurs several hours after anti-MPO antibody administration.[9] Both higher doses of MPO and pre-treatment of mice with LPS (to simulate

the effect of infection which may precipitate this disease) increase the neutrophil influx and the severity of renal injury.[23, 34] The variable degree of injury observed with passive transfer models is probably due at least partly to differences in the titer and pathogenicity of polyclonal anti-MPO antibodies generated in *Mpo*^{-/-} mice.[7, 10] The number of recruited neutrophils and capacity for neutrophils to be activated by ANCA may also account for some of this variability. Xiao et al found that 129S6/SvEv and CAST/Eij mouse strains developed more severe disease in their passive transfer model, and 129S6 neutrophils were subsequently shown to be more strongly activated by TNF and anti-MPO antibodies compared to neutrophils from other studied mouse strains.[75] The observation that humans with ANCA vasculitis had higher serum levels of granulocyte colony stimulating factor (G-CSF) compared to age matched controls led to an interest in using G-CSF to prime and recruit neutrophils in an anti-MPO transfer model. Mice given G-CSF had significantly worse disease, with increased glomerular crescents and macrophage infiltration compared to control mice.[76] Passive transfer models have been used to determine the role of complement in AAV,[8, 52, 77] (discussed above) and to study the pathogenicity of the human B cell epitope identified by Roth et al as associated with active disease.[37]

Anti-MPO splenocyte transfer

Another passive transfer approach identified by Xiao et al involves the transfer of whole splenocytes into *Rag2*^{-/-} mice. Immune responses are induced in *Mpo*^{-/-} mice by immunization with MPO, and GN is then induced in *Rag2*^{-/-} mice by splenocyte transfer from the MPO immunized *Mpo*^{-/-} mice.[7] Although it is not a truly autoimmune model, one advantage of this approach is that it allows both humoral and cellular immunity to be studied. It has been used to in experiments demonstrating the key role that the alternative pathway of complement plays in disease.[77] However, immune complex deposition in glomeruli does occur in this model.

MPO specific T cell clone transfer: CD4+ and CD8+ cells

Although humoral immunity is important in AAV, evidence for the participation of T cells exists in both experimental models and observations in human disease.[78] Gan et al created a T cell transfer model based on experimental evidence indicating that autoreactive CD4+ T cells play a key role in disease pathogenesis.[10] CD4+ T cells from MPO-immunized B cell deficient *Mpo*^{-/-} mice were transferred into *Rag1*^{-/-} mice. When passive MPO-ANCA was

administered to trigger renal injury, mice immunized with MPO specific CD4+ T cells had significantly greater renal injury than those immunized with OVA specific CD4+ T cells, indicating that CD4+ T cells contribute to ANCA-mediated renal injury.[56] Ooi et al defined the immunodominant CD4+ MPO peptide in mice. Immunization with the peptide alone induced autoimmunity to whole MPO and focal and segmental GN when MPO was planted in glomeruli. Subsequently, CD4+ clone were developed, with T cell receptor specificity for the nephritogenic immunodominant MPO peptide. Transfer of these clones to *Rag1*^{-/-} mice induced focal and segmental GN when MPO was planted by using subnephritogenic doses of anti-GBM globulin, passive ANCA or a non-nephritogenic monoclonal IgG1 anti-mouse GBM antibody/MPO peptide conjugate. Although the role of CD8+ T cells in AAV is less defined, Chang et al identified a potentially pathogenic CD8+ T cell MPO epitope ($MPO_{431-439}$) and showed that CD8+ T cell clones specific for this epitope could induce disease in mice immunized with the cognate MPO peptide and triggered with anti-GBM globulin.[11] Collectively, these data confirm that T cells can mediate GN in these models. Human studies in MPO-AAV show that there is prominent diffuse extracellular deposition of MPO in glomeruli and interstitium that could act as a planted antigen target for anti-MPO CD4+ cells. NETs are a prominent but not exclusive method of planting MPO in glomeruli.[15]

<u>Active models of AAV</u>

MPO-immunized rats

The first experimental model indicating that AAV may have an autoimmune etiology was undertaken by Brouwer et al. Brown Norway rats were immunized with human MPO, resulting in anti-human MPO antibodies which cross react with rat MPO.[79] Following this, one kidney was perfused with products of activated neutrophils, including elastase, PR3, MPO and H_2O_2. MPO-immunized rats developed severe necrotizing, crescentic GN, whereas control-immunized or control perfused rats did not.[79] Further evidence for the pathogenic role of MPO-ANCA was then seen in a model where Wistar-Kyoto (WKY) rats were immunized with purified human MPO. Rats developed circulating MPO-ANCA that cross-reacted with rat MPO. GN with similar histological appearances to those seen in human disease and pulmonary vasculitis developed after 6 weeks. However, disease was relatively mild, and disease was limited to WKY rats only.[80] Genetically modified WKY rats are not yet generally available, but a protective effect of TNF blockade has been observed using this model. [81]

Immunization of transgenic mice with the pathogenic B cell epitope

Roth et al[37] performed an active immunization model using the pathogenic B cell epitope they had identified in humans. Humanized HLA-DR15 transgenic mice were immunized with a peptide containing $MPO_{442-460}$, homologous to the human B cell epitope $MPO_{447-459}$. At 28 days, $MPO_{442-460}$-immunized mice developed mild proliferative GN and significantly increased renal injury compared to control mice injected with a peptide from ovalbumin.

Active autoimmunity to MPO, with disease triggered by anti-GBM globulin
Heeringa et al immunized Brown Norway rats with human MPO in Freund's adjuvant, with control rats receiving adjuvant alone. Two weeks after immunization, when MPO-immunized rats had developed an anti-MPO response, anti-rat GBM globulin was administered. In contrast to control rats, which developed mild GN at day ten, MPO-immunized rats developed severe proliferative GN, characterized by crescent formation and fibrinoid necrosis. [82] This model has been established and used in mice, discussed below.

In this murine model, after ANCA recruit neutrophils to glomeruli where they degranulate and deposit MPO, antigen-specific T cells recognizing the deposited MPO instigate further renal injury.[9, 78] Immunization of mice with MPO causes them to break tolerance and generates active anti-MPO T cell autoimmunity. Although MPO-ANCA develop, their titer is low. Consequently, neutrophil recruitment and MPO deposition in the glomerulus is necessary for renal disease to occur. A technically simple way to achieve deposition of MPO in glomerular is a non-nephritogenic dose of heterologous anti-GBM globulin. Higher doses of anti-GBM globulin may in themselves cause significant renal injury. However at the low doses used in this model, the anti-GBM globulin itself does not cause significant glomerular injury, but it is a device for recruiting sufficient neutrophils to plant MPO in the glomerulus and induce T cell responses.[78]

This model has been used to define the importance of CD4+ and CD8+ T cell epitope specificity in ANCA vasculitis.[10, 11, 56] The role of TLR2 and TLR9, Th1 and Th17 cells[25, 30], and the importance of mast cells as effectors of disease [83] have also been illuminated using this model.

Evidence for the critical role that cellular, rather than humoral immune responses play in this model is that that CD4+ T cell depletion significantly attenuates disease but does not change ANCA titers, and that μ-chain deficient mice, although unable to make antibody, are still

susceptible to disease.[9] The commonly used 19 day model causes moderate disease, with necrotizing GN, and is a true 'autoimmune model.' Disadvantages include the need for production of MPO and the use of foreign globulin to trigger disease, presumably because the MPO-ANCA generated in MPO intact mice are not themselves directly capable of inducing neutrophil recruitment to glomeruli so that disease is induced or MPO is lodged in glomeruli.

Bone marrow transplant into MPO-immunized Mpo$^{-/-}$ mice

Schreiber et al developed an alternative model of MPO-ANCA GN.[84] First, anti-MPO antibodies were generated by immunizing *Mpo$^{-/-}$* mice with MPO and adjuvant. Because of the lack of a target autoantigen, immunized mice did not develop disease despite the high MPO-ANCA titer. On day 42, mice were irradiated and underwent transplant with bone marrow from genetically intact mice, with the subsequent development of necrotizing GN and pulmonary hemorrhage. Irradiated, MPO-immunized *Mpo$^{-/-}$* mice transplanted with *Mpo$^{-/-}$* bone marrow did not develop disease, demonstrating the need for endogenous MPO in this model. Studies in this system have implicated several neutrophil proteins, including the signaling molecule phosphoinositol kinase 3 gamma, which facilitates ANCA mediated neutrophil activation, [85] and neutrophil serine protease derived IL-1β in AAV.[86]

Advantages of this approach include not needing to reconstitute anti-MPO antibodies, and the ability to use genetically modified mice for bone marrow transfer. Practical disadvantages are the need to isolate mouse MPO for immunizations, and the 15-week length of the model. Although this model has allowed significant insights into disease pathogenesis, it is not truly autoimmune as in *Mpo$^{-/-}$* mice endogenous anti-MPO reactive T and B cells are unlikely to have been deleted via central tolerance.

Models of PR3-AAV

Generating models of PR3-AAV has been fraught with difficulty. Initial attempts by Pfister et al[87] to create a passive transfer model involved the generation of PR3-ANCA by immunizing PR3/elastase deficient mice with PR3 and then transferring these antibodies to PR3 intact 129Sv/Ev mice. Although this was directly analogous to Xiao's approach using anti-MPO antibodies, it did not cause clinical features of vasculitis, and only a subtle increase in inflammation in TNF exposed skin was observed. Possible reasons for the lack of success of this approach is the lack of expression of mouse PR3 on the surface of unstimulated

neutrophils, and the more substantial difference between murine PR3 and human PR3 compared to MPO (murine PR3 is only 68% homologous to human PR3, whereas in mouse MPO is 86% identical to human MPO).[88]

Considering the lack of success with a passive transfer model in PR3 disease, Van der Geld et al generated a series of chimeric PR3 molecules for immunization into both mice and WKY/Brown Norway rats; although animals developed PR3-ANCA, they did not develop clinical features of vasculitis.[89] Another attempt to reconcile the differences between mouse and human PR3 is to generate mice with a human immune system by engrafting human hematopoietic stem cells into immunodeficient mice. After doing this, Little et al infused these humanized mice with human PR3-ANCA and low dose LPS, causing pulmonary hemorrhage and mild proliferative GN.[90] A splenocyte transfer model, similar to that described in MPO-AAV above,[7] was successful in causing severe focal necrotizing GN in immunodeficient NOD/SCID mice. All mice developed acute kidney injury and died twenty to forty days after adoptive transfer. Interestingly, splenocyte transfer into WT NOD mice caused anti-PR3 responses but no sign of histological injury in the kidney.[91] Whilst the presence of immune complex deposition in the kidneys of the NOD/SCID mice was not reported, presumably this does occur as it did in the MPO-ANCA splenocyte transfer model.

Anti-LAMP-2 models and molecular mimicry

Two experimental models have been created to support the hypothesis of molecular mimicry generated by the discovery of LAMP-2 antibodies in humans. As rat and human LAMP-2 show significant homology, but this sequence is quite distinct in mice, WKY rats were used in these experiments. These models accurately represent the phenotype of human disease, an important aspect of its pathogenesis. The initial finding of Kain et al was that over 90% of patients with biopsy-proven pauci-immune GN had LAMP-2 antibodies; nine of thirteen patients had urinary tract infections with FimH bearing organisms such as *E.Coli* prior to the development of GN, substantiating the molecular mimicry hypothesis.[22] However, anti-LAMP-2 antibodies have not reproducibly found in to be prevalent humans with AAV.[92] In a LAMP-2 passive transfer model, WKY rats injected with with human anti-LAMP-2 rabbit IgG (which cross-reacts with rat LAMP-2) developed severe renal injury. To strengthen the hypothesis of molecular mimicry, rats were immunized with recombinant FimH fusion protein, provoking the development of antibodies that cross reacted with human LAMP-2.

Most rats developed antibodies to rat LAMP-2 and pauci-immune, crescentic GN, similar to human disease, with some additionally developing pulmonary vasculitis.[22]

Other models

Immunization with NET-loaded dendritic cells

Dying neutrophils extrude NETs, lacelike structures of chromatin and antimicrobial peptides such as neutrophil elastase, PR3 and MPO, to kill extracellular bacteria.[12] This form of neutrophil death has emerged as an important mechanism in the pathogenesis of both endothelial damage and potentially the loss of tolerance in AAV. Sangaletti et al[14] generated a model to test the hypothesis that NET formation facilitates the loss of tolerance towards PR3 and MPO by using DCs which were loaded with NETs. Controls included dendritic cells that underwent co-culture with apoptotic or necrotic neutrophils, DCs without NETs, and NETotic neutrophils in the presence of DNAse, which specifically breaks down NETs. Mice immunized with NET-loaded DCs developed significantly increased ANCA and anti-dsDNA titers compared to those treated with DCs or neutrophils alone. Although mice that were injected with apoptotic neutrophil-loaded DCs also developed MPO-ANCA, this was at a significantly lower titer compared to those immunized with NET-loaded DCs. Importantly, mice immunized with NET-loaded DCs developed a phenotype reminiscent of systemic vasculitis, with renal and pulmonary damage, which was not present in the mice immunized with apoptotic neutrophil-loaded DCs. It is possible that these apoptotic neutrophil-loaded DCs induced non-pathogenic ANCA. This model has the advantage of mimicking some of the complexity of the loss of tolerance that occurs in disease, however it is labor intensive and results in the formation of dsDNA antibodies complicating the attribution of anti-MPO autoimmunity to the renal phenotype.

Spontaneous disease in SCG/Kinjoh mice

Spontaneous autoimmune disease with anti-MPO antibodies and crescentic GN develops in SCG/Kinjoh mice, a strain derived from two lupus prone strains, selected for the presence of glomerular crescent formation.[93] These mice start to develop MPO-ANCA at week 6 of life, with all mice being positive for MPO-ANCA by week 20. This model induces crescentic renal disease, but a significant limitation is the presence of concurrent lupus type autoimmunity, with immune complexes, as seen in lupus nephritis (albeit at a lower level in glomeruli), as opposed to the pauci-immune renal pathology seen in human AAV, as well as the fact that genetically modified mice cannot be used,. This model may have some

application for exploring pathogenesis in the infrequently studied subgroup of patients with ANCA-positive lupus nephritis.

Nephrotoxic serum nephritis or autologous phase anti-GBM glomerulonephritis

The most commonly used model of rapidly progressive glomerulonephritis has several names, including nephrotoxic serum nephritis (NTS or NTN), Magusi nephritis and "anti-GBM" glomerulonephritis. However, although histologically this model features crescentic and necrotizing GN, this process does not model AAV. While the label NTN implies the administration of an ill-defined nephrotoxin, clear and well-defined immunological processes direct renal injury in this model.[94] Administration of globulin derived from an animal (usually sheep or rabbit) immunized with a renal basement membrane fraction induces two phases of injury. The first phase, occurring within hours and lasting several days, is dose-dependent acute glomerular injury caused by the foreign immunoglobulin (largely IgG) acting as a nephritogenic antibody, known as the heterologous phase. This models in situ immune complex disease. The second phase, the autologous phase, is mediated by an adaptive cellular and humoral immune response against the bound globulin as a planted foreign antigen. Some effector mechanisms may be similar to that seen in lupus nephritis and possibly the cell-mediated aspects of anti-MPO GN. However, autoimmunity does not occur in this model, and anti-MPO responses and ANCA are absent.[95] This model results from adaptive immunity to a xenoantigen (a foreign immunoglobulin). These mechanisms of injury do not occur in human anti-GBM GN or ANCA-associated GN.

Drawbacks of currently available models

While AAV has been modelled in a number of different ways, with different models making key contributions to our understanding of the pathogenesis of this condition, each model has its own drawbacks. Given the complexity of the disease, it is not surprising that it has been modelled using different approaches. Nonetheless, individually and collectively models of AAV remain imperfect. While models of MPO-AAV exist, there is as yet no consistently used model of PR3-AAV. This seems to be at least in part due to the significant differences between human PR3 and its rodent homologues. In contrast, MPO is better conserved between rodents and humans, and modelling MPO-AAV has been much more successful. Conversely, the majority of *in vitro* studies with human ANCA have used PR3-ANCA. Do these differences between animal models and human studies matter? Perhaps not, in the areas where MPO-AAV and PR3-AAV share pathogenesis or effector mechanisms. Nonetheless,

with the increasing evidence that MPO-AAV and PR3-AAV may be different diseases with, for example different genetic susceptibilities and different relapse rates, we should be mindful of the dominance of anti-MPO animal models and continue to aim to establish tractable *in vivo* models of AAV directed by autoimmunity to PR3.

Generally speaking, most of the models have not been standardized. Key reagents vary and/or are very expensive to produce and maintain. The large amounts of native or recombinant MPO protein required in active models of anti-MPO autoimmunity are expensive and sometimes difficult to generate. Anti-MPO antibodies induced by immunizing $Mpo^{-/-}$ mice are variably pathogenic: their production requires colonies of $Mpo^{-/-}$ mice that need to be immunized with MPO protein. The use of monoclonal anti-MPO antibodies, while a logical approach, has not yet proven to be the answer to these issues. The "anti-GBM" antibody that results in transient neutrophil recruitment in some models is polyclonal – these antibodies are well known to differ in their capacity to engage immune effectors. For these and potentially other reasons, many of the current models are difficult to reproduce, with consistent disease in some laboratories but not in others.

Animal models of AAV have understandably focused on acute effector mechanisms of injury. While we now have a relatively detailed knowledge of how ANCA activates neutrophils and how these activated neutrophils mediate injury, we are also starting to understand the role of effector T cells in disease. A number of models are passive transfer models, ideal for studying effector mechanisms but of little use in defining the nature of loss of tolerance and the development of injurious autoimmunity to neutrophil cytoplasmic proteins. Thus, we have only clues to what systems might be involved in loss of tolerance and in disease relapse. Even in active models their relatively short duration counts against examining memory T and B cells, cells that are likely to be critical in relapsing disease.

Future Directions

In order to address the key questions in pathogenesis and management, researchers in the field will need to continue to use and develop *in vitro* models of AAV, and also to make careful human observations, both in ongoing studies using GWAS and other genetic approaches, and in studies that address more focused questions. In any rare disease, multicenter collaborations are important. Continuing to align human observations with hypotheses that can only be tested experimentally, while avoiding being constrained by

current paradigms, is critical to advancing out understanding. Models, by their very definition are only "models" and current AAV models are difficult and complex. Nonetheless, thought could be given to how they could be improved and made even more relevant to human disease. For example, the introduction of human proteins into rodent systems has been used in other autoimmune diseases and in this disease.[52] The difficulties in using rodent PR3 outlined above emphasize the need for these kinds of approaches. Although an initial attempt in introducing human PR3 into a mouse has not as yet yielded a new disease model,[96] this remains an approach worth pursuing. Boosting the reduced numbers of neutrophils in mice expressing lower amounts of autoantigen might help generate more robust disease models. Studying older rodents, or even inbred strains co-housed with immune experienced pet shop mice would assist in examining immune memory and relapse in the context of autoimmunity in AAV.

On a practical nature, collaborative research consortia in pre-clinical work may facilitate better availability of and the standardization of, critical reagents and techniques. Although current funding models nationally and internationally are not entirely conducive to this sort of approach, the formation of networks in the so called "basic science" area of AAV research could, in ways analogous to clinical research networks, help advance the field considerably. Particularly in studies focused on pre-clinical drug testing, more emphasis could be given to pre-specifying primary endpoints, study design and power. In addition, a recent suggestion by Anders et al,[97] having several research laboratories performing the same drug study, with coordinated tissue and other endpoint assessment at central laboratories bears further consideration.

Conclusion

ANCA-associated vasculitis, a disease that commonly involves that kidney, is a severe autoimmune disease with a unique pathogenesis. The current suite of *in vivo* and *in vitro* models have allowed us to understand this complex disease and point the way towards further advances in understanding and potentially better and more targeted therapies.

Acknowledgements

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Table 1: Key questions for future study in AAV research

	What we know	What we need to know
Classification of Disease		
Should AAV be classified as two genetically distinct but clinically overlapping distinct subtypes?	GPA and MPA (and PR3-AAV and MPO-AAV) have a number of similarities but significant differences.[16]	Will reclassification make a difference to understanding, clinical trial outcomes and management?
Differing geographical distribution of MPO and PR3 subtypes	They may be associated with environmental factors or geographic distribution of certain HLA alleles.[16]	Are there environmental/genetic factors that can be targeted in treatment?
Loss of tolerance		
What is the basis for loss of tolerance?	MPO is thymically expressed under the AIRE promotor,[28] natural ANCA exist,[29] complementary PR3 peptides and NETs may be involved in loss of tolerance.[14]	What are the primary events that cause loss of tolerance and can these be targeted for therapy?
What is the contribution of infection to loss of tolerance, disease triggering and relapses?	Nasal carriage of s. aureus is associated with increased relapses in MPA.[98] TLRs are important in disease pathogenesis.[24, 30] Some evidence suggests molecular mimicry may be involved.[22]	Is molecular mimicry relevant to the pathogenesis of some/all disease? Why do some infections trigger disease and not others? Can TLRs be targeted for therapeutics?
What is the role of silica?	Occupational silica exposure is associated epidemiologically with an increased risk of AAV.[26]	How is silica mechanistically involved in loss of tolerance and/or the induction of disease?
What is the role of regulatory T and B cells?	B regs inhibit Th1 and Th17 and promote T regulatory cell differentiation. Defects in T regulatory function occur in AAV.[59]	Are abnormalities in these cells primary or secondary events? What is the significance of regulatory cells to disease initiation and therapy?
T and B epitopes	Some MPO T[10, 11] and B cell[37] epitopes are known.	What is the relevance of these epitopes to disease? Could

		antibodies against these epitopes be used to create less labor intensive, robust rodent models?
ANCA		
ANCA pathogenicity	ANCA activate neutrophils and in experimental models;[5] anti-MPO antibodies can induce disease.[74]	Are particular subclasses or ANCA directed against particular epitopes more pathogenic than others?
What is the significance of epitope specificity?	In MPO-AAV, one B cell epitope was shown to be more associated with active disease.[37]	Can this finding be further replicated in diverse cohorts? Can monitoring of 'pathogenic epitopes' be used to more accurately assess disease flares?
What are effects of PR3-ANCA and MPO-ANCA on neutrophils?	Aberrantly increased PR3 and MPO expression on neutrophils is seen in patients with AAV; epigenetics may play a role.[20, 31]	Do PR-3 ANCA and MPO-ANCA activate different pathways of inflammation? Is complement activation different between PR3 and MPO ANCA?
What is the significance of LAMP2-ANCA?	LAMP 2 ANCA has been shown to be prevalent in a European[22] but not a US[92] AAV cohort. LAMP 2 ANCA cross reacts with antibodies to bacterial FimH; Passive transfer can cause disease.[22]	What is the true prevalence of LAMP2 ANCA? What is its contribution to disease?
Effects of neutrophils and macrophages		
What is the role of NETs?	NETs are potentially involved in loss of tolerance as well as mediating endothelial injury.[14, 15]	Can NETs be targeted for therapeutics? What contribution do NETs make to loss of tolerance?
What is the role of macrophages?	Macrophage infiltration occurs in AAV lesions ANCA can activate macrophages.[6]	What is the contribution of macrophages to disease, and are they are an appropriate target?
Effector T cells in tissue injury		

Which effector T cells play an important role?	Th1 and Th17 cells are important in disease pathogenesis.[10, 58]	Can these T cell subsets be targeted for therapy, or modified to differentiate into immunosuppressive T cell subsets? What is the role of memory cells?
What is the role of CD8+ cells?	CD8+ T cells play a pathogenic role in mouse models of AAV[11] and there are increased intrarenal CD8+ cells in human disease.[15] CD8+ T cell gene signature predicts relapse in AAV.[65]	How do CD8+ cells effect injury?
Tissue injury		
How does leukocyte recruitment occur?	Anti-MPO antibodies induce leukocyte recruitment to glomeruli in an FcγR and integrin dependent manner.[34, 64]	Why are particular microvascular beds, such as the kidney, more vulnerable?

Abbreviations: AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis;MPO, myeloperoxidase;PR3, proteinase 3; NETs, neutrophil extracellular traps; LAMP 2, lysosome-associated membrane protein 2; TLR, Toll-like receptor

Table 2: Selected models of ANCA associated vasculitis

Model	Active/passive immunity	Species	Putative mechanism/ effectors	Reference
Anti-MPO ANCA transfer	Passive	C57BL/6 or *Rag2*^{-/-} mice	ANCA induces neutrophil degranulation. Dependent on neutrophils and alternative pathway of complement	Xiao, 2002[7]
MPO specific splenocyte transfer	Passive	*Rag2*^{-/-} mice	Anti-MPO T and B cells mediate injury; Dependent on alternative pathway of complement	Xiao, 2002[7]
MPO specific CD4+ T cell transfer	Passive	*Rag1*^{-/-} mice	MPO-specific effector CD4+ cells localize to glomeruli and induce a delayed type hypersensitivity-like lesion.	Gan, 2013[56]
MPO specific CD8+ T cell transfer	Passive	*Rag1*^{-/-} mice	MPO specific effector CD8+ cells recognize MPO deposited in the glomerulus and induce injury.	Chang, 2016[11]
Hu-MPO immunization in WKY rats	Active	WKY rats	Induction of anti-MPO antibody response	Little, 2009[80]
Active autoimmunity with anti-GBM trigger	Active	C57BL/6 mice	Delayed-type hypersensitivity response to planted antigen; T cell dependent	Ruth 2006 [9]
Bone marrow transplant model	Active	Immunized *Mpo*^{-/-} mice transplanted with *Mpo*^{+/+} bone marrow	Circulating anti-MPO antibodies form and induce disease when they recognize MPO on MPO intact neutrophils	Schreiber 2006[84]

PR3 specific splenocyte transfer	Passive	NOD/SCID mice	Anti-PR3 T and B cells mediate injury	Primo, 2010[91]
Human anti PR3 ANCA transfer	Passive	Humanized NOD-scid-IL2Rγ−/− chimeric mice	Chimerism generates neutrophil and monocyte targets for Hu-PR3-ANCA	Little, 2012[90]
Hu-LAMP 2 passive transfer	Passive	WKY rats	LAMP-2 ANCA mediates injury by binding to glomerular endothelium	Kain 2008[22]
Fim H immunization	Active	WKY rats	Fim H provokes development of antibodies that cross react with LAMP-2 and cause injury	Kain 2008[22]
NET loaded DCs	passive	BALB/c; C57BL/6 mice	NET loaded DCs present MPO/PR3 to T cells, cause loss of tolerance and ANCA production	Sangaletti 2012[14]
Autoimmune prone mice	N/A	SCG/Kinjoh mice	Autoreactive T/B cells result in the production of ANCA; glomerular injury occurs from immune complex deposition +/- ANCA	Neumann, 2003[93]

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibody; MPO, myeloperoxidase; PR3, proteinase 3; LAMP 2, lysosome-associated membrane protein 2; NETs, neutrophil extracellular traps.

Table 3: Potential therapeutic targets identified in experimental models of vasculitis

Target	Experimental evidence	Ref
Leukocytes		
Neutrophil	Neutrophil depletion attenuates disease in the anti-MPO Ab passive transfer model.	[74]
CD4+ T cell	Depletion of CD4+ T cells attenuates disease in experimental autoimmune anti-MPO GN; transfer of CD4+ MPO specific clones mediate GN post LPS/anti-MPO Ab trigger	[9,10]
CD8 + T cell	Depletion of CD4+ T cells attenuates disease experimental autoimmune anti-MPO GN; MPO specific CD8+ T cell clone transfer mediates GN	[11]
Mast cell	Mast cell deficiency enhances disease (mast cells net protective effect). Inhibiting mast cell degranulation limits disease (mast cell degranulation is pathogenic) in experimental autoimmune anti-MPO GN	[83]
Plasma cells	Bortezomib attenuates disease in the MPO bone marrow transfer model	[99]
Complement		
Alternative pathway of complement	Mice deficient in C5 (common pathway) and Factor B have attenuated disease in the passive anti-MPO Ab transfer model	[77]
C5a	Pre-treatment with anti-C5a antibodies abrogated renal injury; treatment one day after transfer also resulted in decreased renal injury in the anti-MPO Ab passive transfer model	[8]
	CCX168 administration in mice with a humanized C5aR attenuated injury in the anti-MPO passive transfer model	[52]
Adhesion molecules		
NB1 (CD177)/Mac-1 (CD11b/CD18)	Stimulation with NB1 activating mAb triggers neutrophil degranulation CD11b blockade inhibits PR3-ANCA mediated neutrophil activation	[71]
$\alpha 4$ integrin	Inhibition of $\alpha 4$ integrin attenuates acute glomerular nephrophil recruitment after passive transfer of anti-MPO Ab to unprimed mice	[34]

Leukocyte signaling molecules		
PI3-kinase γ	Inhibitors attenuate disease in the anti-MPO bone marrow transplant model	[85]
p38 MAP kinase	Inhibitors attenuate disease in passive transfer of anti-MPO Ab	[100]
Toll-like receptors		
TLR2	TLR2 ligands promote autoimmunity and disease in experimental autoimmune anti-MPO GN	[25]
TLR4	LPS augments disease in the anti-MPO Ab passive transfer model; TLR4 expressed by both leukocytes and endothelial cells is important	[30]
TLR9	TLR9 ligands promote autoimmunity and disease in experimental autoimmune anti-MPO GN	[25]
Cytokines		
TNF	Anti-TNF antibodies attenuate disease in the MPO immunisation WKY rat model	[81]
IL-17A	IL-17A deficiency attenuates experimental autoimmune anti-MPO GN	[58]
IL-10	Mast cell derived IL-10 is protective in experimental autoimmune anti-MPO GN	[83]
IL-1β	IL-1RA attenuates disease in the anti-MPO bone marrow transplant model	[86]
IFN-γ	Transfer of CD4+ and CD8+ MPO-specific clones secreting IFN-γ can mediate glomerular injury	[10,11]

Figure 1: Simplified diagram of the pathogenesis of ANCA associated vasculitis