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Title

Emerging concepts in the pathogenesis of ANCA-associated vasculitis.

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Abstract

Purpose of review:

Anti-neutrophil cytoplasmic antibodies (ANCA) remain central to our current understanding of the pathogenesis of ANCA-associated vasculitis (AAV), and this review considers recent developments in the context of four key questions: (1) are there targets for ANCA beyond myeloperoxidase (MPO) and proteinase 3 (PR3); (2) are all ANCA pathogenic; (3) how are ANCA generated; and (4) how do ANCA cause disease?

Recent findings:

B-cell epitope mapping raises the possibility that only a subset of ANCA may be pathogenic. Anti-LAMP-2 autoantibodies have recently emerged as novel form of ANCA, and can be found in anti-MPO and anti-PR3-negative disease. These also provide recent evidence for molecular mimicry in the pathogenesis of AAV, but definitive proof in human AAV remains elusive. Neutrophil extracellular traps may represent an important mechanism by which MPO and PR3 are taken up by dendritic cells for presentation to the adaptive immune system, and the role of the alternative pathway of complement in AAV has recently been emphasized, with therapeutic implications.

Summary:

Our current understanding of the pathogenesis of AAV reinforces the central role of neutrophils, but also provides a sound rationale for B-cell and complement-directed therapies.

Keywords: (3-5)

Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a life-threatening, small vessel vasculitis characterized by the presence of pathogenic anti-proteinase 3 (PR3) and anti-myeloperoxidase (MPO) autoantibodies and renal, lung and upper respiratory tract involvement. Clinically and pathologically, three forms are traditionally recognized: granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), which is distinguished by necrotizing granulomas on biopsy and frequent upper respiratory tract involvement; microscopic polyangiitis (MPA); and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss Syndrome), which is much less common and distinguished by a prominent eosinophilia and allergic features.[1] There is significant clinical and pathological overlap between these diseases, and evidence is accumulating that sub-classification of AAV using ANCA antigen specificity may better reflect both prognosis and disease pathogenesis than traditional clinical definitions, reinforcing that fundamentally AAV remains an ANCA-centric disease.[2–4] This review, therefore, considers current research in the context of four key questions: (1) are there targets for ANCA beyond MPO and PR3; (2) are all ANCA pathogenic; (3) how are ANCA generated; and (4) how do ANCA cause disease?

Are there targets for ANCA beyond myeloperoxidase and proteinase-3, and are all

ANCA pathogenic?

ANCA were first recognized for their ability to label neutrophils by indirect

immunofluorescence.[5] The staining conformed to two patterns: perinuclear, and

cytoplasmic, and MPO and PR3 were soon identified as their predominant target

autoantigens, respectively. Multiple additional targets of ANCA were identified

during the 1990s, including cathepsin-G and neutrophil elastase,[6] but these lack

the specificity and the associated evidence of pathogenicity that has since

accumulated for anti-PR3 and anti-MPO ANCA. The latter ranges from a case report

of neonatal vasculitis in the context of exposure to maternal anti-MPO ANCA to

evidence of glomerulonephritis after passive transfer of anti-MPO and anti-PR3

ANCA in murine models of disease.[7–9] On the other hand, there are also numerous

examples of detectable anti-MPO and anti-PR3 ANCA in otherwise healthy

individuals or individuals with a range of non-vasculitic diseases (e.g. systemic

lupus erythematosus (SLE), ulcerative colitis), which demonstrates that ANCA are

not always pathogenic.[10,11] In addition, up to a third of patients with a clinical

diagnosis of AAV are ANCA-negative by conventional assays,[6] suggesting there

may be additional targets for autoantibodies.

The question of ANCA specificities and pathogenicity has been addressed by recent

studies. Using a mass spectrometry-based approach, Roth and colleagues identified

25 epitopes bound by anti-MPO autoantibodies.[12] Autoantibodies binding one of

the linear epitopes, MPO[447-459], were exclusively found in patients with active

disease, suggesting that these autoantibodies may be pathogenic. This was supported by evidence that ANCA binding this epitope activated neutrophils *in vitro* and by the observation that DR2 transgenic mice immunized with the corresponding murine epitope developed a pauci-immune glomerulonephritis. However, complicating this, these immunized mice developed a polyclonal anti-MPO ANCA that bound a range of epitopes including but not limited to the MPO[447-459]. The remaining MPO epitopes were classified according to whether they bound ANCA from patients with active disease, remission or healthy volunteers, and this was used as a basis to infer potential pathogenicity. However, where ANCA were detectable in healthy volunteers, it was at extremely low levels. It remains unclear, therefore, whether ANCA targeting these epitopes are truly non-pathogenic at the higher concentrations observed in disease.

The same study also found that purified immunoglobulin from patients with ANCA-negative disease was able to bind the pathogenic MPO[447-459] epitope, implying that detection of ANCA in a proportion of ANCA-negative patients may be masked by a serum factor (perhaps a fragment of ceruloplasmin) interfering with standard assays. Larger studies are needed to validate these findings and determine the proportion of ANCA negative patients accounted for by this proposed mechanism.

There are other potential mechanisms for ANCA-negative vasculitis. Espéli and colleagues have demonstrated that most auto-antibody producing plasma cells in mice with active SLE are found in inflammatory lesions, suggesting local production

of autoantibodies might help drive disease[13]; this potential mechanism has not

been investigated in ANCA-negative AAV. Studies of anti-LAMP-2 autoantibodies

performed by Kain and colleagues also suggest that these may represent a novel

form of ANCA present in anti-PR3 and anti-MPO ANCA-negative disease. LAMP-2, a

lysosomal membrane protein, was first identified as an antigenic target of ANCA in

1995 as part of a broader screen.[14] Follow-up publications in 2008 and 2012

reported that anti-LAMP-2 autoantibodies were widely present in patients with

ANCA-positive vasculitis (14/16 patients in the 2008 study and 78/84 patients in

2012), coexisting with anti-MPO or anti-PR3 in most cases. These studies were not

without controversy: the complex, highly glycosylated nature of native LAMP-2

meant that assays for anti-LAMP-2 antibodies were difficult to replicate,[15,16]

however Kain argues plausibly that this is due to differences in patient selection

(anti-LAMP-2 antibodies are sensitive to immunosuppression and disease activity)

and the source of LAMP-2 antigen used.[17] More recently, anti-LAMP2 antibodies

were found in eight of eleven ANCA-negative patients presenting with a pauci-

immune glomerulonephritis.[18*] These autoantibodies were notable for binding

the less-glycosylated glomerular form of LAMP-2, not the highly-glycosylated

neutrophil form. Evidence for the pathogenicity of anti-LAMP-2 autoantibodies

comes from their association with disease in animal models and their correlation

with active disease in the human studies discussed above. Animal models include a

2013 Japanese study in which transgenic rats overexpressing the human T-cell

lymphocyte virus type 1 gene env-pX develop a cutaneous vasculitis and

spontaneous anti-LAMP-2 autoantibodies (without anti-PR3 or anti-MPO

ANCA).[19] When these antibodies were transferred into disease-free env-pX rats they progressed to develop an accelerated neutrophil-rich cutaneous vasculitis in comparison to non-treated control rats. The same study also identified anti-LAMP-2 autoantibodies in a subset of patients with cutaneous polyarteritis nodosa, and with Henoch-Schonlein purpura, diseases not classically associated with ANCA. This implies that, as with anti-MPO and anti-PR3 ANCA, anti-LAMP-2 autoantibodies may not wholly specific for AAV.

Antibodies targeting moesin have also recently been described in a Japanese cohort of patients with anti-MPO AAV with some capacity to stimulate monocytes and neutrophils *in vitro*.[20]

How are ANCA generated?

Multiple hypotheses have been presented for the formation of ANCA, with molecular mimicry one of the most discussed. However, a pathogen may contribute to the risk of autoimmunity without necessarily being present at the onset of that disease (the so-called 'fertile field' hypothesis),[21] which makes establishing its role particularly challenging. Yet there is increasing evidence of heterologous immune responses to multiple pathogens making molecular mimicry a plausible hypothesis.[22] The most convincing evidence for its role in AAV comes from homology between FimH, a component of type 1 fimbriae found on a range of gram-negative pathogens, and LAMP-2.[23] Here, rats immunized with FimH progressed

to develop cross-reactive anti-LAMP-2 antibodies and a pauci-immune

glomerulonephritis.

Another hypothesis is that homology between certain *Staphylococcus aureus*

proteins and a peptide encoded by the reverse DNA strand to PR3 ('complementary'

PR3, cPR3) results in an immune response to *S. aureus* generating antibodies that

are also cross-reactive with cPR3.[24] These anti-cPR3 autoantibodies are then said

to seed the development of idiotypic, PR3-reactive antibodies, ultimately leading to

the development of clinical GPA. However, there is no direct evidence that anti-cPR3

antibodies cross-react with bacterial proteins, and others have not been able to

confirm the presence of elevated anti-cPR3 antibody titres in GPA, nor establish a

relationship between the presence of anti-cPR3 antibodies and *S. aureus*

carriage.[25]

Nonetheless, an association between *S. aureus* carriage and AAV is well

described,[26] and a role for *S. aureus* in the pathogenesis of AAV continues to be

sought. Potential mechanisms include the ability of *S. aureus* to potently induce the

formation of neutrophil extracellular traps (NETs),[27] discussed below or, more

generally, bacterial activation of neutrophils through the activation of toll-like

receptors. TLR4 and TLR9, whose ligands are gram-positive bacterial components

and bacterial DNA respectively, have been shown to trigger MPO release and

increased surface expression of PR3.[28] A recent candidate gene study has also

identified polymorphisms at the *TLR9* locus as modulating susceptibility to GPA.[29]

This circumstantial evidence suggests a plausible mechanism by which *S. aureus* could drive GPA, but is remains far from conclusive.

Non-infectious triggers for ANCA formation have been described. Medications are chief among these and include levamisole (used as a 'cutting' agent for cocaine), one of the few instances in which both anti-PR3 and anti-MPO autoantibodies coexist, and propylthiouracil as recently reviewed in [30].

A role for T-cells in the generation of ANCA is suggested by their high affinity, class-switched nature and underscored by long-reported associations between MHC class II variants and AAV.[31,32] Recent GWAS studies have begun to define the latter more closely. Anti-MPO AAV and anti-PR3 AAV were strongly associated with HLA-DQ and HLA-DP variants, respectively.[3] The HLA-DP association was replicated in a second AAV GWAS, likely driven by the high anti-PR3 prevalence in this predominantly GPA cohort.[32] Precisely how a specific HLA risk allele predisposes to autoimmunity in AAV has not been determined, but in other diseases with a Class II HLA association it has been possible to map the genetic association onto key amino acid residues in the peptide-binding groove.[33] The ability of these HLA risk alleles to specifically bind peptides that have undergone post-translational modification seems to be an important property that may predispose to autoimmunity.[34]

What has also become clear is that the generation of ANCA may be facilitated by positive feedback loops within the innate immune system whereby, for example, ANCA stimulates the formation of NETs and which then facilitate the further generation of ANCA. These are discussed further in the next section, which examines recent developments in some of the effector pathways downstream of ANCA.

How do ANCA cause disease?

Recent research into the role of NETs and complement in AAV has added significantly to our understanding of how ANCA cause disease.

Neutrophil extracellular traps, and their role in AAV

A unique feature of neutrophils, which are key players in AAV, is their ability to generate NETs, a 'sticky mesh' of extruded chromatin, nucleic acid and a range of cytoplasmic proteins. These serve antibacterial functions, but also represent a means by which cytoplasmic proteins may be exposed to the immune system.[35] In 2009, AAV was one of the first autoimmune diseases to be linked to excessive NET formation.[36] This initial study demonstrated that NETs contain MPO and PR3 and are induced by ANCA. Moreover, extracellular DNA, histones and neutrophil granule components including PR3 and MPO co-localised in the glomeruli of patients with an active ANCA-associated glomerulonephritis, placing NETs at the site of tissue damage *in vivo*. Since then, increasing circumstantial evidence suggests that NETs represent an important means for autoantigen uptake by antigen presenting cells (APCs) in AAV. First, Sangaletti and colleagues showed in an elegant study that

murine NETs specifically interact with myeloid dendritic cells (mDCs) to facilitate

the uptake of MPO and PR3. Moreover, when these NET-cultured mDCs were then

transferred into healthy mice both new ANCA formation and an active

glomerulonephritis were observed,[37] although exactly how an interaction with

NETs licenses APCs to provide enhanced costimulation to B- and T-cells in

subsequent interactions is unclear. Recent human studies provide additional

evidence for the importance of NETs in AAV, showing that that the serum of patients

with MPO+ vasculitis potently induces NET formation and retards their degradation,

the latter potentially through an inhibitory action of anti-NET antibodies.[38*] It is

hypothesized, but remains to be proven, that excessive or inappropriate NET

formation or delayed NET degradation represents an important mechanism by

which the key AAV autoantigens MPO and PR3 are taken up by APCs in a pro-

inflammatory context for presentation to B- and T-cells (figure 1).

Complement

Our understanding of the role of complement in tissue damage in AAV has also

undergone a substantial shift. The pauci-immune nature of the characteristic lesions

had given rise to an initial belief that complement did not play a significant role in

AAV pathogenesis.[39] Since then, a number of studies have convincingly shown

that activation of the alternative pathway of complement and an intact C5a receptor

(C5aR) are necessary for the development of glomerulonephritis in murine models

of vasculitis, prompting a re-assessment of the role of complement in human

AAV.[40,41] Experiments *in vitro* showed that ANCA-stimulated healthy human

neutrophils are able to activate complement and that complement acting through

the C5aR is able to prime neutrophils and upregulate PR3 surface expression.[42]

Moreover, C3F, a common coding genetic variant of C3, was found to be

overrepresented in patients with AAV in small candidate gene studies.[43–45]

Despite the pauci-immune tag, a number of studies have also demonstrated low

level glomerular immune-complex deposition, including complement

components.[46,47]

A Chinese group reported that complement deposition in renal biopsies of AAV

patients correlated with worse renal injury, and that glomerular deposition and

urine levels of the alternative pathway complement fragment Bb, in particular,

correlate well with AAV activity. [48*] Other complement components including

C3a, C5a and soluble C5b-9 were also found to be elevated and, separately, they

reported increased plasma levels of activated complement components C3a, C5a

and Bb in patients with active AAV.[49] C5aR (CD88) expression in renal biopsy

specimens was predominantly tubular, was reduced in active vasculitis and absent

on infiltrating glomerular neutrophils, raising questions about the importance of

this receptor in AAV.[50] However, given that C5aR on neutrophils is internalized

upon activation, and cleaved by neutrophil serine proteases from the surface of

other cell types, this is not necessarily inconsistent with a role for C5aR in AAV.[51]

Taken together these observations support a role for the alternative pathway of

complement in human AAV, consistent with the murine data. An inappropriate

positive feedback loop in which ANCA-activated neutrophils themselves activate

complement, further increasing the amount of C5a present, may be important in

sustaining the inflammatory process (figure 2). These observations, especially if

replicated, provide a rationale for the use of eculizumab, a C5a inhibitor, in AAV,

with a preliminary report providing an early suggestion of clinical efficacy.[52]

Other notable recent findings – in brief

Under certain conditions it is clear that B cells can have a regulatory function, driven

at least in part by the production of IL-10. These "regulatory B-cells" can be hard to

define phenotypically, and there is only limited evidence that they comprise a

distinct "lineage". Four small recent clinical studies suggest that in AAV, especially

GPA, a sub-population of B cells retains an ability to produce IL-10 and suppress T-

cell differentiation and proliferation, but is reduced in number.[53–56] Further

work is needed to clarify the role of B-cell regulation of AAV, however, as these

changes may be secondary to changes in effector populations, and none of the

studies established a convincing dose relationship between AAV disease activity and

these cells. Regulatory T-cells (Tregs) also have the capacity to suppress immune

responses. Studies of Treg populations in AAV are conflicting, and there is no

consensus regarding changes in their suppressive ability or the size of the T-reg

population in active disease.[57–59]. The most recent study to examine their role in

AAV enrolled 62 patients in a cross-sectional fashion, finding that during active

disease this population was expanded relative to other CD4+ T-cell populations but,

again, it is difficult to know whether this is secondary to changes in the effector

compartment given that the controls were paired remission samples and healthy

volunteers.[60]

Conclusions

In the last few years, some controversies in the pathogenesis of AAV have moved closer to resolution but others remain hotly debated. NETs have emerged as a likely mechanism by which MPO and PR3 are taken up by APCs for presentation to the adaptive immune system, but the precise mechanisms by which tolerance to these self-antigens is broken remains unclear. Molecular mimicry remains an attractive hypothesis, and the most convincing evidence for this comes from controversial studies into the development of anti-LAMP-2 autoantibodies. Recent publications suggest an increasing acceptance of their role in AAV, but further research is needed. Large-scale genetics studies have identified a number of loci, including MHC Class II, that confer genetic susceptibility to AAV, and an important topic for future research will be determining how polymorphisms at these loci contribute to disease pathogenesis. An increasing appreciation of the importance of complement in generating tissue inflammation in AAV underscores the exciting potential of the complement pathway inhibitor eculizumab as a novel therapeutic for active disease. This demonstrates that the difficult process of teasing out the key pathogenic features driving AAV can provide real patient benefit.

Key Points: (3-5 bullet points)

- Emerging evidence suggests that epitope specificity may contribute to the pathogenicity of anti-neutrophil cytoplasmic antibodies (ANCA), and that anti-LAMP-2 antibodies represent a novel form of ANCA.
- The alternative pathway of complement is increasingly recognized as being

important in mediating the pathogenicity of ANCA.

- Neutrophils remain central to the pathogenesis of ANCA-associated vasculitis, with neutrophil extracellular traps playing a potentially important role in initiating the immune response.

Conflicts of interest:

The authors declare that there are no conflicts of interest relevant to this publication.

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Figure titles and legends:

Figure 1.

Excessive neutrophil extracellular trap (NET) formation promotes the development of ANCA by allowing uptake of MPO and PR3 by antigen presenting cells (APC) in a stimulatory context. ANCA further stimulates neutrophils to generate NETs through NETosis.

Figure 2.

Activation of the alternative complement pathway results in the generation of C5a, which exerts a broad range of downstream effects on neutrophils through the ligation of its receptor, C5aR, including the further generation of complement in a positive feedback loop.