B cell phenotype and function in granulomatosis with polyangiitis
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chapter 2

ANCA pathogenicity revisited: pathogenic versus non-pathogenic ANCA

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Abstract

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is strongly associated with autoantibodies against myeloperoxidase (MPO) and proteinase 3 (PR3). No clear consensus has been reached on the pathogenicity of these autoantibodies. Animal models for MPO-ANCA, in vitro data suggesting pathogenicity of ANCA, and one case of a neonate showing symptoms of vasculitis after transplacental transfer of MPO, argue in favour of a pathogenic role for ANCA. On the other hand, the presence of natural MPO and PR3 autoantibodies in healthy individuals, lack of a strong correlation between ANCA titers and disease activity, and the occurrence of ANCA-negative AAV patients argue against pathogenicity of ANCA. Recent papers have drawn attention to the possibility of epitope specificity defining ANCA pathogenicity. Certain MPO epitopes were found to be specific for active disease, and others remained present during remission or were also present in healthy individuals. One linear epitope, aa447-459, was not only exclusive for active disease, but also detected in the total Ig fraction of ANCA-negative patients, reactivity being masked in serum by ceruloplasmin. So, not all ANCA seems to be equal, some could be pathogenic while others are not. For development of an autoimmune response, a specific ANCA repertoire is required, which may occur through intra-molecular epitope spreading in patients.
Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a primary systemic small-vessel vasculitis. The three main diseases that fall under its definition are granulomatosis with polyangiitis (GPA, formerly Wegener’s granulomatosis), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome). The autoantibodies that are characteristic for these diseases are mainly directed against the antigens myeloperoxidase (MPO) and proteinase 3 (PR3) [1]. ANCA were first described in 1982 [2] and have proven invaluable for the diagnosis of AAV. Generally, GPA is mostly associated with PR3-ANCA and MPA with MPO-ANCA and, for both diseases, a small subgroup of patients is ANCA-negative [3]. A recent genome-wide association study has shown that PR3-ANCA versus MPO-ANCA positive AAV patients display distinct genetic associations, even more than their associated diseases, GPA and MPA, respectively [4]. PR3-ANCA and MPO-ANCA disease are also characterised by clinical and histopathological differences. Renal-limited disease occurs mainly in MPO-ANCA patients, while PR3-ANCA is strongly associated with upper and lower respiratory tract granulomatous inflammation whether or not in combination with renal disease. Furthermore, PR3-ANCA patients show more widespread extra-renal organ involvement compared to MPO-ANCA patients [5]. These differences have led to proposals to categorise AAV based on autoantibody specificity rather than on disease phenotype. Recently, it was suggested that a prefix should be added to the diagnosis of GPA or MPA to specify ANCA reactivity, indicating the importance of ANCA specificity in AAV [6]. Aside from anti-PR3 and anti-MPO-ANCA, a small number of patients are positive for anti-human neutrophil elastase ANCA. Its occurrence is particularly related to the use of cocaine contaminated with levamizole inducing midline destructive lesions [7]. Recently, another possible autoantigen has entered the scene in AAV. The presence of lysosomal membrane protein 2 (LAMP-2) autoantibodies was described in almost all patients with pauci-immune necrotising glomerulonephritis and their prevalence was higher than that of ANCA [8]. In addition, these autoantibodies were suggested to be pathogenic and induced by molecular mimicry with bacterial antigens [8]. While in a European cohort a high prevalence of anti-LAMP-2 was described [9], a US cohort showed a far lower frequency of anti-LAMP-2 autoantibodies, and its occurrence was not specific for patients with AAV [10]. Before a conclusion can be drawn on the clinical significance of LAMP-2 antibodies these two studies have to be reconciled. Several differences between the two studies could help explain the discordance. Kain et al. [9] have reported that the highest anti-LAMP-2 titers are found in patients that are newly diagnosed and untreated, with the difference in frequency of anti-LAMP-2 antibodies being significant between treated and untreated patients [9]. Roth et al. [10] included only a restricted number of newly diagnosed, untreated patients. The antigen substrates used in both studies were
not identical, which could affect assay performance. Standardisation of methods to detect LAMP-2 antibodies is required in order to compare data from different groups. Thus, at the moment the clinical significance of LAMP-2 autoantibodies, in contrast to PR3-ANCA and MPO-ANCA, has not yet been established. However, the pathogenic role of MPO and PR3 autoantibodies in AAV is also not fully understood. In this review, we will discuss the evidence for and against the pathogenicity of ANCA, including the most recent findings on epitope specificity.

**Proof for pathogenicity of ANCA**

Evidence for the pathogenicity of ANCA is derived from animal models, in vitro experiments and clinical observations. For MPO-ANCA disease, several animal models have been developed. In 2002, a model was described using immunisation with mouse-MPO in MPO-knockout mice, followed by transfer of their splenocytes or anti-MPO containing IgG-fraction into wild-type mice. This resulted in severe vasculitic disease after splenocyte transfer in kidneys and lungs, but also the transfer of anti-MPO containing IgG induced urinary abnormalities, early glomerular neutrophil accumulation and focal necrotising crescentic glomerulonephritis [11]. Another model was based on the induction of an immune response to human MPO in rats cross-reacting with rat MPO, resulting in pauci-immune crescentic glomerulonephritis and lung haemorrhage [12]. The development of a model for PR3-ANCA disease has not been as successful, although promising models are being developed. Transfer of PR3 antibodies generated in PR3-deficient mice into lipopolysaccharide-primed mice did not cause significant renal or pulmonary pathology [13]. Immunisation of rodents with either human or murine PR3 resulted in induction of PR3-antibodies, but not in development of disease [14]. Splenocyte transfer from autoimmunity-prone non-obese diabetic (NOD) mice immunised with murine PR3 to NOD–severe combined immunodeficiency mice resulted in the development of vasculitis and severe glomerulonephritis, but not in granuloma formation [15]. More recently, a humanised model was proposed in which mice received human haematopoietic stem cells, which resulted in a human-mouse chimeric immune system. After passive transfer of PR3-ANCA, the mice developed mild glomerulonephritis and lung haemorrhage but no granuloma formation [16]. The animal models for MPO-ANCA indicate that the autoantibodies are pathogenic in rodents in producing an MPA-like syndrome. Current animal models do, however, not proof that PR3-ANCA are pathogenic in terms of producing a GPA-like syndrome including granulomatous inflammation.

*In vitro* data also points towards a pathogenic role for ANCA. ANCA were found capable of activating primed neutrophils *in vitro*. Interaction of ANCA with primed neutrophils resulted in neutrophil degranulation and their production of reactive oxygen species (ROS) [17]. It was further shown that activation of neutrophils by ANCA at the vascular wall resulted in endothelial cell injury [18], and ANCA promote stable adhesion and
Pathogenic vs non-Pathogenic ANCA

Subsequent migration of neutrophils into the endothelium [19]. More recently, activation of neutrophils by ANCA was shown to result in the formation of neutrophil extracellular traps (NETs) [20]. Neutrophils were primed with tumour necrosis factor (TNF)α and subsequently incubated with purified IgG from healthy controls or AAV patients. Robust NET formation was observed only with patient IgG. On the NETs, the autoantigens PR3 and MPO were detected, indicating that netosis is one of the ways PR3 and MPO are presented to the immune system. These ANCA-induced NETs can activate dendritic cells and autoreactive B cells in a Toll-like receptor 9–dependent manner [20]. These in vitro effects of ANCA on neutrophils are dependent on priming of the neutrophils, usually with TNFα. This suggests anti-TNFα therapy may be effective in AAV patients; however, a randomised, placebo-controlled trial using etanercept did not find any beneficial effect of this drug in AAV patients regarding induction of remission [21]. So, substantial in vitro experimental data underscore different pathogenic roles of ANCA in AAV.

Clinical proof for the pathogenicity of ANCA in humans is limited. An often referred single case report describes a neonate born to a mother with a history of MPA. MPO-ANCA were observed at birth in the neonate, followed by pulmonary haemorrhage and renal involvement [22]. This implies that transplacental transfer of MPO-ANCA was directly responsible for the clinical symptoms. However, no similar cases have been reported since and another neonate was perfectly healthy despite transplacental transfer of high levels of MPO-ANCA [23]. Further indirect clinical proof is the efficacy of plasma exchange, this treatment increases the rate of renal recovery in severe ANCA-associated systemic vasculitis compared with intravenous methylprednisolone [24].

A clear relationship between ANCA titers and disease activity is not consistently found. While certain studies indicate that a rise in ANCA titer can be predictive of an ensuing relapse [25], others fail to confirm these observations. In a study on 156 GPA patients, PR3-ANCA titers were only weakly associated with disease activity, decreases in PR3-ANCA were not related to a shorter time to remission and increasing PR3-ANCA titers were not associated with relapse in the following year [26]. A recent meta-analysis on this topic concluded that a rise in ANCA during remission is only modestly predictive of future relapse [27]. So, although in vitro and in vivo experimental data are strongly suggestive for a pathogenic role of ANCA in the AAV, clinical observations are insufficient to proof the pathogenicity of ANCA.

ANCA pathogenicity questioned

One discovery that questions the pathogenicity of ANCA is the presence of natural autoantibodies (NAAs) directed against PR3 and MPO as described in healthy individuals. Antibodies were purified from isolated IgG from 20 healthy controls by antigen-specific affinity columns, showing titers of natural MPO- and PR3 autoantibodies, albeit at significantly lower levels than those from patients with vasculitis. All individuals also
had anti-glomerular basement membrane NAAs, which are also found in ~10% of AAV patients [28]. The MPO-NAA were characterised further and compared with MPO-ANCA from AAV patients. MPO-NAA recognised conformational epitopes in the heavy chain of MPO, similar to MPO-ANCA. The median titer for MPO-NAA was 1:40 compared to 1:4800 for MPO-ANCA in AAV patients, and the avidity of the NAA was significantly lower. Finally, an IgG subclass analysis was performed, showing that NAA are mainly restricted to the IgG1-subclass and lack the presence of IgG3 [29]. ANCA in AAV have been found in all four IgG subclasses, of which IgG3-ANCA were initially thought to be most pathogenic and increased during active disease compared with remission [30]. However, rises in IgG3-ANCA were not predictive of relapse [25, 31]. In vitro, MPO-ANCA-positive IgG rich in IgG3 induced a higher level of respiratory burst of neutrophils than MPO-ANCA-positive IgG poor in IgG3 [32], and IgG3-ANCA were capable of capturing rolling neutrophils far more than any other subclass, converting neutrophils to a static adhesive state [33]. The latter study reported that super oxide production was induced similarly by both IgG1- and IgG3-ANCA [33], suggesting that IgG1-ANCA in healthy controls are certainly not intrinsically harmless.

Secondly, a subset of patients test negative for both MPO- and PR3-ANCA despite fulfilling criteria for AAV [34, 35]. Comparing ANCA-negative pauci-immune renal vasculitis patients with MPO- or PR3-ANCA positive patients, no differences in active renal lesions were observed, while severe interstitial fibrosis and glomerulosclerosis were more prominent in ANCA-negative and MPO-ANCA patients compared with PR3-ANCA patients. Overall mortality rates were not different between ANCA-positive and ANCA-negative patients [35]. Interestingly, rituximab was still effective in ANCA-negative patients, indicating that other functions of B cells than ANCA production might play a role in the pathogenesis of AAV [36].Thirdly, as mentioned earlier, the relation between ANCA titers and disease activity is not very strong. So, while ANCA pathogenicity is a currently accepted paradigm, clinical evidence is certainly not complete (reviewed in [37]). One explanation for the discrepancies between clinical and experimental data may be based on the concept that epitope specificity determines pathogenicity of ANCA.

Epitope specificity determines pathogenicity

Epitope mapping of MPO and PR3 has been performed extensively over the years (Figure 1A-B). For PR3, a number of different studies have attempted to map its epitopes [38–40]. The most recent PR3 epitope study used overlapping octapeptides and identified seven common antigenic regions, three of which were in direct proximity to amino acids that form the catalytic triad of the protein [41]. The results are not completely consistent between studies, likely due to different methods and patient groups.
For MPO, similar studies have been done. Early on, it was established that MPO-ANCA do not target a single epitope, but rather a small number of MPO regions, primarily in the carboxy-terminus of the heavy chain [42]. Using overlapping decapeptides to represent the MPO protein, 12 patient samples and matched controls were tested for reactivity. Seven major epitopes were identified to which at least 33% of patients exhibited reactivity. Although patients shared these epitopes, the response was highly variable. Six of the identified reactive epitopes were found on the heavy chain of the mature MPO protein structure, and the remaining epitope was located within the pro-peptide region of the protein [43].

Figure 1. (A) Three-dimensional (3D) crystal structure of PR3 (pdb 1FUJ) with highlighted epitopes. Epitopes identified in a single study are coloured blue [38], orange [39], purple [40] or green [41]. PR3 epitopes found in multiple studies are highlighted in red. (B) 3D crystal structure of MPO (pdb 3F9P) with highlighted epitopes. Epitopes identified in a single study are coloured blue [43] or green [44], whereas epitopes identified in both studies are highlighted in red. (C) 3D crystal structure of MPO (pdb 3F9P). Focused on the Roth et al. epitopes that were either specific for disease or also present in healthy controls [44]. Epitopes specific for ANCA disease (red, with aa447-459 highlighted in blue) are spatially close to or next to natural epitopes (green). For construction of the image, Swiss-pdbViewer was utilised [46].
A recent paper by Roth et al. [44] examined MPO epitope specificity using a high sensitivity epitope excision and mass spectrometry approach. They investigated patients in remission, patients with active disease, ANCA-negative patients, and healthy controls, and identified a total of 25 different epitopes, 12 of which were exclusive to active disease and 8 were also present in healthy subjects (termed natural epitopes). The majority of these identified epitopes, 20 of 25, were confirmed to be conformational. One linear epitope (aa447-459) was exclusive to active disease, and reactivity to this specific epitope declined with remission. Total Ig fractions from serum samples of ANCA-negative patients, confirmed to be negative as tested by multiple laboratories, were tested for reactivity against these different peptides. Reactivity was observed against a single peptide that corresponded to the MPO peptide aa447-459, identified earlier as an epitope specific for active disease. This discrepancy between serum and total Ig was explained by masking of the epitope by a fragment of ceruloplasmin, an MPO inhibiting serum protein. Functionally, anti-MPO aa447-459 antibodies could induce the release of ROS from neutrophils, whereas antibodies specific for natural epitopes barely induced ROS release. The epitope is conserved between humans and mice, and following IgG transfer from mice immunised with a murine MPO peptide that overlaps with peptide aa447-459, mice developed glomerular injury and neutrophil accumulation. These results emphasise the pathogenic capacity of MPO aa447-459 autoantibodies. This study concludes that epitope specificity defines pathogenicity of ANCA and explains the existence of NAA in healthy individuals. Moreover, it states that ANCA-negative patients may be ANCA-positive patients responding to masked or currently unidentified epitopes [44].

Another study has also examined the connection between epitope specificity and disease activity. Gou et al. [45] studied the association between linear epitopes of MPO-ANCA and clinicopathological features of 77 AAV patients. Six linear peptides were constructed, all of which were recognised by at least one patient and reactivity was observed throughout the MPO molecule. During disease remission, the number of fragments recognised was significantly lower than at initial onset. Interestingly, 50% of patients recognised the aa399-519 region at initial disease onset but during remission all reactivity to aa399-519 turned negative, confirming that certain epitopes are recognised specifically during active disease. This region also encompasses the aa447-459 epitope; however, as the region is much more extensive these results cannot be ascribed to the aa447-459 epitope. While earlier studies showed only reactivity to the heavy chain, reactivity to the light chain of MPO was also observed. Patients exhibiting light chain reactivity had more severe disease and reactivity to a higher number of other MPO fragments compared with those without light chain reactivity. The light chain is less accessible for antibody binding, indicating that in these patients intra-molecular epitope spreading is more extensive [45]. So, it seems that not all ANCA are pathogenic, but only those against certain epitopes. Spatially, the epitopes specific to ANCA disease were found in close proximity to
the epitopes on the MPO molecule recognised by natural antibodies [44](Figure 1C), suggesting the possibility that reaction first develops against natural epitopes of the molecule and subsequently spreads to pathogenic epitopes. The possibility that development of pathogenic ANCA is due to dysregulation of natural ANCA rather than a newly developed autoimmune capability is intriguing. While the mechanisms behind this are unknown, it could be due to dysregulation of immune regulatory mechanisms in patients with AAV.

**Regulation of the ANCA-related immune response**

Regarding the regulation of the immune response in AAV patients, many studies focus on the role of regulatory T and B cells (T\textsubscript{reg}, B\textsubscript{reg}). T\textsubscript{reg} cells are CD4+ cells expressing the interleukin (IL)2 receptor \(\alpha\)-chain (CD25) and high levels of the transcription factor forkhead box P3 (FoxP3). They are indispensable for maintenance of immune self-tolerance by suppressing aberrant or excessive immune responses harmful to the host [47]. The percentage of FoxP3\textsuperscript{+}CD25\textsuperscript{high}CD4\textsuperscript{+} cells with a memory phenotype was increased in AAV patients, and their suppressive capacity was diminished [48]. This defective suppressive capacity was associated with increased usage of an exon 2-deficient splice variant of FoxP3 in AAV compared with healthy controls [49].

Currently, also B\textsubscript{reg} cells are under investigation in patients with AAV. Studies in mice models have demonstrated that B cell deficiency can lead to exacerbation of autoimmune diseases. T cell receptor knockout mice, which spontaneously develop colitis, showed a more severe and earlier developing course of disease when they were B cell deficient [50], leading to the conclusion that B cells can play a suppressive role, a capacity which was later linked to the production of IL10. Recovery of induced experimental autoimmune encephalomyelitis was dependent on the presence of B cells. However, after using a bone marrow chimeric system resulting in the deficiency of IL10 in B cells, animals were unable to recover and the immune response persisted [51]. IL10 producing B cells have been described in humans as well, although no definitive phenotypical markers have been determined for these cells. B\textsubscript{reg} cells were proposed to be mainly contained within the CD24\textsuperscript{high}CD38\textsuperscript{high} B cell population, although this population certainly does not exclusively consist of B\textsubscript{reg} cells [52]. Others, however, found B\textsubscript{reg} cells enriched in the CD24\textsuperscript{high}CD27\textsuperscript{+} B cell population [53]. As these two populations are non-overlapping, the proper phenotypic description for B\textsubscript{reg} cells is still uncertain.

For B\textsubscript{reg} research in AAV mainly the CD24\textsuperscript{high}CD38\textsuperscript{high} population has been investigated so far. A decrease in CD5\textsuperscript{+} B cells, a marker also present on the CD24\textsuperscript{high}CD38\textsuperscript{high} population, was described during active disease [54]. Differences in percentages of CD24\textsuperscript{high}CD38\textsuperscript{high} B cells within the total B cell population have been demonstrated between patient groups and healthy controls, in part relating to disease activity. Results do not show a consistent, clear answer (Table 1). One attempt to identify B\textsubscript{reg} surface markers is based on capturing IL10\textsuperscript{+} cells and running whole-genome expression...
analysis resulting in a new phenotypic proposal. While no unique transcription factor was detected, B\textsubscript{reg} cells were shown to be CD73\textsuperscript{−}CD25\textsuperscript{+}CD71\textsuperscript{+} [55]. If no clear phenotypic characterisation is possible due to plasticity of B cells other ways to look at B\textsubscript{reg} cells should be investigated such as focusing on production of IL10. IL10 production in stimulated total CD19\textsuperscript{+} B cells was diminished in patients with active disease and during remission [56]. However, other studies found only a decrease of IL10 production during active disease [58] or no difference in IL10 production between patients and controls [59 (Table 1). The possibility that dysregulation of regulatory cell populations contributes to epitope spreading is interesting and should be further investigated.

Conclusions and future research
The recent findings on epitope specificity of ANCA may give answers for questions regarding the pathogenicity of ANCA. If only a specific subset of ANCA is pathogenic, then the presence of NAA is not incompatible with ANCA pathogenicity. It explains why ANCA titers and disease activity do not correlate well, as routine tests measure all ANCA, also non-pathogenic ones. Moreover, these experiments question the existence of ANCA-negative patients, as at least some ANCA-negative patients have now been confirmed to have MPO-ANCA. An important limiting factor is, however, the characterisation and detection of antibodies to conformational epitopes. Most MPO-epitopes are conformationally dependent, as reflected by the data of Gou et al. [45], showing that > 40% of MPO-ANCA-positive patients do not recognise linear peptides. Critical epitopes may be missed by only studying linear epitopes. It seems unlikely that the ‘ANCA-negative’ patients would only have ANCA against a single epitope, while other patients have ANCA against a range of epitopes. Studying conformational epitopes is, however, more complicated due to their tertiary and quaternary structures. Besides MPO, epitope specificity of PR3 should be examined using a similar strategy. This could reveal whether masked epitopes occur also for PR3, therewith explaining the occurrence of more ANCA-negative patients. First, however,

Table 1. Regulatory B cells in ANCA associated vasculitis patients compared to healthy controls.

<table>
<thead>
<tr>
<th>Phenotype studied</th>
<th>% of total B cells</th>
<th>IL10 production by total B cells</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24\textsuperscript{hi}CD38\textsuperscript{hi}</td>
<td>decreased % in remission, similar % in active disease</td>
<td>-</td>
<td>[57]</td>
</tr>
<tr>
<td>CD24\textsuperscript{hi}CD38\textsuperscript{lo}</td>
<td>similar % in remission, decreased % in active disease</td>
<td>similar in remission and active disease</td>
<td>[59]</td>
</tr>
<tr>
<td>CD24\textsuperscript{lo}CD38\textsuperscript{hi}</td>
<td>similar %, only decreased % in active MPO patients</td>
<td>similar in remission, decreased in active disease</td>
<td>[58]</td>
</tr>
<tr>
<td>CD5\textsuperscript{+}</td>
<td>similar % in remission, decreased % in active disease</td>
<td>-</td>
<td>[54]</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>decreased in both remission and active disease</td>
<td>[56]</td>
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the experiments done by Roth et al. [44] should be confirmed in other institutions and population groups, to attest that the data can be replicated and to determine possible differences between patient cohorts. Besides this, effort should be focused on further characterisation of pathogenic and non-pathogenic MPO- and PR3 epitopes. This will not only increase our understanding of the pathogenesis of AAV, but may also give possibilities for targeted treatment. However, not all active disease patients displayed reactivity to the pathogenic epitope described by Roth et al. [44]. So, further analysis of epitope specificity in relation to disease activity should be explored, also in longitudinal studies. To further clarify the occurrence of epitope spreading, its mechanisms should be elucidated, including the role of regulatory T and B lymphocytes. Better characterisation of these cell populations is mandatory. What can be concluded is that not all ANCA are equal, some seem clearly pathogenic whilst others appear innocuous. For development of AAV, a specific ANCA repertoire is required. This repertoire may develop through intra-molecular epitope spreading. An insight into epitope specificity and their regulatory mechanisms may lead to better understanding of AAV pathogenesis with possibilities for targeted treatment. When both the field on epitope specificity and regulatory mechanisms are more extensively studied, the pathogenesis of AAV and the role of ANCA may be significantly better understood.

**Take home messages**

- Controversial data exists regarding pathogenicity of ANCA:
  - Certain clinical data, in vitro studies and in vivo experimental studies point towards pathogenicity
  - Presence of natural ANCA in healthy individuals, ANCA-negative patients and lack of a strong association between ANCA titers and disease activity argue against pathogenicity

- Epitope specificity of ANCA could explain:
  - Lack of a strong correlation between ANCA titers and disease activity due to the occurrence of reactivity against pathogenic epitopes during active disease and non-pathogenic epitopes during remission.
  - The occurrence of ANCA negative patients due to masking of certain MPO epitopes by serum factors.
  - The occurrence of natural ANCA in healthy persons due to reactivity against non-pathogenic epitopes.

- ANCA against natural epitopes may precede extension to pathogenic epitopes, via intra-molecular epitope spreading.
- Disturbances in regulatory mechanisms may be involved in this process.
- In conclusion: not all MPO-ANCA are pathogenic, only those against specific epitopes.
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