Autoantibodies against Linear Epitopes of Myeloperoxidase in Anti–Glomerular Basement Membrane Disease

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Abstract
Background and objectives Approximately 20%–30% of patients with anti–glomerular basement membrane disease present coexisting anti-myeloperoxidase (MPO) autoantibodies. We previously showed the recognition of a linear fragment of the MPO heavy chain N-terminus (H, MPO_{279–409}) in plasma from most double-positive patients. Herein, we investigated the frequency of autoantibodies against overlapping H-derived linear peptides in plasma from patients with anti–glomerular basement membrane disease.

Design, setting, participants, & measurements We synthesized 13 overlapping linear peptides (1H_{1–13} covering MPO_{279–409}. We retrospectively collected plasma samples from 67 patients with anti–glomerular basement membrane disease from 1996 to 2012, and we screened them for IgG autoantibodies by ELISA using intact human MPO and the overlapping peptides as antigens, and we further investigated the clinical significance. Autoantibody binding to the linear MPO structure was confirmed by Western blotting.

Results We followed up the 67 patients until 2015, with a median follow-up time of 10.0 (2.3–36.0) months, and 56 ESRD events occurred among the 67 patients with follow-up data. Plasma from 23.9% (16) of the patients recognized intact human MPO, whereas 62.7% (42) plasma samples recognized MPO_{279–409} linear peptides. Of the 13 linear peptides, 1H_{4} (44.8%, 30 patients) and 1H_{12} (40.3%, 27 patients) exhibited the highest recognition frequencies. Patients with autoantibodies against 1H_{11} or 1H_{12} (MPO_{371–409}) were older (46.1±18.8 versus 34.1±16.6 years; P<0.01), had higher serum creatinine upon diagnosis (median 7.8 mg/dl, interquartile range 4.9–12.6 mg/dl versus median 5.4 mg/dl, interquartile range 2.4–7.3 mg/dl; P=0.02), and had a higher probability of progressing to ESRD; however, multivariate Cox regression analysis showed that 1H_{11} or 12 reaction was not an independent risk factor for renal failure (hazard ratio, 1.2; 95% confidence interval, 0.8 to 2.8; P=0.19).

Conclusions Autoantibodies against linear peptides of MPO can be detected in the majority of patients with anti–glomerular basement membrane disease, and several are associated with disease severity. The potential common pathogenic mechanism between anti–glomerular basement membrane antibodies and anti-MPO autoantibodies in anti–glomerular basement membrane disease requires further investigation.


Introduction
Goodpasture syndrome is an autoimmune disorder characterized by the presence of anti–glomerular basement membrane (GBM) autoantibodies, resulting in rapidly progressive glomerulonephritis and severe lung hemorrhage, with a high frequency of ESRD and death (1–3). The target antigen of the anti-GBM antibodies is noncollagenous domain 1 of the α3 chain of type IV collagen [α3(IV)NC1], which has two major epitopes: E_{A} and E_{B} (4,5).

Approximately 20%–30% of patients with anti-GBM disease present serum ANCA, which are mainly directed against myeloperoxidase (MPO) (6). These patients are defined as “double positive” and have severe clinical manifestations and poor outcomes (7–9). The considerable overlap of the two rare diseases might indicate an underlying common pathogenic mechanism. Several studies have suggested that MPO-ANCA could cause glomerular injury, leading to the exposure of cryptic epitopes on α3(IV)NC1 and thereby inducing anti-GBM autoantibody production (10). However, only 5%–10% of patients with ANCA-associated vasculitis have coexisting anti-GBM disease (6,7,11), which casts doubt on this hypothesis.

As confirmed by previous studies, no cross-reaction occurs between autoantibodies against α3(IV)NC1 and MPO (12). These autoantibodies are detected on the basis of recognition of the conformational epitopes on intact antigen molecules. In a recent study...
on ANCA-associated vasculitis, several MPO linear epitopes were identified as pathogenic, even in ANCA-negative disease (13). These findings implicate diverse autoantibodies against different MPO epitopes in the pathogenesis of ANCA-associated vasculitis. We recently screened autoantibodies against six fragments of the MPO molecule, covering both the light and the heavy chains, in plasma from patients with MPO-ANCA-associated vasculitis; a linear fragment of the N-terminal portion of the MPO heavy chain, $^{1}\text{H}279$–$^{409}$, was recognized in the majority of samples (61.5%, eight out of 13) from double-positive patients (14).

We hypothesized that double positivity in anti-GBM disease might be largely underestimated. Therefore, in this study, we designed a panel of overlapping synthetic linear peptides covering the N-terminus of the MPO heavy chain. Serum autoantibodies against these linear peptides were detected in a large cohort of patients with anti-GBM disease. Clinical and pathologic associations were also analyzed, with the aim of exploring potential mechanisms for MPO-ANCA generation in anti-GBM disease.

Materials and Methods

Patients and Plasma

This study included 67 patients with anti-GBM disease who visited Peking University First Hospital from 1996 through 2012. Clinical data were collected at the time of diagnosis and during follow-up. The follow-up was started in 2001, with visits every 1–3 months after the patients were discharged. The end date of follow-up in this study was 2015. All patients were positive for circulating anti-GBM autoantibodies, as detected by ELISA using purified bovine $\alpha$ (IV)NC1 (Euroimmun, Lubeck, Germany) and recombinant human $\alpha$3(IV)NC1 as solid-phase antigens. Sixteen of the 67 patients were positive for MPO-ANCA, as detected by an indirect immunofluorescence assay and antigen-specific ELISA using purified MPO (Euroimmun). Plasma was collected before immunosuppressive therapy or plasma exchange was initiated. Plasma from 44 patients with MPO-ANCA-associated vasculitis without coexisting anti-GBM antibodies served as a disease control, and plasma from 55 healthy blood donors served as a normal control. Plasma samples were stored at $-20^\circ$C until further use. Multiple freeze-thaws were avoided, and none of the plasma samples was heat treated before use. The research was in compliance with the Declaration of Helsinki and was approved by the ethics committee of Peking University First Hospital. Written informed consent was also obtained from each participant.

Preparation of Linear Peptides

A panel of 13 sequential overlapping peptides was synthesized, covering amino acids (aa) 279–410 of MPO (14). Each peptide was 20 aa in length, with ten overlapping aa. The peptides were synthesized using an automatic peptide synthesizer with 9-fluorenylmethoxycarbonyl chemistry (Beijing Sclight Biotechnology Ltd Co., Beijing, China), and purified on a reverse-phase C18 column by preparative HPLC. Purified peptides were analyzed by HPLC for purity and by mass spectrometry to verify that they had the correct sequence. Peptides with purity >98% were used for further tests. The sequence of each peptide is listed in Table 1. Twenty peptides unrelated to MPO or GBM were also synthesized as peptide controls. In addition, a linear fragment of the MPO C-terminus (H4; aa 622–745) was produced in Escherichia coli, as previously described (14).

Determination of Antigen Specificity by ELISA

The synthetic peptides were diluted to 10 $\mu$g/ml with coating buffer (0.05 M bicarbonate buffer, pH 9.6), used to coat the wells of half of a polystyrene microtiter plate (Nunc-Immuno plate; Nunc, Roskilde, Denmark) and incubated overnight at 4°C. The other half of the plate was coated with coating buffer alone to establish antigen-free wells. Plasma was diluted to 1:100 in PBS containing 0.1% Tween-20 and 1% BSA, added in duplicate, and incubated at 37°C for 60 minutes. Binding was detected with alkaline phosphatase-conjugated goat anti-human IgG (Fc specific; Sigma-Aldrich, St. Louis, MO) at a dilution of 1:5000. P-nitrophenyl phosphate (1 mg/ml; Sigma) was used in substrate buffer (1 M diethanolamine and 0.5 mM magnesium chloride [pH 9.8]), and color development was measured spectrophotometrically at 405 nm (Bio-Rad, Tokyo, Japan) after 30 minutes. Each plate contained positive, negative, and blank controls. The plasma from the 55 healthy blood donors was used to generate cutoff values (mean+2SDs) for each peptide. The samples were re-examined when a SEM>10% was found. The results were normalized to the positive control and are expressed as a percentage of the OD value of the positive control.

Cross-Reaction between Autoantibodies against $\alpha$3(IV)NC1 and MPO Peptides

Cross-reactions between autoantibodies against the linear peptides of MPO and $\alpha$3(IV)NC1 were investigated using an inhibition ELISA. Briefly, polystyrene microtiter plates were coated with soluble linear peptides (H1–H13) at a concentration of 10 $\mu$g/ml. Meanwhile, diluted plasma was preincubated with the soluble linear peptides (H1–H13) or recombinant human $\alpha$3(IV)NC1 at concentrations ranging from 0.5 to 2 mg/ml at 37°C for 60 minutes. The mixtures were then transferred to the linear peptide-coated microtiter plates, and the bound autoantibodies were detected with alkaline phosphatase-conjugated secondary antibodies, as described above.

Detection of Circulating anti-MPO Autoantibodies by Western Blot Analysis

Intact human MPO (Merck Millipore, Billerica, MA) was boiled in sample buffer and subjected to 12.5% SDS-PAGE at 80 V under reducing conditions with 5% $\beta$-mercaptoethanol. The protein was then transferred to a nitrocellulose membrane (Merck Millipore) and blocked in Tris-buffered saline containing Tween-20 and 20 g/l skimmed milk (TBSTM; 0.01 M Tris hydrochloride [pH 7.2], 0.15 M sodium chloride, 0.1% Tween-20, and 20 g/l skimmed milk) for 60 minutes at room temperature. Strips were then incubated with plasma diluted to 1:50 in TBSTM at 4°C overnight, followed by incubation with peroxidase-conjugated secondary antibodies. The proteins were then revealed on autoradiographic film using the Enhanced Chemiluminescence Plus Western blotting detection system (GE Healthcare,
**Statistical Analysis**

The normally distributed quantitative parameters were expressed as the mean±SD, and the quantitative parameters that were not normally distributed were expressed as the median and interquartile range. Differences in quantitative parameters were assessed using the t test or nonparametric tests. Differences in qualitative data were analyzed using the chi-squared test or Fisher exact test. Moreover, Pearson and Spearman rank correlations were performed to analyze the association between the levels of autoantibodies against linear peptides and the clinical data, as appropriate. Peptides with <5% recognition were excluded from further statistical analyses. Kaplan–Meier analysis was used to investigate patient survival, and renal survival was assessed using the log-rank test. A P value <0.05 was considered significant. The analyses were performed using SPSS statistical software (version 10.0; SPSS Inc., Chicago, IL).

**Results**

**Patient Data**

Sixteen (23.9%) of the 67 patients had coexisting MPO-ANCA. These patients were significantly older than those without ANCA s (57.7±14.8 versus 34.5±16.1 years; P<0.001). The median duration of follow-up was 10.0 (2.3–36.0) months, and 56 (n=67; 83.6%) ESRD events occurred. At the end of 1 year of follow-up, 86.6% (58 out of 67) of patients survived, and 19.4% (13 out of 67) of patients had normal renal function. The outcomes of the double-positive patients were similar to those of the patients with anti-GBM autoantibodies alone.

In total, 32 of the 67 patients underwent a renal biopsy, and direct immunofluorescence showed linear IgG deposition along the GBM, with or without complement C3. Patients with ESRD and very old individuals (>75 years old) did not undergo kidney biopsy. The presence of anti-GBM antibodies was confirmed by ELISA and Western blot analysis using recombinant human α3(IV)NC1 as a solid-phase antigen.

**Frequency of Serum Autoantibodies Recognizing Linear Peptides of MPO**

We previously reported that MPO peptide recognition was restricted to the 1H fragment in double-positive patients (14). Therefore, we synthesized 13 sequential linear peptides covering the entire length of MPO 1H279–409, or 1H–1 to 1H–13, with each overlapping by ten aa (Figure 1, Table 1).

Plasma from 42 (62.7%) of the 67 patients with anti-GBM disease demonstrated reactivity with at least one peptide. Among the 16 patients with coexisting MPO-ANCA, 13 (81.3%) showed plasma reactivity with at least one linear peptide; for the 51 patients without ANCA, 29 (56.9%) had plasma reactivity with at least one peptide. Autoantibodies against linear peptides were more common in classic double-positive patients (3, 1–9 versus 1, 0–4; P=0.04).

In total, 44 patients with MPO-ANCA–associated vasculitis served as disease controls. Plasma from 39 of these patients (88.6%) demonstrated reactivity with at least one linear peptide, which was significantly higher reactivity than that observed for anti-GBM plasma (P<0.001) (Figure 1).

Circulating autoantibodies binding to the linear structure of MPO in patients with anti-GBM disease were further

**Table 1. Amino acid sequences of the linear peptides on the MPO 1H fragment and the frequencies and levels of serum autoantibodies from patients with anti-GBM disease**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence from the N-Terminus of the MPO Heavy Chain</th>
<th>Position from the N-Terminus</th>
<th>Recognition by Anti-GBM Plasma, % (no. out of 67)</th>
<th>Median Autoantibody Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H–1</td>
<td>VNCETSCVQPPCFPLKIPP</td>
<td>279–298</td>
<td>25.4% (17)</td>
<td>0.63</td>
</tr>
<tr>
<td>1H–2</td>
<td>PPCFPLKIPPNDNPRKQNAD</td>
<td>289–308</td>
<td>13.4% (9)</td>
<td>0.65</td>
</tr>
<tr>
<td>1H–3</td>
<td>NDPRKIQADACIPFFRSCPA</td>
<td>299–318</td>
<td>10.4% (7)</td>
<td>0.43</td>
</tr>
<tr>
<td>1H–4</td>
<td>CIPFFRSCPAACPGISNITRN</td>
<td>309–328</td>
<td>44.8% (30)</td>
<td>0.63</td>
</tr>
<tr>
<td>1H–5</td>
<td>CPGISNITRNQINAL</td>
<td>319–333</td>
<td>16.4% (11)</td>
<td>0.56</td>
</tr>
<tr>
<td>1H–6</td>
<td>ITINQINALTSFVSF</td>
<td>324–338</td>
<td>17.9% (12)</td>
<td>0.39</td>
</tr>
<tr>
<td>1H–7</td>
<td>QINALTSFVDAASMVYGSEEPLA</td>
<td>329–350</td>
<td>19.4% (13)</td>
<td>0.31</td>
</tr>
<tr>
<td>1H–8</td>
<td>ASMVYGSEEPLARNLRNNSNQL</td>
<td>339–360</td>
<td>20.9% (14)</td>
<td>0.37</td>
</tr>
<tr>
<td>1H–9</td>
<td>RNLRNMSNQLGLAVNORFQ</td>
<td>351–370</td>
<td>16.4% (11)</td>
<td>0.48</td>
</tr>
<tr>
<td>1H–10</td>
<td>GLAVNORFQDNGRAWLPFD</td>
<td>361–380</td>
<td>19.4% (13)</td>
<td>0.44</td>
</tr>
<tr>
<td>1H–11</td>
<td>DNGRAWLPFDLNHDDPCLLT</td>
<td>371–390</td>
<td>35.8% (24)</td>
<td>0.45</td>
</tr>
<tr>
<td>1H–12</td>
<td>NLHDPCCLTNRSARIPCFL</td>
<td>381–400</td>
<td>40.3% (27)</td>
<td>0.66</td>
</tr>
<tr>
<td>1H–13</td>
<td>NRSARIPCFLAGDTRSSEP</td>
<td>391–410</td>
<td>10.4% (7)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

GBM, glomerular basement membrane; MPO, myeloperoxidase.
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fi
rmed by Western blot analysis using intact human MPO as the ligand under reducing conditions (Figure 2).

Plasma from patients with anti-GBM disease recognizing the linear peptides of MPO could also bind to a band representing the MPO heavy chain.

The specificity of circulating autoantibodies against the linear peptides of MPO in all 67 patients with anti-GBM disease was further confirmed by ELISA against 20 linear peptides unrelated to MPO or α3(IV)NC1. Only two of the 20 peptides were recognized, by two of the 67 (3.0%) plasma samples. In addition, the specificity of the autoantibodies against the N-terminus of the MPO heavy chain was confirmed by coating antigen-free wells with H4 (MPO622-745). The difference in net OD values between H4 (MPO622-745)-coated wells and antigen-free wells was not significant (P=0.99).

**Frequencies of Serum Autoantibodies Recognizing Each Linear Peptide of MPO**

Among the 13 peptides, 1H–4, 1H–11, and 1H–12 were most commonly recognized by plasma from patients with
anti-GBM disease, with prevalence of 44.8% (30 out of 67 patients), 35.8% (24 of 67) and 40.3% (27 of 67), respectively (Figure 1). The binding levels for these peptides are shown in Figure 3.

Compared with patients with anti-GBM autoantibodies alone, classic double-positive patients showed a significantly higher frequency of $^{1}H$–5 (43.8% versus 9.8%; $P=0.001$) and $^{1}H$–6 (50.0% versus 7.8%; $P<0.001$) recognition (Figure 1).

Clinical Association of Autoantibodies Recognizing Linear Peptides of MPO

In general, patients with autoantibodies against any linear peptide were older than patients without the autoantibodies ($45.2\pm19.4$ versus $31.3\pm13.6$ years; $P<0.01$).

The clinical association of autoantibodies recognizing each linear peptide was also analyzed. Because $^{1}H$–11 and $^{1}H$–12 are two adjacent peptides, both on the MPO surface, we combined the two peptides as $^{1}H$–11 to 12 (defined as the detection of autoantibodies against $^{1}H$–11 or $^{1}H$–12).

Compared with patients lacking autoantibodies against $^{1}H$–11 to 12 (Table 2), patients with autoantibodies against $^{1}H$–11 to 12 showed significantly higher serum creatinine at the time of diagnosis (7.8 mg/dl, 4.9–12.6 mg/dl, versus 5.4 mg/dl, 2.4–7.3 mg/dl; $P=0.02$) and more linear peptides were recognized by their sera (4, 3–9 versus 0, 0–1; $P<0.001$).

In comparison with patients lacking antibodies against $^{1}H$–11 to 12, patients with these autoantibodies showed a significantly higher probability of progression to ESRD (hazard ratio [HR], 1.8; 95% confidence interval [95% CI], 1.1 to 3.0; $P=0.03$) (Figure 4); however, multivariate Cox regression analysis showed that $^{1}H$–11 to 12 reaction was not an independent risk factor for renal failure (HR, 1.2; 95% CI, 0.8 to 2.8, $P=0.19$).

Cross-Reaction between Autoantibodies against $\alpha$3(IV)NC1 and MPO Peptides

In the inhibition ELISA, binding to MPO peptides could be inhibited by the corresponding MPO peptides in a dose-dependent manner, but not by soluble $\alpha$3(IV)NC1, indicating that the autoantibodies against the linear peptides of MPO and $\alpha$3(IV)NC1 were distinct populations (see Supplemental Figure 1).

Measurement of Complement Activation by Anti-$^{1}H$–4, Anti-$^{1}H$–11, and Anti-$^{1}H$–12 Autoantibodies

Autoantibodies against $^{1}H$–4, $^{1}H$–11, and $^{1}H$–12 from the plasma of six patients with anti-GBM disease were affinity purified in microtiter plates, with an average concentration of 0.03±0.02 mg/ml.

$C3c$ deposition activated by the $^{1}H$–4, $^{1}H$–11, and $^{1}H$–12 immune complexes was significantly higher than deposition in the healthy controls ($P<0.001$) (see Supplemental Figure 2).

Discussion

In our previous study, plasma from eight of 13 (61.5%) patients with anti-GBM disease with coexisting MPO-ANCA could recognize MPO$^{279–409}$ (14). In this study, we further investigated the recognition of linear peptides covering MPO$^{279–410}$ by plasma from patients with anti-GBM disease. Plasma from 42 patients (62.7%) with anti-GBM disease recognized linear peptides of MPO$^{279–410}$.
which was a much higher degree of recognition than for native MPO (23.9%, 16 of 67 patients). No cross-reaction was found between autoantibodies against a3(IV)NC1 and MPO peptides; thus, the autoantibodies against linear peptides of MPO279–410 were distinct from those against a3(IV)NC1.

The large proportion of patients with anti-GBM disease with coexisting autoantibodies against linear peptides of MPO279–410 indicates that “double positivity” should not be regarded as a coincidence. In general, autoreactive T cells, recognizing linear peptides presented by antigen-presenting cells, initially activate B cells to produce autoantibodies against the same linear peptide. Subsequently, intra- and interepitope spreading occurs (17,18) through somatic recombination to produce autoantibodies with high affinity to various conformational epitopes (19,20).

The discovery of autoantibodies specific to linear peptides of MPO indicates that autoreactive T cells against MPO might appear in the majority of patients with anti-GBM disease, inducing autoreactive B cell activation against linear MPO. During the development and progression of anti-GBM disease, epitope spreading from linear peptides to conformational a3(IV)NC1 has been shown in vivo in rats and confirmed in patients (21–23). We speculated that intramolecular epitope spreading might also occur from autoreactive B cells against the linear peptides of MPO.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1H–11 To 12 Positive</th>
<th>1H–11 To 12 Negative</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>33</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>46.1±18.8</td>
<td>34.1±16.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>18/15</td>
<td>21/11</td>
<td>0.36</td>
</tr>
<tr>
<td>Hemoptysis, n (%)</td>
<td>7 (21.2)</td>
<td>13 (38.2)</td>
<td>0.11</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>8.3±1.9</td>
<td>9.2±2.1</td>
<td>0.23</td>
</tr>
<tr>
<td>Oliguria/anuria, n (%)</td>
<td>16 (30.3)</td>
<td>13 (42.7)</td>
<td>0.42</td>
</tr>
<tr>
<td>Urinary protein (g/24 h)</td>
<td>1.7 (0.9–3.5)</td>
<td>2.8 (1.7–5.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Gross hematuria, n (%)</td>
<td>7 (21.2)</td>
<td>13 (38.2)</td>
<td>0.09</td>
</tr>
<tr>
<td>Serum creatinine upon diagnosis</td>
<td>7.8 (4.9–12.6)</td>
<td>5.4 (2.4–7.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Crescents in glomeruli (%)</td>
<td>90.2±15.9</td>
<td>74.6±26.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Number of linear peptides</td>
<td>4 (3–9)</td>
<td>0 (0–1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>recognized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal survival at 1 year, n (%)</td>
<td>7 (21.2)</td>
<td>4 (12.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Patient survival at 1 year, n (%)</td>
<td>28 (84.8)</td>
<td>30 (90.1)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1H–11 to 12, combination of 1H–11 and 1H–12.

Figure 4. | Renal outcomes of patients with anti-GBM disease with or without autoantibodies against MPO 1H–11 to 12. Cum., cumulative; GBM, glomerular basement membrane; 1H–11 to 12, combination of 1H–11 and 1H–12; H1-11 to 12, 1H–11 to 12; MPO, myeloperoxidase.
to autoreactive B cells against conformational MPO. In fact, autoantibodies against the linear peptides of MPO were detected more commonly in “double-positive” patients who possessed autoantibodies against conformational MPO; thus, the appearance of MPO-ANCA might be associated with autoantibodies reactive to linear MPO peptides through the epitope-spreading process.

Another attractive hypothesis is that anti-GBM autoantibodies and MPO-ANCA, at least in anti-GBM disease, may arise from the same unknown origin. An initial linear epitope in either MPO or the α3(IV)NC1 molecule might be responsible for the production of both autoantibodies through intra- and intermolecular epitope-spreading processes. According to our previous study on anti-GBM disease, P14 (α3129–130) is a major linear B cell epitope on α3(IV)NC1, which is the mutual B and T cell epitope in the development of anti-GBM disease (24). Interestingly, autoantibodies against P14 were also found more frequently in double-positive patients. In this study, autoantibodies against 1H4–6 (MPO300–330) and 1H11–12 (MPO371–400) were also more commonly detected in double-positive patients. In addition, most of the peptides in 1H279–409, and particularly 1H4 and 1H12, are on the surface of MPO (see Supplemental Figure 3). Although we failed to identify any aa sequence similarity among the three linear peptides, the homology of the critical aa of the T cell epitope could be within the linear peptide but without continuity. Exploring the critical motif of the T cell epitope in anti-GBM disease might be helpful in searching for cross-reactivity between anti-GBM antibodies and MPO-ANCA at the T cell level.

Interestingly, in this study, the presence of serum autoantibodies against the linear peptide 1H11–12 (MPO371–400) was associated with the severity of anti-GBM disease. In particular, patients with autoantibodies against 1H11–12 presented higher serum creatinine upon diagnosis and worse renal outcome during follow-up. Thus, 1H11–12 (MPO381–400) might constitute a risk epitope. This possibility was supported by a recent study, in which a sole linear epitope on the MPO molecule (MPO447–459) recognized by plasma from ANCA-negative vasculitis patients correlated with disease severity. Autoantibodies against this epitope had pathogenic properties, as demonstrated by their capacity to activate neutrophils in vitro and to induce nephritis in mice (13). In this study, autoantibodies against 1H11 and 1H12 resulted in complement fixation, which might be a mechanism by which the autoantibodies induce kidney injury. The frequent recognition of the linear peptide 1H11–12 (MPO371–400) might suggest that extensive epitope spreading occurred in these patients. The patients with positive anti-1H11 to 12 autoantibodies also showed more autoantibodies against other linear peptides of MPO. Moreover, the close relationship between extensive epitope spreading and severe kidney injury has been shown in human anti-GBM disease (13).

A limitation of our study is that we did not synthesize overlapping linear peptides covering the entire length of MPO. Because the 1H fragment was detected with the highest frequency among the light and heavy chains of MPO and was more detectable in double-positive patients, we focused on this fragment. As a result, we might have missed several potential linear epitopes on the MPO molecule; this possibility deserves further investigation.

In conclusion, autoantibodies against linear peptides of MPO can be detected in the majority of patients with anti-GBM disease, and several are associated with disease severity, suggesting a potential common pathogenic mechanism for anti-GBM antibodies and MPO-ANCA in anti-GBM disease.

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Disclosures
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