







Activation of a Latent Epitope Causing Differential Binding of Antineutrophil Cytoplasmic Antibodies to Proteinase 3

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Objective. Proteinase 3 (PR3) is the major antigen for antineutrophil cytoplasmic antibodies (ANCA) in the systemic autoimmune vasculitis, granulomatosis with polyangiitis (GPA). PR3-targeting ANCA (PR3-ANCA) recognize different epitopes on PR3. This study was undertaken to study the effect of mutations on PR3 antigenicity.

Methods. The recombinant PR3 variants, iPR3 (clinically used to detect PR3-ANCA) and iHm5 (containing 3 point mutations in epitopes 1 and 5 generated for epitope mapping studies) immunoassays and serum samples from patients enrolled in ANCA-associated vasculitis (AAV) trials were used to screen for differential PR3-ANCA binding. A patient-derived monoclonal ANCA 518 (moANCA518) that selectively binds to iHm5 within the mutation-free epitope 3 and is distant from the point mutations of iHm5 was used as a gauge for remote epitope activation. Selective binding was determined using inhibition experiments.

Results. Rather than reduced binding of PR3-ANCA to iHm5, we found substantially increased binding of the majority of PR3-ANCA to iHm5 compared to iPR3. This differential binding of PR3-ANCA to iHm5 is similar to the selective moANCA518 binding to iHm5. Binding of iPR3 to monoclonal antibody MCPR3-2 also induced recognition by moANCA518.

Conclusion. The preferential binding of PR3-ANCA from patients, such as the selective binding of moANCA518 to iHm5, is conferred by increased antigenicity of epitope 3 on iHm5. This can also be induced on iPR3 when captured by monoclonal antibody MCPR2. This previously unrecognized characteristic of PR3-ANCA interactions with its target antigen has implications for studying antibody-mediated autoimmune diseases, understanding variable performance characteristics of immunoassays, and design of potential novel treatment approaches.

INTRODUCTION

Antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) comprises a group of systemic small vessel

vasculitis syndromes including granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (1). The 2 major target antigens for the ANCA in vasculitis are proteinase 3 (PR3) and

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Research Foundation, Genentech, and Biogen Idec. At the Mayo Clinic, the RAVE trial was supported by a Clinical and Translational Science Award (CTSA) grant (UL1-RR-024150-01) from the National Center for Research Resources (NCR). At Johns Hopkins, the RAVE trial was supported by a CTSA grant (UL1-RR025005) from the NCR and by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH (grants K23-AR-052820 to Dr. Seo and K24-AR-049185 to Dr. Stone). At Boston University, the RAVE trial was supported by CTSA grants (UL1-RR-025771 and NIH-M01-RR00533) from the NCR, an Arthritis Foundation Investigator Award (to Dr. Monach) an Arthritis Foundation Investigator Award (to Dr. Monach), and the National Institute of Arthritis and Musculoskeletal and Skin Diseases (grant K24-AR-02224 to Dr. Merkel).

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myeloperoxidase (MPO) (2–4). Testing for the presence of ANCAs has become indispensable in the evaluation of patients suspected of having AAV (5). Patients with PR3-targeting ANCAs (PR3-ANCAs) are at higher risk for relapses than patients with MPO-targeting ANCAs (MPO-ANCAs). In addition, PR3-ANCA positivity and rising titers following treatment portend relapses, particularly for patients with renal disease and other disease manifestations of capillaritis (6–8). The binding of PR3-ANCAs to PR3 has many well-documented proinflammatory effects, and PR3-ANCAs are thought to play a pathogenic role for the development of necrotizing vasculitis (9–11).

The oligoclonal PR3-ANCAs from patients with GPA are known to bind to different epitopes of the folded PR3 antigen, whereas denatured PR3 or improperly folded recombinant PR3 generated in nonmammalian expression systems do not reliably bind PR3-ANCAs (12–14). Consistently, studies of continuous epitope mapping of PR3-ANCAs using oligopeptides have generated inconclusive results (15–18).

In this context, we instead performed discontinuous epitope mapping of anti-PR3 monoclonal antibodies (mAb) and PR3-ANCAs using human–murine chimeric recombinant PR3 molecules with surface epitope–specific point mutations to obtain mechanistic insights into how interactions of PR3 with its environment during inflammation are modified by PR3-ANCAs and potentially targetable by therapeutics (19). Herein, the iPR3 mutant represents the most prevalent wild-type PR3 conformation (Val¹⁰³) and contains the Ser^{195Ala} mutation at the active site (Figure 1) to avoid potential enzymatic degradation of ANCAs or PR3-capturing mAb by PR3 in immunoassays or cytotoxicity when recombinant PR3 is expressed in HEK 293 cells (20–23). Because iPR3 has the same folded conformation of wild-type mature PR3, it has been used for 2 decades as a standard PR3 antigen for sensitive and specific PR3-ANCA detection by immunoassay (7,22,24–29).

In our epitope mapping study, we developed a human–murine chimeric mutant of iPR3 (iHm5) (19) to investigate the involvement of epitope 5 on PR3—an epitope defined by binding of a group of mAb including MCPR3-7—in binding PR3 to neutrophil membranes (19,30,31). Because iHm5 has its 3 hydrophobic/aromatic residues (Ala¹⁴⁶, Trp²¹⁸, and Leu²²³) (Figure 1) of human PR3 replaced by their murine hydrophilic counterparts (Thr¹⁴⁶, Arg²¹⁸, and Gln²²³), we expected that the PR3-ANCAs would show reduced binding to the mutated epitopes on iHm5 relative to iPR3. Instead, we serendipitously identified a human mAb

(moANCA518), derived from a patient with GPA, that recognized iHm5 but not iPR3 and demonstrated that this preferential recognition of iHm5 by moANCA518 was caused by the increased mobility of epitope 3, leading to the remote activation of a latent epitope on PR3 by the distant mutation (32).

Informed by this background, we conducted the present study using large well-defined patient cohorts to determine 1) the scope of this preferential binding of PR3-ANCAs to iHm5, 2) the potential utility of iHm5 as an in vitro antigen for PR3-ANCA detection, 3) the epitope(s) involved in this enhanced antigenicity of iHm5 for PR3-ANCAs, and 4) whether remote activation of a latent epitope could also be induced by binding of mAb to distant epitopes of iPR3.

MATERIALS AND METHODS

Reagents were obtained from Sigma unless otherwise specified. The HEK 293 cell line used for the expression of recombinant PR3 mutants was obtained from ATCC.

Recombinant PR3 mutants. The complementary DNA constructs coding for iPR3 and iHm5 and their expression in HEK 293 cells have been described in detail elsewhere (19,26). Both mutants carry a carboxy-terminal c-Myc peptide extension and a poly-His peptide extension for anchoring in solid phase immunoassays and purification using nickel columns from GE Healthcare, as previously described (19,26–28,33). The molecule iPR3 has previously been referred to as rPR3-S176A (22), rPR3S195A (24,26), and iPR3-Val¹⁰³ (32), and iHm5 has been referred to as Hm5 (19) and iHm-Val¹⁰³ (32).

Immunoassays. For Western blots, PR3 mutants were loaded (1 µg/lane) onto 12% Tris-HCl gels from Bio-Rad. Protein samples were not reduced, and sodium dodecyl sulfate gel electrophoresis was performed at 180V for 35 minutes. Proteins were transferred from gels to nitrocellulose membranes. Membranes were subsequently washed with Tris buffered saline (TBS) buffer and blocked for 45 minutes at room temperature (RT) with TBS/0.2% nonfat dry milk. Next, membranes were washed twice with TBS/0.1% Tween 20. Monoclonal antibodies, diluted to 0.5–1.0 µg/ml as indicated, were incubated on the membrane overnight at 4°C. Next, membranes were washed twice with TBS/0.1% Tween 20 and incubated with goat anti-human or anti-mouse IgG horseradish peroxidase (HRP) conjugates and

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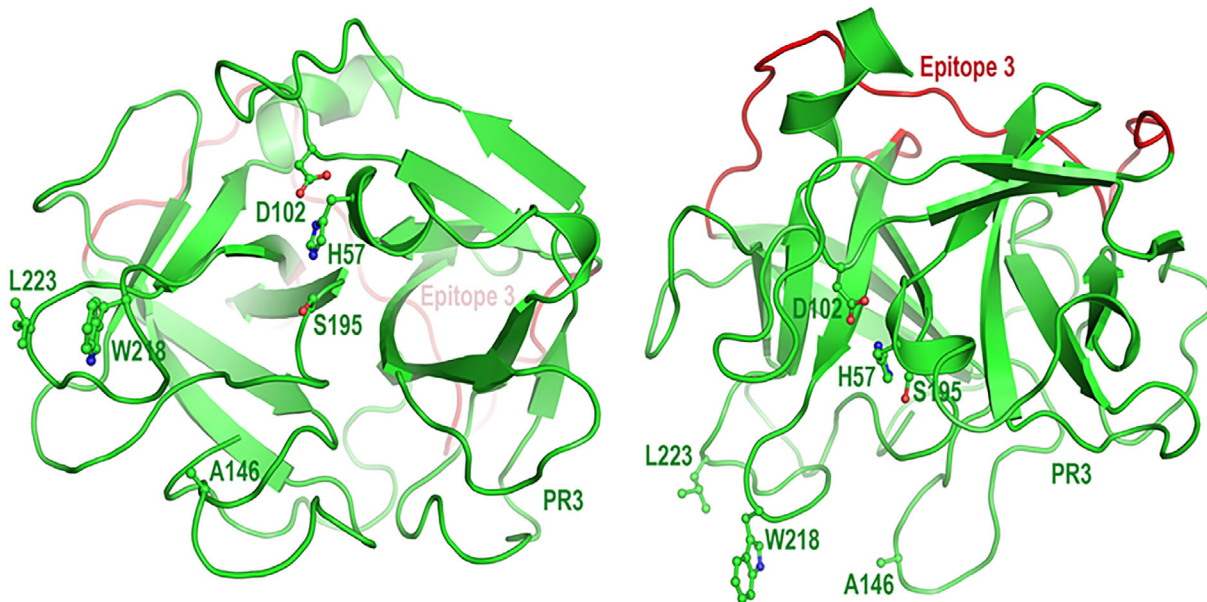


Figure 1. Model of human proteinase 3 (PR3). Left, PR3 in the standard orientation facing the active site pocket. Right, PR3 with ~90-degree rotation. The catalytic triad of this neutrophil serine protease comprises His⁵⁷, Asp¹⁰², and Ser¹⁹⁵. Ala¹⁴⁶, Trp²¹⁸, and Leu²²³ in PR3 shown here are replaced by their hydrophilic murine counterparts (Thr¹⁴⁶, Arg²¹⁸, and Gln²²³) in iHm5. Epitope 3 is shown in red on the PR3 structure side opposite where the mutations were introduced. This illustration was generated using PyMOL version 1.7.0.3. Amino acid numbering is based on the method described in reference 50.

diluted to 1:20,000 for 20 minutes at RT. Membranes were washed again and developed with a Pierce ECL Western Blotting Substrate kit according to instructions of the manufacturer (Thermo Fisher Scientific) and exposed as indicated.

For the direct enzyme-linked immunosorbent assay (ELISA) experiments, MaxiSorp (Invitrogen) plates were coated with iHm5 or iPR3 (1.0 $\mu\text{g/ml}$) in NaHCO_3 (pH 9.5), overnight at 4°C. In between steps, plates were washed 3 times with 200 μl of phosphate buffered saline (PBS) with 0.05% Tween 20 (10 mM sodium phosphate, 0.15M NaCl, 0.05% Tween 20, and pH 7.5). The plates were then blocked with 200 μl of PBS with 1% Tween 20 and 10% bovine serum albumin (BSA) diluted to 1:10 for 2 hours at RT and protected from direct light exposure. Monoclonal ANCA 518 or epitope-specific anti-PR3 mAb (1.0 $\mu\text{g/ml}$) were diluted to 1:20 in PBS with 1% Tween 20 and 10% BSA and used as primary antibodies. The antibody binding to the PR3 mutants was probed with HRP-conjugated anti-human IgG antibody (1:250 dilution) incubated for 1 hour at RT. As substrate, 100 μl of 3,3',5,5'-tetramethylbenzidine (Thermo Fisher Scientific) was developed for 15 minutes and stopped with 100 μl 2 N H_2SO_4 . The absorbance in optical density (OD) was measured using spectrophotometry at 450 nm. Results are expressed as the net absorbance in OD after subtraction of the absorbance readings of the background wells coated with NaHCO_3 only. For the inhibition experiments, epitope-specific anti-PR3 mAb were coated to MaxiSorp plates at concentrations of 2.0–4.0 $\mu\text{g/ml}$ in NaHCO_3 overnight at 4°C to capture the PR3 mutants.

The anchor ELISA method for IgG PR3-ANCAs has previously been described for IgA PR3-ANCAs (33) and is conceptually similar to the capture ELISA utilizing the C-terminal c-Myc-peptide extension of a PR3 mutant (26,27). Either the purified PR3 mutants or culture media supernatants from PR3 mutant expressing HEK 293 cell clones diluted in immunoradiometric assay (IRMA) buffer (50 mM Tris, 0.1M NaCl, pH 7.4, and 0.1% BSA) were incubated in Pierce nickel-coated plates from Thermo Fisher Scientific for 1 hour at RT; the background wells were incubated with serum-free media instead (26,27,33). Plates were washed 3 times with TBS wash buffer (20 mM Tris, 500 mM NaCl, pH 7.5, 0.05% Tween 20) in between steps. Serum samples were diluted 1:20 in TBS buffer containing 0.5% BSA and incubated with the antigen for 1 hour at RT. PR3-ANCAs were detected after incubation for 1 hour at RT with alkaline phosphatase-conjugated goat anti-human IgG (1:10,000 dilution). When required, IgA and IgM PR3-ANCAs were detected using alkaline phosphatase-conjugated goat anti-human IgA (1:2,000 dilution) and alkaline phosphatase-conjugated goat anti-human IgM (μ -chain-specific) (1:20,000 dilution), respectively. *P*-nitrophenyl phosphate was used as substrate at a concentration of 1 mg/ml. The net absorbance was obtained by spectrophotometry at 405 nm after 30 minutes of exposure. The assay's cutoff value for a positive result was defined as the mean net absorbance obtained from 30 normal control serum samples plus 2 SD of the mean. The net absorbance cutoff values for IgG PR3-ANCA detection using iPR3 and iHm5 were determined as 0.105 and 0.107 OD, respectively.

The MCPR3-2 capture ELISA method for PR3-ANCAs detection has been described previously (24) and is used here to assess the binding of moANCA518 to iHm5 and iPR3. In brief, microtiter wells (Immulon 1B; Thermo Fisher) were incubated with 100 μ l of 4 mg/ml MCPR3-2, in NaHCO₃ (pH 9.5), at 4°C overnight. After washing 3 times with TBS wash buffer (20 mM Tris, 500 mM NaCl, pH 7.5, 0.05% Tween 20), 100 μ l of culture media supernatants from PR3 mutant-expressing HEK 293 cell clones diluted in IRMA buffer (0.05 mM Tris, 0.1M NaCl, pH 7.4, 0.1% BSA) were incubated in the wells for 1 hour at RT. Plates were washed 3 times with TBS wash buffer (20 mM Tris, 500 mM NaCl, pH 7.5, and 0.05% Tween 20) in between steps. Control wells were incubated in parallel with buffer alone. The moANCA518 solution (1 μ g/ml) was prepared in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) with 0.5% BSA and used as the primary antibody in incubation with the antigen for 1 hour at RT. The moANCA518 binding to the antigen was detected after incubation (1 hour at RT) of alkaline phosphatase-conjugated goat anti-human IgG antibody (A-9544; Sigma) in a 1:10,000 dilution in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) with 0.5% BSA. *P*-nitrophenyl phosphate was used as substrate at a concentration of 1 mg/ml. The net OD was obtained by spectrophotometry at 405 nm after 30 minutes of exposure.

Serum and plasma samples. The 30 serum samples from normal donors used for the determination of the normal (negative) range of the anchor ELISA for PR3-ANCA detection using the PR3 mutants as antigen were obtained from the Clinical Immunology Laboratory of the Mayo Clinic in Rochester, MN. Three hundred serum samples from healthy octogenarians used for the specificity analyses were obtained from the Mayo Clinic Biospecimen repository. No patient identifiers or clinical data about these individuals were available to the investigators. The use of these serum samples for this study was approved by the Mayo Clinic Institutional Review Board.

Serum samples from patients with AAV used in this study were collected during the Wegener's Granulomatosis Etanercept Trial (WGET) and during the Rituximab versus Cyclophosphamide for AAV trial (RAVE). Details of the WGET and RAVE protocols, patient characteristics, and trial results have been described elsewhere (34–36). Participants in both trials provided written consent for the use of their serum samples in ancillary studies.

For inhibition experiments with Fab fragments, we used PR3-ANCA-positive plasma samples from patients with GPA collected for a biospecimen repository approved by the Mayo Clinic Institutional Review Board. We have previously shown that the agreement of PR3-ANCA levels determined by immunoassay in matched serum and plasma samples is excellent (37).

Generation of moANCA518. DNA barcode-enabled sequencing of the antibody repertoire was performed on plasmablasts derived from 5 participants in the RAVE study at baseline,

at the time of remission and at the time of subsequent relapse, as described for rheumatoid arthritis and Sjögren's syndrome, which has been described elsewhere (32,38,39). The generated recombinant human monoclonal antibodies were tested for reactivity with ANCA target antigens, including MPO (33), human neutrophil elastase (HNE) (40–42), iPR3, and iHm5 in parallel, via the anchor ELISA using recombinant antigens carrying a C-terminal poly-His tag. We used moANCA518 to comparatively gauge the binding of PR3-ANCA to iHm5 and the activation of the latent epitope on iPR3 by remote antibody binding.

Monoclonal antibodies and Fab fragments. The mAb PR3G-2 (targeting epitope 1) was a gift from Prof. C.G.M. Kallenberg from the University of Groningen, and the mAb WGM2 (targeting epitope 3) was purchased from Hycult Biotech Inc. (19,43,44). The mAb MCPR3-2 (targeting epitope 4), MCPR3-3 (targeting epitope 3), and MCPR3-7 (targeting epitope 5) that were generated by Dr. Specks' group at the Mayo Clinic have been described and characterized in detail elsewhere (19,24,26). A Pierce Fab Preparation Kit from was used to generate the Fab fragments from 1.0 mg of a mAb (moANCA518, WGM2, MCPR3-2, MCPR3-3, or MCPR3-7) following the protocol of the manufacturer (Thermo Fisher Scientific).

Statistical analysis. IBM SPSS Statistics for MacOS, version 25, was used to calculate the mean and SE of 3–5 repeat experiments and to compare the means between groups with Student's 2-tailed paired *t*-test.

RESULTS

Preferential binding of PR3-ANCAs to iHm5 over iPR3. Using the net absorbance as a measure of the reactivity of a serum sample with an antigen, we found that the reactivities of IgG PR3-ANCA-positive serum samples with iHm5 were either equal or higher than those with iPR3 in an anchor ELISA (Figure 2). Of the 178 serum samples obtained from the WGET participants at enrollment, 148 had previously tested positive for PR3-ANCA in ≥ 1 of several immunoassays for PR3-ANCAs (34). Using the anchor ELISA, 144 samples tested positive for IgG PR3-ANCAs with iHm5, and 135 were positive with iPR3 (Figures 3 and 4). Of the 135 samples that reacted with both antigens, 108 (80%) had higher reactivities with iHm5 than with iPR3, and 41 (30%) of the samples had reactivities with iHm5 that were more than double the reactivities with iPR3 (Figures 3 and 4).

To confirm these results in an independent cohort, we also analyzed 129 serum samples, which had previously tested positive for PR3-ANCAs in ≥ 1 immunoassay and were obtained from the participants in the RAVE study at the time of enrollment (35,36). Of these, 128 samples showed reactivity with iHm5, and 126 samples reacted with both iHm5 and iPR3. The reactivities with iHm5 in 33 (26%) of

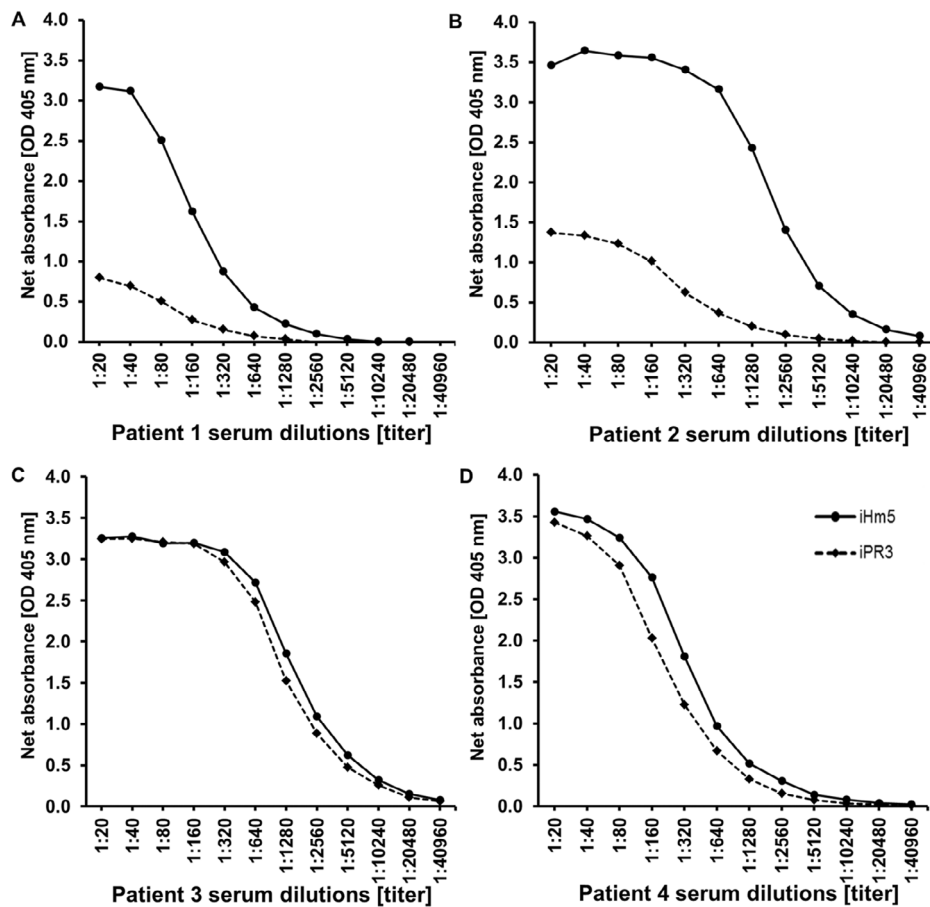


Figure 2. Reactivity of proteinase 3–targeting antineutrophil cytoplasmic antibody (PR3-ANCA)–positive serum samples with iHm5 and iPR3. Representative examples of serum samples from patients with PR3-ANCA–associated vasculitis showing higher (A and B) or equal (C and D) binding to iHm5 compared to iPR3 in the anchor enzyme-linked immunosorbent assay.

these 126 samples were more than double the reactivities with iPR3 (Figures 3 and 4). The single serum sample from the WGET cohort and the 3 serum samples from the RAVE cohort that showed higher reactivity with iPR3 than iHm5 all yielded very high net absorbance values for both antigens, and on repeat testing results were consistently slightly lower with iHm5, with differences for each sample just above the intraassay coefficient of variance of the assay of 5% (data not shown).

When comparing the differential PR3-ANCA binding to iHm5 versus iPR3 with disease activity scores or specific organ involvement, we found no association in either the WGET or RAVE cohort. To further evaluate whether the preferential binding of PR3-ANCA to iHm5 over iPR3 is of potential clinical utility, we explored the sensitivity and specificity of the anchor ELISA using iHm5 in defined cohorts.

Of the 14 WGET participants who had previously consistently tested negative for PR3-ANCAs in other assays (28), enrollment serum samples from 2 patients (14%) were positive in the anchor ELISA using iHm5, indicating that in a sizable number of patients with GPA who were previously labeled as “ANCA-negative,” PR3-ANCA can be detected with the iHm5

mutant. These 2 patients had disease manifestations associated with necrotizing granulomatous inflammation rather than capillaritis. By contrast, among 52 MPO-ANCA–positive participants in the RAVE study, a patient population not expected to have PR3-ANCA, 2 serum samples (3.8%) tested positive (net absorbance 0.625 OD and 0.125 OD, respectively) via the anchor ELISA using iHm5 and tested negative when using iPR3.

We also determined the prevalence of PR3-ANCAs in a population of 300 octogenarians without AAV via the anchor ELISA using iHm5 and iPR3, because the frequency of autoantibodies without a corresponding disease is reportedly increasing with age (45). Only 1 sample from this population showed weak IgG PR3-ANCA reactivity with iHm5 using the anchor ELISA (net absorbance 0.173 OD), and 2 different patients showed reactivity with iPR3 (net absorbance 0.115 OD and 0.245 OD, respectively).

To determine whether using iHm5 in the anchor ELISA would allow an earlier detection of PR3-ANCA seroconversion during serial follow-up of patients with AAV, we compared the seroconversion patterns of 33 participants from the WGET and found that PR3-ANCA seroconversion detected by the anchor ELISA using

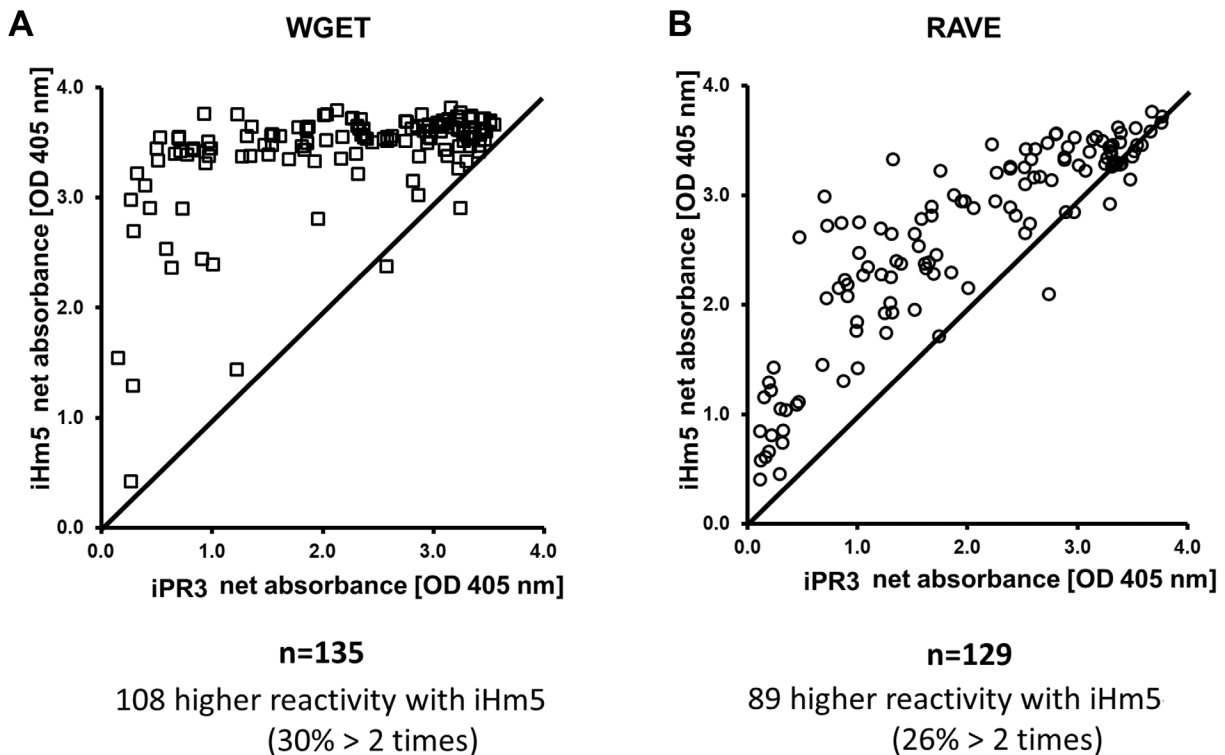


Figure 3. Proteinase 3–targeting antineutrophil cytoplasmic antibody (PR3-ANCA) reactivity with iHm5 and iPR3 determined by anchor enzyme-linked immunosorbent assay (ELISA) using serum from patients included in the Wegener’s Granulomatosis Etanercept Trial (WGET) and the Rituximab versus Cyclophosphamide for ANCA-associated Vasculitis (RAVE). Scatterplot shows net absorbance values of iHm5 recognition compared to iPR3 recognition using the anchor ELISA with plasma and sera from patients of the 2 ANCA-associated vasculitis (AAV) cohorts, WGET (A) and RAVE (B). There is a shift in net absorbance values toward the binding of patient PR3-ANCA to iHm5. In the RAVE trial, rituximab was compared to cyclophosphamide for remission induction in AAV, and in the WGET, etanercept was compared to placebo for remission induction in AAV.

iPR3 could not be detected at an earlier quarterly study visit when using iHm5. We also compared the utility of iHm5 to iPR3 for IgA and IgM PR3-ANCA detections using the anchor ELISA and found similar preferential binding of IgA and IgM PR3-ANCAs to iHm5 (data not shown).

Taken together, these results indicate preferential reactivity of the majority of PR3-ANCA–positive serum samples to iHm5 compared to iPR3 and suggest that iHm5 may enable better detection of low levels of PR3-ANCAs in patients with AAV, compared to iPR3, without significantly increasing the frequency of false-positive results in patients without AAV.

Selective binding of moANCA518 to iHm5. Twenty-five human mAb derived from plasmablasts of patients with PR3-ANCA–positive relapsing AAV were screened for binding to ANCA target antigens using the anchor ELISA with iPR3, iHm5, and 2 other ANCA-targeting antigens (MPO and HNE). Interestingly, we found that one such mAb (moANCA518) exhibited selective binding to iHm5 in the anchor ELISA and by Western blot (32), whereas none of these mAb bound to iPR3, MPO, or HNE. Here we confirmed the selective binding of moANCA518 to iHm5 over iPR3 in a direct ELISA (Supplementary Figure 1A, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42418>).

By contrast, murine anti-PR3 mAb (PR3G-2, MCPR3-3, and WGM2) exhibited equal binding to both antigens in the direct ELISA (Supplementary Figure 1B).

Remote mutation–induced selective binding of PR3-ANCA to epitope 3 on iHm5. To identify the epitope recognized by moANCA518 on iHm5 in a capture ELISA, we used epitope-specific mAb to capture iHm5 and block or modulate the binding of moANCA518 to the epitope that the mAb recognize (19). As previously described by our group, we found that PR3G-2, a mAb that recognizes epitope 1 of PR3 (43), did not affect binding of moANCA518 to iHm5 (32), whereas MCPR3-3 and WGM2, both of which recognize epitope 3 of PR3 (19), respectively decreased and abolished the moANCA518 binding (32) ($P < 0.01$; Figure 5A<<F5>>). When using a PR3-ANCA–containing serum sample obtained from the same patient at the same study visit, from which the plasmablasts expressing moANCA518 were generated, we observed similar effects of these mAb on the reactivity of the PR3-ANCA serum sample with iHm5 (Figure 5B). These findings identify epitope 3 on iHm5 as the epitope for moANCA518 and for a significant proportion of PR3-ANCA present in the serum obtained at the same time as the moANCA518–generating plasmablasts.

The WGET Study* 148 PR3-ANCA-positive samples*		
iHm5 positive n = 144		iPR3 positive n = 135
Comparison of reactivity in 135 samples positive for both antigens		
iHm5 > iPR3 n = 108 80%	iHm5 = iPR3 n = 26 19%	iHm5 < iPR3 n = 1 1%
The RAVE Trial 129 PR3-ANCA-positive samples**		
iHm5 positive n = 128		iPR3 positive n = 126
Comparison of reactivity in 126 samples positive for both antigens		
iHm5 > iPR3 n = 89 71%	iHm5 = iPR3 n = 34 27%	iHm5 < iPR3 n = 3 2%

Figure 4. Comparison of the net absorbance values of the PR3-ANCA reactivity with iHm5 and iPR3. In 71–80% of the samples, a higher PR3-ANCA recognition of iHm5 was documented. Additionally, in 19–27% of the patients, iHm5 and iPR3 were equally recognized. Very few patients displayed higher recognition of iPR3: 1 in the RAVE trial and 3 in the WGET. When the confidence interval overlapped, the determinations were considered equal. * Positive for PR3-ANCA in ≥ 1 antigen-specific immunoassay (ref. 28). ** Positive for PR3-ANCA in ≥ 1 antigen-specific immunoassay (ref. 7). See Figure 3 for definitions.

We further confirmed that epitope 3 of iHm5 was the primary target for moANCA518 by using Fab from epitope-specific mAb as inhibitors of binding of moANCA518 to iHm5, via the anchor ELISA (Figure 5C). As expected, we observed the strongest inhibition when the Fab fragment from moANCA518 was used to inhibit moANCA518 binding. A strong inhibition was observed with the Fab fragments from MCPR3-3 and WGM2 that target epitope 3 of PR3 (19,24). In addition, we found no effect of the Fab fragments from MCPR3-7, which recognizes epitope 5 (19,31), on the moANCA518 binding.

The above results demonstrate that moANCA518 recognizes epitope 3 on iHm5. More importantly, because epitope 3 is on the opposite side of the chimeric mutation sites in epitopes 1 and 5 on iHm5 (Figure 1), and has the same amino acid sequence as that of epitope 3 on iPR3, our observation that moANCA518 recognizes epitope 3 instead of epitope 1 or 5 further indicates that the moANCA518 binding is conferred by an unexpected increase of antigenicity in epitope 3 induced by the distal mutations in epitopes 1 and 5.

To determine whether other PR3-ANCAs would preferentially bind to epitope 3 on iHm5 over iPR3 due to the distal mutations in iHm5, we further evaluated serum or plasma samples from patients with AAV that had exhibited significant preferential reactivity with iHm5 over iPR3 as described above, and found that the Fab fragments from MCPR3-3 and moANCA518 significantly inhibited the reactivity of a representative PR3-ANCA-containing

plasma sample to iHm5 in the anchor ELISA ($P = 0.001$ and $P = 0.017$, respectively) (Figure 5D).

These findings indicate that the preferential reactivity of PR3-ANCA-containing serum or plasma samples with iHm5 can at least in part be explained by some PR3-ANCAs in these samples being sensitive to the change of antigenicity in epitope 3 of iHm5 induced by the distant mutations, just like moANCA518.

Capture by monoclonal antibody activates latent epitope on iPR3 for PR3-ANCA recognition.

Finally, we wanted to know whether binding of PR3 to antibodies can also induce the activation of distant latent epitopes. Therefore, we evaluated the effect of the binding of the mAb MCPR3-2 on the recognition of iPR3 and iHm5 by moANCA518. MCPR3-2 binds to epitope 4 and does not compete for epitopes recognized by a sizable proportion of PR3-ANCA from patients with AAV (24). In contrast to the selective binding of moANCA518, the recognition of iHm5 but not iPR3 demonstrated in the immunoassays, in which the PR3 antigen is presented in isolation (anchor ELISA, direct ELISA, Western blot), i.e., not bound to another protein, the capture of both constructs iHm5 and iPR3 by MCPR3-2 in the capture ELISA induced the recognition of iPR3 by moANCA518 and further enhanced that of iHm5, compared to in the anchor ELISA (Figure 6A<<F6>>). The amount of antigen coating available for antibody binding was comparable between constructs in both assays (Figure 6B).

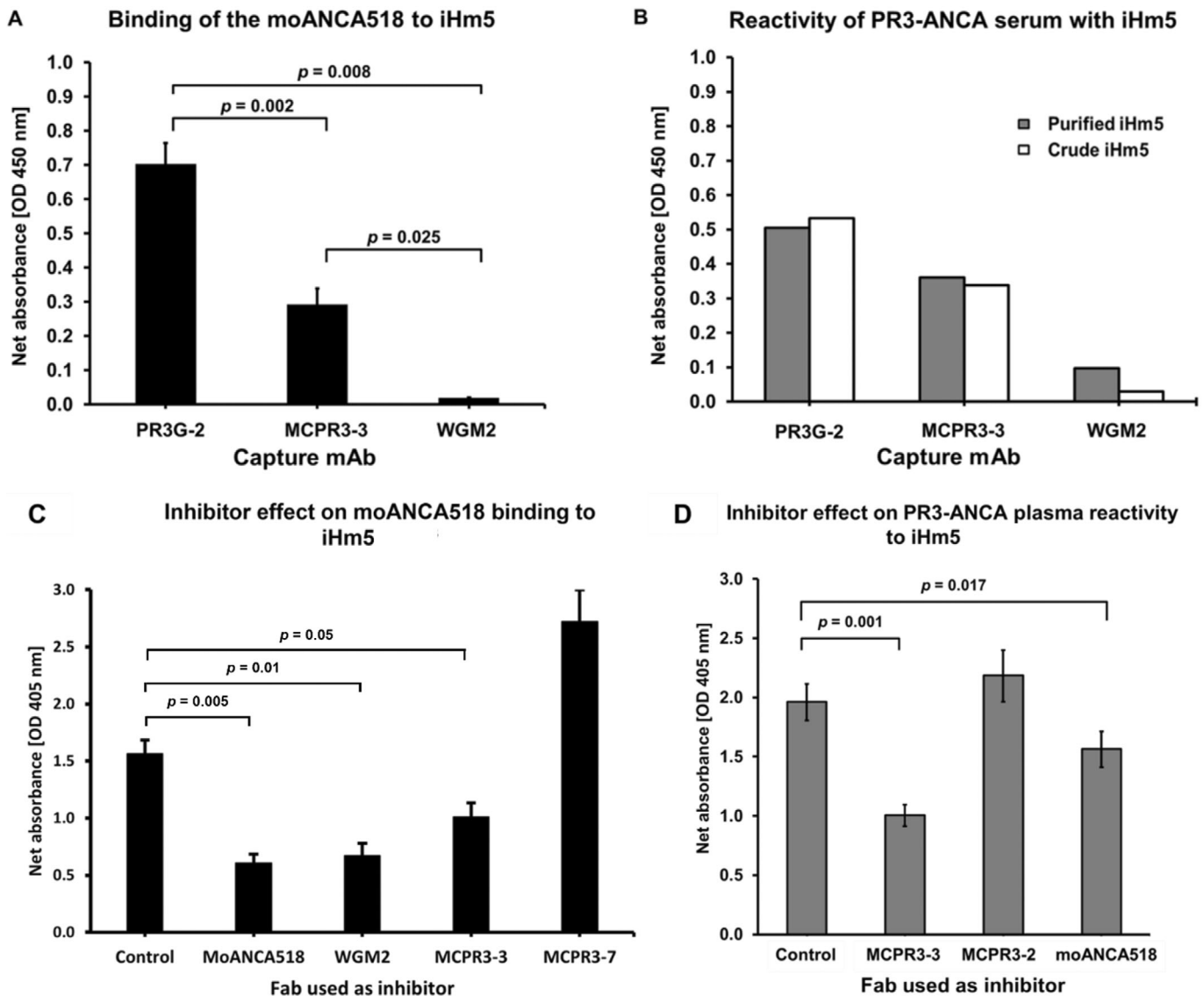


Figure 5. Identification of iHm5 epitope 3 as a target of monoclonal ANCA 518 (moANCA518) and PR3-ANCA. **A**, Inhibition of moANCA518 binding to iHm5 using different monoclonal antibodies (mAb) to capture antigen iHm5 is shown. Binding of moANCA518 to iHm5 was impaired significantly when the mAb MCPR3-3 and WGM2 (recognizing epitope 3) were used to capture the antigen, compared to PR3G-2, which binds (recognizing epitope 1). This previously shown data (ref. 32) is included to illustrate similarities with the PR3-ANCA serum results. **B**, Serum PR3-ANCA from the same patient/study visit from which moANCA518 was derived from plasmablasts yields similar differential reactivity of PR3-ANCA with iHm5 captured by mAb. Results for purified or crude iHm5 contained in media supernatant from cells expressing the recombinant antigen were similar. **C**, Fab fragments from epitope-specific mAb as competitors of moANCA518 binding to iHm5 using the anchor ELISA are shown. Compared to the control, binding of moANCA518 to iHm5 was reduced significantly by moANCA518 Fab fragment ($P = 0.005$) and Fab fragments from MCPR3-3 ($P = 0.005$) and WGM2 ($P = 0.01$) but not MCPR3-7 Fab fragment (recognizing epitope 5). **D**, Binding of PR3-ANCA to iHm5 in the anchor ELISA was similarly inhibited by Fab fragments recognizing epitope 3 (MCPR3-3 and moANCA518). In **C** and **D**, comparisons were made with control mouse IgG1 Fab fragments. Bars show the mean \pm SE. See Figure 3 for other definitions.

These results indicate that the binding of iPR3 to proteins, in this case to the mAb MCPR3-2 (binding to epitope 4), can induce mobility changes in epitope 3 of iPR3 required for the binding of moANCA518 that mimic the effect of the distant 3 point mutations of iHm5 on epitope 3, which favor the recognition by moANCA518 and by a sizable proportion of PR3-ANCAs.

DISCUSSION

In this study, we show that stronger binding of PR3-ANCAs to PR3 can be induced by the activation of a latent epitope caused by a distant mutation or binding of a mAb to a distant epitope. About half of all PR3-ANCA-positive sera displayed stronger binding to iHm5 than to iPR3, indicating that iHm5 has

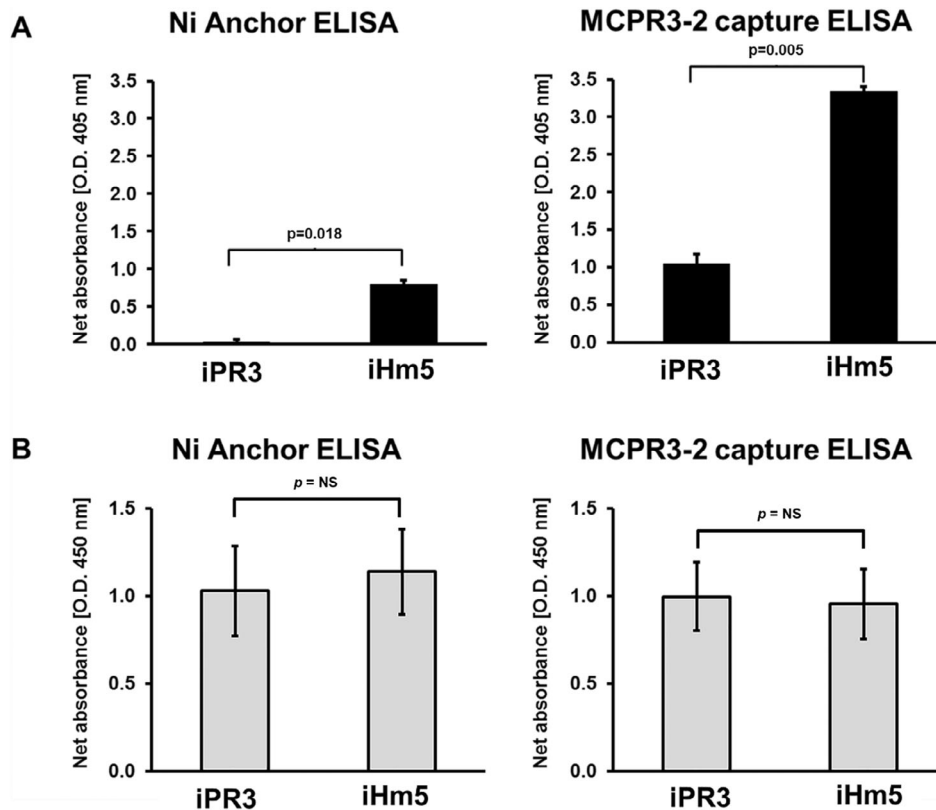


Figure 6. Binding to mAb facilitates iPR3 recognition by moANCA518. **A**, Detection of the binding of moANCA518 to iHm5 and iPR3 using anchor ELISA and MCPR3-2 capture ELISA. Binding of moANCA518 to iHm5 was detected by anchor ELISA, whereas no binding to iPR3 could be detected ($P = 0.018$). In contrast, on the MCPR3-2 capture ELISA, moANCA518 bound to both constructs, iHm5 and iPR3, but the signal of the binding to iHm5 was higher when compared to the signal of the binding to iPR3 ($P = 0.005$). **B**, Control experiments documenting comparable coating of iPR3 and iHm5, using the anchor ELISA, and comparable coating of MCPR3-2 bound to iPR3 and iHm5, using the MCPR3-2 capture ELISA, detected by polyclonal rabbit anti-PR3 antibody. Bars show the mean \pm SE. Ni = nickel; NS = not significant (see Figure 3 for other definitions).

clinical relevance as an antigen for more sensitive immunoassays for PR3-ANCA detection relative to the use of iPR3. Using iHm5, we discovered a human monoclonal PR3-ANCA, moANCA518, which binds preferentially to epitope 3 on iHm5 (compared to iPR3) as a consequence of an unexpected increase of the antigenicity in epitope 3 of iHm5 that was induced by the 3 remote chimeric mutations in epitopes 1 and 5. Furthermore, we found that the majority of PR3-ANCA-positive serum or plasma samples from patients with AAV with higher reactivity to iHm5 than to iPR3 also displayed similar preferential binding to epitope 3 of iHm5 as moANCA518. Finally, the effect of the mutations causing activation of a latent PR3 epitope could be emulated by capturing iPR3 with the epitope 4-specific mAb MCPR3-2, as this induced binding of moANCA518 to iPR3.

The PR3 variant iPR3 has been used for 2 decades as a standard PR3 antigen for sensitive and specific PR3-ANCA detection by immunoassay (7,22,24–29). The iHm5 variant containing 3 point mutations and its “inverse” chimeric counterpart, iMh5 (formerly referred to as Mh5) were originally designed for discontinuous epitope mapping and structure–function analysis studies (19). When

we used iHm5 for the screening of anti-PR3 mAb and PR3-ANCAs, we expected that some PR3-ANCA-positive sera would display reduced binding to iHm5, which would have allowed the conclusion that some PR3-ANCAs in such a serum sample target epitope 5. Instead, we identified no loss of binding to iHm5 compared to iPR3 by any PR3-ANCA-positive sera from patients with PR3-ANCA-positive AAV, but there was preferential reactivity of the majority of PR3-ANCA-positive serum samples to iHm5 compared to iPR3; this indicates that iHm5 may enable better detection of low levels of PR3-ANCAs in patients with AAV.

Consequently, we investigated whether iHm5 represents a useful antigen for more sensitive immunoassays for PR3-ANCA detection. We found that low levels of PR3-ANCAs can be detected more readily in some patients with PR3-ANCA-positive AAV without generating a significant number of false-positive results in patients with MPO-ANCA-positive vasculitis or octogenarians without AAV. Here, it is of interest that the 2 patients from the WGET cohort who had previously consistently tested negative for PR3-ANCA in all assays but were positive when iHm5 was used as the antigen had no clinical features attributable to capillaritis;

they had only upper and lower respiratory tract manifestations or orbital involvement attributable to granulomatous inflammation (28). The fact that PR3-ANCA could be detected using the most sensitive assay in these patients is consistent with the hypothesis that PR3-ANCAs originally emerge in the inflammatory environment of the granulomatous tissue lesions, where selection and affinity maturation of PR3-specific autoreactive B cells occur (46,47).

However, we were unable to detect recurrent PR3-ANCAs earlier using iHm5 versus iPR3 as antigen when testing the serial follow-up serum samples obtained from trial participants at quarterly intervals (34–36). Whether the use of iHm5 might be clinically useful for early detection of PR3-ANCA seroconversion after rituximab therapy in patients with chronically relapsing PR3-ANCA-associated AAV would require a dedicated prospective study with PR3-ANCA measurements at narrower intervals.

The identification of moANCA518 with selective binding to iHm5 was a fortuitous finding that allowed us to further investigate the observation of increased reactivity of many PR3-ANCA-positive sera with iHm5. The PR3-ANCA response is thought to be an oligoclonal immune response, as serum samples from patients have been documented to contain PR3-ANCAs binding to more than 1 epitope (19,48). The systematic analysis of the binding of moANCA518 to iHm5 using mAb with defined PR3-ANCA epitope recognition as inhibitors of binding led to another surprise, namely, that moANCA518 does not bind to the surface region of PR3 where the mutations that differentiate iHm5 from iPR3 are located; instead, it binds to epitope 3 of iHm5, which is located on the side of the iHm5 structure opposite the side where the mutations were introduced (32) (Figure 1). This allowed the use of moANCA518 binding to iHm5 and iPR3 as a gauge to determine that serum PR3-ANCAs from patients do indeed contain antibodies that can only bind to PR3 when the latent epitope is activated.

It has previously been described for antiphospholipid syndrome that changes in the conformation of the target antigen induced by the binding to cardiolipin vesicles result in the exposure of previously inaccessible epitopes, rendering them available for recognition by pathogenic autoantibodies (49). Here, we demonstrated that the binding of mAb MCPR3-2 to iPR3 on epitope 4 induced recognition of iPR3 by moANCA518. This finding has 2 implications. First, it may explain why the MCPR3-2 capture ELISA method for PR3-ANCA detection has consistently been more sensitive than other methods without loss of specificity, as some PR3-ANCA can only bind if a latent epitope is activated by an antibody-antigen interaction. Second, it implies a previously unrecognized level of complexity and variability of the oligoclonal PR3-ANCA interactions with its target antigen in individual patients. Therefore, one can assume that the functional impact including pathogenicity of PR3-ANCA may change as the components of the oligoclonal mixture change in patients over time.

In conclusion, this study shows that iHm5 is a clinically relevant PR3 mutant that can be used as an antigen for sensitive

PR3-ANCA detection in patients with AAV, without significant false-positive PR3-ANCA detection in patients without AAV. More importantly, our results demonstrate that the preferential binding of PR3-ANCAs to iHm5 over iPR3 is the result of an increase of the antigenicity of epitope 3 on iHm5 induced by the 3 distant point mutations, and a similar effect can be induced in epitope 3 by engagement of the separate epitope 4 by a mAb. Consequently, the present work suggests that remote activation or potentiation of a latent epitope can be achieved not only by distant point mutations but also by the binding of antibodies or possibly other ligands to PR3. Further investigations are needed to determine whether these mechanisms play a role in etiologies of antibody-mediated autoimmune diseases and whether they can be utilized for the development of novel treatment strategies for these diseases.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Casal Moura had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Casal Moura, Thompson, Nelson, Fussner, Hummel, Specks.

Acquisition of data. Casal Moura, Thompson, Nelson, Fussner, Hummel, Specks.

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