Identification of a Major Epitope Recognized by PLA2R Autoantibodies in Primary Membranous Nephropathy

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ABSTRACT
Phospholipase A2 receptor 1 (PLA2R) is a target autoantigen in 70% of patients with idiopathic membranous nephropathy. We describe the location of a major epitope in the N-terminal cysteine-rich ricin domain of PLA2R that is recognized by 90% of human anti-PLA2R autoantibodies. The epitope was sensitive to reduction and SDS denaturation in the isolated ricin domain and the larger fragment containing the ricin, fibronectin type II, and first and second C-type lectin domains (CTLD). However, in nondenaturing conditions the epitope was protected against reduction in larger fragments, including the full-length extracellular region of PLA2R. To determine the composition of the epitope, we isolated immunoreactive tryptic fragments by Western blotting and analyzed them by mass spectrometry. The identified peptides were tested as inhibitors of autoantibody binding to PLA2R by surface plasmon resonance. Two peptides from the ricin domain showed strong inhibition, with a longer sequence covering both peptides (31-mer) producing 85% inhibition of autoantibody binding to PLA2R. Anti-PLA2R antibody directly bound this 31-mer peptide under nondenaturing conditions and binding was sensitive to reduction. Analysis of PLA2R and the PLA2R-anti-PLA2R complex using electron microscopy and homology-based representations allowed us to generate a structural model of this major epitope and its antibody binding site, which is independent of pH-induced conformational change in PLA2R. Identification of this major PLA2R epitope will enable further therapeutic advances for patients with idiopathic membranous nephropathy, including antibody inhibition therapy and immunoadsorption of circulating autoantibodies.


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Idiopathic membranous nephropathy (IMN) is a rare form of GN affecting 10–12 cases per million population.1 The important discovery in 2009 that circulating antibodies to phospholipase A2 receptor 1 (PLA2R) are present in 70% of patients with IMN identified the autoimmune nature of this pathologic abnormality.2 Genetic evidence of the involvement of PLA2R in IMN came from the genome-wide association study identifying PLA2R and DQA1 as genes accountable for the genetic susceptibility to IMN.3 Clinical confirmation that anti-PLA2R antibodies are relevant in MN is evident from studies showing an association between high levels of anti-PLA2R and active disease,4,5 poor clinical outcome at 5 years,5,6 and less chance of spontaneous remission.7 Failure to render patients anti-PLA2R seronegative by immunosuppression therapy is associated with high risk of relapse.8
In other autoimmune kidney diseases, such as anti–glomerular basement membrane (GBM) disease, which is characterized by anti–collagen IV (α3NC1) autoantibodies and ANCA vasculitis characterized by antimyeloperoxidase autoantibodies, the antigenic epitopes include both linear peptide sequences and three-dimensional (3D) conformational structural. Knowledge of these antigen epitopes in these conditions has been important for understanding the pathologic disease mechanisms and may help to stratify patient subgroups and disease severity.

PLA2R is a member of the mannose receptor (MR) family, which shares a common domain structure and include the mannose receptor (CD206), Endo180, DEC-205, PLA2R, and FcRY. Detailed structure based on x-ray crystallography for CD206 and conformational studies performed on FcRY have so far been used to predict the structure and function of the domains in PLA2R. However, specific experimental structural data for PLA2R are currently lacking, although this is required to improve the prospect of targeted treatments for patients with IMN.

The interactions between anti-PLA2R and PLA2R are reported to be sensitive to chemical reducing agents, stable in the detergent SDS, and independent of glycosylation. It has been suggested that the pH-dependent extended and bent conformations of the MR family may play a role in exposure of epitopes in PLA2R. It is unlikely that genetically determined amino acid changes in PLA2R specific to cases of IMN could influence immunogenicity or contribute to a 3D conformational disease epitope. One study described linear epitopes within PLA2R using an overlapping array of short peptide sequences derived from PLA2R. Four linear peptides were tested using ELISA, but no difference was seen between IMN-seropositive patients and control sera. This suggests that 3D conformational structure as described by Beck et al. is a critical feature of PLA2R epitopes.

Our strategy was to identify which variably sized constructs of PLA2R react with anti-PLA2R under denaturing conditions (Western blotting) and under non-denaturating conditions (native blotting). We used trypsin digestion of the smallest domain of extracellular PLA2R recognized in Western blotting (N-C3) by anti-PLA2R antibodies and analyzed the immunoreactive fragments by mass spectrometry–based proteomics to determine the constituent peptides. Using experimental data from electron microscopy, we generated a new multidomain model of PLA2R to understand the interaction of anti-PLA2R with PLA2R. We measured the affinity and subclass composition of different anti-PLA2R antibodies. Finally, we confirmed the identity of a major PLA2R epitope by using synthesized candidate peptides to inhibit the binding of autoantibodies to PLA2R as measured by surface plasmon resonance (SPR).

RESULTS

Identification of the Smallest Fragment of PLA2R Reacting by Western Blotting

We generated a series of recombinant PLA2R protein fragments, including full-length extracellular PLA2R (N-C8), the N-terminus to C-type lectin domain (CTLD) (N-C3), the N-terminus to CTLD2 (N-C2), and an isolated ricin domain (Figure 1A). Using Western blotting from SDS-PAGE gels, we compared the reactivity of rabbit anti-PLA2R (characterized in Supplemental Figure 1) and human anti-PLA2R autoantibodies (a pool of 5 sera) against these PLA2R fragments. We found that all of the recombinant fragments were recognized by the rabbit antibody under non-denaturing conditions and in the presence of reducing agent (Figure 1B). By contrast, the human anti-PLA2R recognized all fragments in non-denaturing conditions in the absence of reducing agent but only N-C8 and N-C3 in the presence of reducing agent. Reactivity of the N-C2 fragment and the ricin domain was significantly ablated or abolished, respectively, by reduction in non-denaturing conditions (Figure 1B). We show clearly in the Western blot under denaturing conditions that autoantibodies recognize only N-C8 and N-C3 and that SDS alone destroys the epitope structure in N-C2 and ricin fragments. Finally, to confirm the location of this major epitope to the N-terminal region, we determined the ability of N-C3 to inhibit autoantibody binding using an inhibition ELISA; we found the N-C3 fragment competed effectively for the binding of autoantibodies to N-C8 in approximately 90% of the patient sera tested, confirming the presence of a major epitope within the N-C3 fragment of the receptor (Supplemental Figure 2).

Comparison of the Binding Affinity of Autoantibodies to PLA2R Fragments

We tested sera from four anti-PLA2R–positive patients and showed that the autoantibodies targeted the same conformational epitope as seen on the Western blots of trypsin-fragmented N-C3 (Figure 2A, Supplemental Figure 3). We next determined the affinity of interaction between purified human anti-PLA2R (Supplemental Figure 4) and the four PLA2R constructs (N-C8, N-C3, N-C2, and ricin). The autoantibodies bound with equally strong affinity of approximately 0.1 nM to N-C8, N-C3, N-C2, and the ricin domain, confirming the existence of a single epitope in the protein (Figure 2B). The kinetic constants derived from the analysis of purified antibodies were similar to those obtained from the patient sera, demonstrating the utility of unpurified antibodies in sera and their potential use in a diagnostic assay (Table 1). Moreover, the antibody affinity was similar in all patient sera (all predominantly IgG4) tested at approximately $0.5 \times 10^{-10}$ M (Supplemental Figure 5, Table 1) and higher than the dissociation constant described for α3NC1 antibodies in anti-GBM disease ($2 \times 10^{-9}$ M). The N-terminus to C-type lectin domain (CTLD) (N-C3), the N-terminus to CTLD2 (N-C2), and an isolated ricin domain (Figure 1A). Using Western blotting from SDS-PAGE gels, we compared the reactivity of rabbit anti-PLA2R (characterized in Supplemental Figure 1) and human anti-PLA2R autoantibodies (a pool of 5 sera) against these PLA2R fragments. We found that all of the recombinant fragments were recognized by the rabbit antibody under non-denaturing conditions and in the presence of reducing agent (Figure 1B). By contrast, the human anti-PLA2R recognized all fragments in non-denaturing conditions in the absence of reducing agent but only N-C8 and N-C3 in the presence of reducing agent. Reactivity of the N-C2 fragment and the ricin domain was significantly ablated or abolished, respectively, by reduction in non-denaturing conditions (Figure 1B). We show clearly in the Western blot under denaturing conditions that autoantibodies recognize only N-C8 and N-C3 and that SDS alone destroys the epitope structure in N-C2 and ricin fragments. Finally, to confirm the location of this major epitope to the N-terminal region, we determined the ability of N-C3 to inhibit autoantibody binding using an inhibition ELISA; we found the N-C3 fragment competed effectively for the binding of autoantibodies to N-C8 in approximately 90% of the patient sera tested, confirming the presence of a major epitope within the N-C3 fragment of the receptor (Supplemental Figure 2).

Effect of pH on PLA2R Conformation and Epitope Availability

PLA2R shares structural similarities with other members of the MR family and one member, FcRY, undergoes conformational change in a pH-dependent manner. We measured the associated conformational change by monitoring the thickness of the N-C8 protein layer in real time using dual polarization interferometry and determined a susceptible pH

region around pH 6.2 where the transition in conformation occurred (Figure 3A). Both N-C3 and N-C8 variants were then incubated in neutral pH (7.2) and a more acidic pH (6.2), and their sedimentation rates, which depend on the shape and size of the protein, were monitored using analytical ultracentrifugation. At a pH of 7.2, N-C8 sedimented as a single species with a sedimentation coefficient of 8.1s (Figure 3B, Table 2).

At a pH of 6.2, the protein became more compact, which was reflected in a larger sedimentation coefficient of 9.2s. The N-C3 data (Figure 3B inset) collected at a pH of 6.2 and 7.2 yielded an identical sedimentation coefficient (5.3s), indicating that compaction of the molecule at lower pH was specific to the N-C8 receptor and not to the shorter fragment N-C3. To test whether anti-PLA2R binding to N-C8 was independent of this conformational change, single cycle kinetics between the anti-PLA2R antibody and the N-C8 receptor were performed at two pHs (pH of 6.2, tight conformation; pH of 7.2, extended conformation) and showed no difference in affinity (Figure 3C). This indicates that the binding of the autoantibody was not affected by altered conformation of the receptor due to the environmental pH.

Defining a Major Epitope in PLA2R
To locate this major epitope, we generated immunoreactive trypsin fragments of PLA2R under nonreducing conditions. Comparing trypsin-digested and intact PLA2R, we confirmed using the five sera (Table 1) that the epitope reactivity was preserved in the digested antigen (mean ± SEM, 44.8% ± 4.2%), data not shown. Trypsin fragmentation of the N-C3 antigen, followed by multidimensional separations (OFFGEL fractionation and
Figure 2. Human anti-PLA2R shows similar binding affinity to N-C8, N-C3, N-C2 and the ricin domain. (A) Western blot analysis of fragmented N-C3 peptides by OFFGEL fractionation using different patient sera. (B) Representative sensorgrams derived from injections of different concentrations of affinity purified anti-PLA2R antibodies over immobilized N-C8 (left panel), N-C3 (right panel), and N-C2 and ricin domain (bottom panels). The tables summarize the kinetic constants of four separate runs for the binding of purified human PLA2R-specific antibody to the PLA2R fragments. Results were obtained after reference subtraction. Kinetics data were fitted to a Langmuir 1:1 interaction model. The association ($k_a$), dissociation ($k_d$), and equilibrium ($K_D$) constants for each run were similar and revealed an overall high binding affinity with an apparent $K_D$ of approximately 0.1 nM.
SDS-PAGE analysis), were performed to identify the smallest reactive polypeptides in the epitope (Supplemental Figure 3). The polypeptides of interest identified by blotting with anti-PLA2R antibody from patient sera were cut from the gel, reduced and identified by mass spectrometry (MS). MS analysis revealed eight peptides potentially constituting part of the PLA2R epitope (Table 3) and these originated from the ricin domain, FNII domain, CTLD3 domain, and interdomain loops between CTLD 1/2 and CTLD 2/3. The distribution of these peptides is discontinuous on the linear sequence but could be in proximity within

### Table 1. Concentrations, affinity, and IgG subclasses of antibodies to PLA2R

<table>
<thead>
<tr>
<th>Patients</th>
<th>ELISA (U/ml)</th>
<th>Concentration (µM)</th>
<th>K_D (M)</th>
<th>IgG1 (%)</th>
<th>IgG2 (%)</th>
<th>IgG3 (%)</th>
<th>IgG4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30,000</td>
<td>1.4±0.06</td>
<td>0.49×10^-10</td>
<td>16</td>
<td>9</td>
<td>2</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>2600</td>
<td>0.32±0.1</td>
<td>0.45×10^-10</td>
<td>4</td>
<td>51</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>30,000</td>
<td>1.0±0.12</td>
<td>0.43×10^-10</td>
<td>10</td>
<td>27</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>2300</td>
<td>0.36±0.19</td>
<td>0.85×10^-10</td>
<td>11</td>
<td>10</td>
<td>0</td>
<td>78</td>
</tr>
<tr>
<td>5</td>
<td>9300</td>
<td>0.51±0.18</td>
<td>0.32×10^-10</td>
<td>17</td>
<td>22</td>
<td>19</td>
<td>42</td>
</tr>
</tbody>
</table>

Absolute concentrations of PLA2R-specific antibody detected in five patient sera tested by ELISA (U/ml) and derived from three kinetic runs (µM). The K_D column is the kinetic constants for the binding of patient autoantibodies to immobilized N-C3. The last four columns indicate the percentage of each IgG subclass (IgG1–4) in the five patient sera measured by ELISA, demonstrating the presence of predominantly IgG4. Values expressed with a plus/minus sign are the mean ± SD.

**Figure 3.** Human anti-PLA2R binding to N-C8 is unaffected by a pH induced conformational change. (A) pH-dependent conformational change of N-C8 determined by dual polarization interferometry. Changes in the thickness of the N-C8 layer as a function of decreasing pH was measured and represented the adsorption behavior of different folded states. (B) Sedimentation velocity of N-C8 and N-C3 (inset) at a pH of 6.2 (black line) and of 7.2 (gray line) analyzed by the distribution of Lam equation solutions c(s) model using the program Sedfit. (C) Single-cycle kinetics between immobilized purified human PLA2R-specific antibody and N-C8 at a pH of 6.2 and of 7.2. A range of 1, 2, 5, 10, and 15 nM of N-C8 was injected, one cycle at a pH of 6.2 and the other at a pH of 7.2. The surface was not regenerated between injections in each cycle.
Table 2. Hydrodynamic parameters determined for N-C8 and N-C3 at a pH of 7.4 and a pH of 6.2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Theoretical Mass (D)</th>
<th>Mass MALLS (D)</th>
<th>Sedimentation Coefficient (S_{20,w})</th>
<th>Frictional Ratio (f/f_0)</th>
<th>Hydrodynamic Radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-C3 (pH 7.4)</td>
<td>79,461</td>
<td>88,820</td>
<td>5.29±0.09</td>
<td>1.38</td>
<td>3.81±0.23</td>
</tr>
<tr>
<td>N-C3 (pH 6.2)</td>
<td>79,461</td>
<td>ND</td>
<td>5.35±0.10</td>
<td>1.39</td>
<td>ND</td>
</tr>
<tr>
<td>N-C8 (pH 7.4)</td>
<td>164,308</td>
<td>188,800</td>
<td>8.08±0.21</td>
<td>1.51</td>
<td>6.25±0.19</td>
</tr>
<tr>
<td>N-C8 (pH 6.2)</td>
<td>164,308</td>
<td>ND</td>
<td>9.23±0.25</td>
<td>1.32</td>
<td>ND</td>
</tr>
</tbody>
</table>

Density, 1.0045 g/ml. Viscosity, 0.01017 poise. 

The folded molecule and held together by intact disulfide bonds. These eight peptides were synthesized, and we incubated the autoantibody with an excess of the candidate peptides and assessed their potential to inhibit the binding between the autoantibody and its receptor using SPR. Of these peptides, only peptide 1 (GIFVIQSESLKKC), representing the β1 strand, and peptide 2 ([W]SVLTLENCK), forming the β3 strands from the ricin domain, showed significant reduction in binding of purified autoantibody to N-C3 (Figure 4A). By extending peptide 1 toward peptide 2, it also encompasses the β2 strand to form the 31-mer (WQDKGIFVIQSESLKKC[QAGK]SVLTLENCK), creating one of the three-dimensional lobes in the β-trefoil structure. This β1,2,3 lobe is also maintained by a disulfide bond connecting the β2 and β3 strands (Figure 5A).11 The two main competing peptides, peptide 2 and the 31-mer peptide, were titrated in a binding experiment to compete with binding of anti-PLA2R antibody to immobilized N-C3. Peptide 2 showed reproducible inhibition up to 47% and the 31-mer peptide inhibited by 85% (Figure 4B). The competing 31-mer peptide bound to the antibody (with an affinity of approximately 5x10^{-10} M) (Figure 4C), and this resulted in reduced affinity between autoantibody and both N-C3 and N-C2 (Figure 5B) confirming the specificity of the binding.

The rabbit anti-PLA2R antibody did not react with the 31-mer peptide under non-denaturing blotting conditions in contrast to the human anti-PLA2R (pool of 5 sera). Moreover, the reactivity of human anti-PLA2R with the 31-mer peptide was sensitive to reduction (Figure 5A, left panel). The 31-mer peptide in solution is a disulfide-bonded structure illustrated by the shift in the elution profile between the reduced and unreduced state (Figure 5A, right panel). The 3D conformation of the 31-mer peptide identifies the disulfide bond bridging between peptides 1 and 2 (Figure 5A). To identify possible sequence homology for peptides 1 and 2, we used the Basic Local Alignment Search Tool (BLAST)15 against a database of microbial proteins and found no homology for the peptide 1 sequence. However, the LTLENCK sequence of peptide 2 (SVLTLENCK) was identified in a cell wall enzyme, D-alanyl-D alanine carboxypeptidase, common in several bacterial strains, including Clostridia species. This finding may be relevant for the pathogenesis of IMN.

3D PLA2R Structure and Arrangement of Domains

There is no multidomain model of PLA2R on which to map these peptide sequences. We therefore produced a 3D structure of the full-length molecule N-C8 using transmission electron microscopy and single particle averaging. We established the relative positioning of the domains and as a result a predicted location of the epitope. Negative stained data were used to visualize the shape of PLA2R (Figure 6A), anti-PLA2R (Supplemental Figure 4) and the preformed isolated immune complex between PLA2R and the autoantibody. Analysis of these datasets allowed us to generate the first 3D structure to a moderate resolution of 20 Angstroms (Supplemental Figure 6) of the extracellular domains of PLA2R (Figure 6B). PLA2R is a flat structure (approximately 4 nm wide) with an overall shape similar to the η symbol, measuring about 12 nm×9 nm. Using homology models of the individual domains, we constructed a structural representation of the domain arrangement informed by the sharp molecular envelope produced from

Table 3. Potential peptides involved in the antibody binding site

<table>
<thead>
<tr>
<th>Peptide ID</th>
<th>Sequences of Identified Peptides (Residue Numbers)</th>
<th>Sequence Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GIFVIQSESLKKC (39–51)</td>
<td>Ricin domain</td>
</tr>
<tr>
<td>2</td>
<td>SVLTLENCK (57–65)</td>
<td>Ricin domain</td>
</tr>
<tr>
<td>3</td>
<td>EDDLWCAITTSR (198–209)</td>
<td>Fibronectin type II domain</td>
</tr>
<tr>
<td>4</td>
<td>YLNHDHEIVERDAWK (357–372)</td>
<td>Linker between CTLD1 and CTLD2</td>
</tr>
<tr>
<td>5</td>
<td>YYANHEPWNPR (373–387)</td>
<td>Linker between CTLD1 and CTLD2</td>
</tr>
<tr>
<td>6</td>
<td>TWHEALR (399–405)</td>
<td>CTLD2</td>
</tr>
<tr>
<td>7</td>
<td>AGHVLSDAESQCOAQGWER (504–521)</td>
<td>Linker between CTLD2 and CTLD3</td>
</tr>
<tr>
<td>8</td>
<td>YSGGCVAMGR (613–623)</td>
<td>CTLD3</td>
</tr>
</tbody>
</table>

List generated from the MS analysis of the potential peptides involved in the antibody binding site. Sequences of the identified peptides, the residue numbers as well as their coverage are described in the table.
transmission electron microscopy (Figure 6B). The resulting models for both N-C3 and N-C8 were analyzed for their hydrodynamic solution properties using the program Hydropro (version 10). The sedimentation coefficients of the models were in good agreement with the compact form of N-C8 and the N-C3 experimental results (Table 2), giving confidence in the overall shape of the molecule. Furthermore, a low-resolution 3D model of the immune complex confirmed the location of the anti-PLA2R binding site in the ricin domain (Supplemental Figure 7).

**DISCUSSION**

We describe the location and composition of a dominant epitope in PLA2R that interacts with human autoantibodies.

Located in the N-terminal CysR ricin domain, the β2-β3 strands of the trefoil structure involve the peptide WQDKGFVIQ-SESLLKKCICGKSLLENCK, which forms the focus of this epitope. The crystal structure of this CysR domain is reported as a β-trefoil form also seen in ricin B17 and is composed of 12 antiparallel β strands: 4 in each part of the 3-lobe -structure. Three disulfide bonds hold these lobes in place, highlighting the structural importance of the disulfide bond present in the 31-mer peptide connecting β2 and β3 strands. Our data show that this epitope in the PLA2R fragments (N-C8, N-C3, N-C2, and ricin domains) in non-denaturing conditions is available to react with human anti-PLA2R autoantibodies. Importantly, the epitope structure is resistant to reduction in the larger species N-C8 and N-C3 but is sensitive to reduction in N-C2, ricin, and the 31-mer peptide. In the SDS-denatured conformations, the
epitope is preserved only in N-C8 and N-C3 but not N-C2 and ricin. This clearly shows that the larger fragments N-C8 and N-C3 possess additional conformational interdomain properties that protect the epitope from SDS denaturation or reduction. However, as described by Beck et al., the combination of SDS denaturation and reduction destroys the epitope in the N-C8 extracellular sequence (Supplemental Figure 8).

Our current best-fit model of the preformed immune complex of PLA2R and anti-PLA2R confirms that the IgG Fab binds to PLA2R in the area of the ricin/CTLD3 interaction. Current knowledge of the PLA2R sequence derived from gene sequencing suggests that the PLA2R protein structure in patients with IMN is not significantly different from that of healthy controls. Other potential mechanisms affecting antigen conformation and aberrant immune processing need exploring to understand how PLA2R becomes immunogenic. We described a pH-dependent conformational change in PLA2R (N-C8), as previously reported for FcγR, and show...
that this is not a property of the N-C3 fragment and does not influence the affinity of antibody interaction. This eliminates conformation change due to pH<6.2, which can be present in inflammatory tissue abnormalities and in subcellular compartments of leukocytes as a major contributor to immunogenicity. However, our evidence-based model describes the adjacency of the ricin and CTLD3 domains in a ring-like configuration, unlike the linear sequence commonly illustrated in cartoons. This 3D conformational structure may be important for stability of the epitope in N-C3 and may explain its resistance to denaturation (SDS) or reduction, to which N-C2 lacking CTLD3 is susceptible. Our current structural model requires further confirmation from x-ray crystallography studies.

Figure 6. 3D PLA2R model structure and arrangement of domains. (A) Representative area micrographs of N-C8 with highlighted images of single molecules within white squares, selected projections obtained and two-dimensional averages of the images within the corresponding class. Scale bar=1000 Å. TEM, transmission electron microscopy; MSA, multi statistical analysis. (B) Electron density of N-C8 solved from approximately 20,000 selected particles at 20-Å resolution and a 3D reconstruction of N-C8 showing the domains arrangement. Individual PLA2R domains were modeled using Phyre2. The domains were aligned in the electron microscopic density map using Chimera and validated using area under the curve hydrodynamics parameters (Table 2). Scale bar=50 Å.
Potentially, the stability of the epitope in soluble forms of PLA2R under physiologic conditions may facilitate selection of B cells for generation of class II peptides to present to T cells. This is a testable hypothesis for immunogenicity and is a significant step toward understanding the initiation of the autoimmune mechanism in IMN. Interestingly, approximately 10% of anti-PLA2R positive sera react variably to another epitope that is not in N-C3 fragment and that by implication is in C4-C8 region of PLA2R. Further work is needed to identify the nature of this second epitope and whether antibodies to this minor epitope are associated with a particular clinical phenotype of IMN.

The affinity of anti-PLA2R antibodies in different patient sera is uniformly high and approximately 2- to 5-fold greater than that described for the classic autoantibody anti–collagen IV (α3NC1). This could explain the clinical paradox of PLA2R-positive biopsy specimens associated with absence of anti-PLA2R antibodies in the serum. We suggest that as anti-PLA2R antibodies are secreted by plasma cells, the high affinity of the antibody will ensure the autoantibody is adsorbed out of serum onto PLA2R positive cells, such as podocytes, until the rate of production of antibody exceeds the rate of removal from the serum; at this point there will be free detectable circulating anti-PLA2R. These biopsy-positive, seronegative cases may, therefore, represent an early phase of the immunologic abnormality seen in primary disease and in recurrent disease after transplantation.

Having identified the β2-β3 strands (31-mer peptide) containing an inherent disulfide bond, we mapped this peptide to the reported ricin domain structure. It will be important to further investigate how the availability of this binding pocket to autoantibodies is controlled by adjacent domains. It has been proposed that the induction of autoimmunity may be mechanistically driven by infection, whereby an immune response against bacterial or viral antigen cross-reacts with a self-component. In this regard, preliminary bioinformatic analysis of peptide 2 in BLAST searches against microbial protein databases revealed complete homology for the sequence LTLENCK, which is part of the bacterial cell wall enzyme D-alanyl-D alanine carboxypeptidase common to Clostridium species and other bacteria. Clonal expansion of B cells driven by common infections may allow MN genetically susceptible individuals to bind PLA2R through the B cell receptor, ingest, degrade, and present PLA2R peptides on class II receptors (coded by pathologic alleles of DQA1) to engage T cell help and promote autoantibody production. In autoimmune mechanisms over time, there is often a change in use of epitopes on the autoantigen, in some cases as proposed for anti-GBM antibodies, the binding of the autoantibody induces neo-epitopes initiating “epitope spreading.” Now that we have defined a major epitope, we can investigate whether use of this epitope changes over time in chronic MN disease, from initiation and first presentation of disease through induction of remission, relapse, and recurrent disease after transplantation.

Knowledge of this PLA2R epitope is potentially useful in developing novel therapies for translation into the clinic for patient benefit. The use of the 31-mer peptide as an inhibitor of anti-PLA2R binding to the podocyte may modulate the consequences of anti-PLA2R–mediated cell biology. Alternatively, the 31-mer peptide coupled to immunoadsorption columns could provide an effective means of specifically removing anti-PLA2R in conjunction with immunosuppression, particularly for patients resistant to standard mainline immunosuppressive drugs.

**CONCISE METHODS**

### Cloning, Expression, and Purification of PLA2R N-C8, N-C3, and N-C2 and the Ricin Domain

The previously described codon-optimized clone of human extracellular PLA2R (N-C8) was modified to generate the smaller PLA2R fragments expression vectors (details in Supplemental Methods). The resulting constructs were transfected into HEK293-EBNA1 cells (human embryonic kidney cells; Invitrogen) using Lipofectamine 2000 reagent (Invitrogen). The secreted proteins were purified using nickel affinity chromatography. Conditioned medium containing 20 mM imidazole was loaded onto a 5-ml HiTrap Excell nickel affinity chromatography column (GE Healthcare). Bound proteins were eluted by using a linear gradient of 20–500 mM imidazole over 10 column volumes in a buffer containing 10 mM BisTris and 150 mM NaCl (pH, 7.2). The PLA2R-containing fractions were desalted using PD-10 columns (GE Healthcare) into 10 mM BisTris (pH, 7.2) and further purified by anion exchange chromatography on a MiniQ column using the Etta purifier HPLC system (GE Healthcare). The proteins were eluted by using a linear gradient of 0–1 M NaCl. Buffer conditions were optimized using an Optim screening instrument (Avacta).

### N-C3 Inhibition ELISA

The ELISA method was a variation on the standard assay previously described. Briefly, PLA2R (N-C8) at 125 ng/ml was coated onto ELISA plates overnight and plates blocked with Superblock (Thermo Fisher Scientific). Fifty microliters of 1:100 dilution of patient serum plus 50 µl of buffer or NC-3 fragment (to give a final concentration in the well of 9.0 µg/ml, 0.9 µg/ml, 0.09 µg/ml, and 0.009 µg/ml) were preincubated for 2 hours at room temperature before adding to the ELISA plate for a further 2 hours with shaking. Following extensive washing, anti-human IgG-horseradish peroxidase at 1:25,000 dilution in assay buffer was added to all wells for 2-hour incubation. Following washing, the plate was developed by adding substrate, and the reaction terminated at 5 minutes by addition of 1 M H2SO4. Plates were read at 450 nm and inhibition curves constructed. The concentration of 9.0 µg/ml was used to compare the inhibition profile of 43 different sera previously reported as anti-PLA2R positive. This experiment could be repeated with just the ricin domain to confirm that there are no adjacent epitopes in FN II or CTLD1–3.

### Binding Studies Using SPR

Purified recombinant N-C8, N-C3, N-C2, and ricin proteins (50–100 µg/ml) diluted in 10 mM sodium acetate at a pH of 4.5 were immobilized on a ProteOn GLM sensor chip using standard amine coupling linking chemistry (GE Healthcare).
chemistry. Ligand densities of 6000 RU, 5000 RU, 5000 RU, and 500 RU, respectively, were achieved. The first set of kinetic runs were performed using 6 concentrations of purified anti-PLA2R antibody as analytes (1, 2, 5, 10, 15, and 20 nM) in HEPES buffer (pH, 7.2) containing 0.005% Tween 20. Each injection lasted 120 seconds at a flow rate of 80 μl/ml with a 900-second dissociation phase. Tightly bound proteins were then dissociated by two successive (40 seconds) injections of 100 mM glycine (pH, 2.2). The second series of kinetic used patient sera. Seven dilutions of sera containing anti-PLA2R antibody (1/100, 1/200, 1/500, 1/800, 1/1000, 1/1500, and 1/2000) were injected onto the same immobilized N-C8 and N-C3 proteins. Samples were all double-referenced with blank row (injected with running buffer) and control serum. The data were analyzed and fitted to a Langmuir 1:1 interaction model. Single cycle kinetics between immobilized purified anti-PLA2R antibody (2000 RU bound onto a CM5 sensor chip) and N-C8 were performed on a Biacore T200 instrument (Biacore AB). Two cycles of 1, 2, 5, 10, and 15 nM of N-C8 were injected, one at a pH of 6.2 and the other at a pH of 7.2. Kinetic parameters were determined with the manufacturer’s Biacoreevaluation 4.1 software. Peptide inhibition assays were carried out as follows. Each individual peptide (5 μM) was incubated with 10 nM of human purified anti-PLA2R antibody for 1 hour; then the complex was injected on the captured N-C3 (5000 RU bound). Peptides 2 and 31-mer peptide were titrated to compete the binding against the immobilized anti-PLA2R antibody complex (10 nM). The concentration of anti-PLA2R in the unknown serum samples.

**Determination of Anti-PLA2R Antibody Concentration in Patient Sera**

Quantitation of serum antibody concentrations was performed on the Proteon XPR-36 (Bio-Rad) SPR instrument, where the initial binding rates of purified human anti-PLA2R binding to N-C3 were used as standard curve to determine the concentration of anti-PLA2R in the unknown serum samples.

**Dual Polarization Interferometry**

Dual polarization interferometry was used to provide quantitative information on the thickness, the refractive index and density of the immobilized PLA2R layer with the capability to measure conformational change in real time. These experiments were performed on a Farfield AnaLight instrument. Purified recombinant N-C8 and N-C3 proteins (at a concentration of 50 μg/ml) were immobilized onto an amine functionalized AnaChip using sulfo-GMBS (Pierce) an amine-to-sulphhydryl crosslinker (2 mg/ml). After stabilizing the immobilized N-C8 and N-C3 with sufficient rinsing with BisTris running buffer (10 mM BisTris [pH, 7.2], 150 mM NaCl), the proteins were then exposed to a decreasing pH range (pH, 7.2, 6.8, 6.4, 6.2, 6, 5.9, 5.8, 5.5, and 5). N-C8 and N-C3 were regenerated between the different pHs with running buffer (pH, 7.2). Analysis of the raw data and determination of density, thickness, and mass per unit area were performed using the AnaLight Bio200 analysis software (Farfield Scientific).

**Structure Analysis by Sedimentation Velocity**

The sedimentation coefficients for recombinant N-C8 and N-C3 proteins (at 150 μg/ml) were determined from velocity experiments using the Optima XL-A ultracentrifuge (Beckman Instruments). The experiments were performed using double sector cells and a rotor speed of 74,500 g for N-C8 and 116,500 g for N-C3, taking 150 scans at 1.5-minute intervals at a wavelength of 280 nm and at a temperature of 20°C. The sedimenting boundaries were analyzed using the program Sedfit, version 8.7.21

**OFFGEL Fractionation and Mass Spectrometry Analysis**

N-C8 and N-C3 purified proteins (300 μg) were digested with 15 μg proteomics grade trypsin (Sigma-Aldrich) overnight at 37°C. Peptides (nonreduced) were separated by isoelectric point and size and the smallest fragments were identified by western blotting with patient sera. Bands of interest corresponding to the reactive bands on the Western blot were excised and in-gel tryptic digestion was performed. Dried gel pieces were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Digested samples were analyzed by liquid chromatography-MS/MS using an UltiMate 3000 Rapid Separation LC (RSLC; Dionex Corporation) coupled to an LTQ Velos Pro (Thermo Fisher Scientific) mass spectrometer. Data produced were searched using Mascot (Matrix Science, London, UK) against the Uniprot_human database, version 2011_05_03. Data were validated using Scaffold (Proteome Software).

**Negatively Stained Transmission Electron Microscopy**

Negatively stained samples of PLA2R, antibody, or the PLA2R-antibody complex (10–20 μg/ml) were absorbed to the surface of a freshly glow-discharged 400 mesh carbon coated grid (EMS) for 30 seconds, washed twice in 10-μl droplets of distilled water, and then placed on a 10-μl droplet of 5% (wt/vol) uranyl acetate for 30 seconds and air dried. Low-dose data (10–20e-/Å) were recorded on a G30 Polara operating at 200 kV and a nominal magnification of 39,000× with a Gatan 4K CCD camera. CCD images were recorded with a 1-second exposure at defocus values between 0.5 and 2.5 μm and at a sampling increment of 3.05 Å/pixel.

**Single-Particle Analysis and 3D structure Determination**

Single-particle averaging was performed using EMAN2.22 Data-sets were selected using a combination of manual and semi-automated swim picking. Following full CTF correction, each dataset was subjected to multi statistical analysis 2D classification using cross correlation coefficient as the main comparator. A selection of uniquely positioned projection averages were selected and used to generate an initial 3D model for refinement (C1 symmetry was applied to the processing for all datasets). This model was used as a start model for eight rounds of iterative refinement, using FRC as the main alignment comparator, to produce a self-consistent 3D structure that was low-pass-Gaussian filtered to 20-Å resolution. Resolution estimates of the final structures were determined using the Fourier shell correlation with a cutoff value of 0.5. 3D volumes were examined and modeling was performed using Chimera.23

**Modeling**

Homology models of the domains were generated using the software Phyre2.24 These were assembled into the electron microscopic density map using Chimera, version 1.8, without the interglobular linkers.
present. The resulting models of both the N-C3 and N-C8 were converted to shell models using the program Hydropro10.16 The hydrodynamic outputs were compared with experimental data for the hydrodynamic radius deduced from light scattering, and sedimentation coefficient determined using analytical ultracentrifugation, in order to establish the best arrangement of the domains.

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DISCLOSURES

None.

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Supplementary Information

Complete methods

Cloning, expression and purification of PLA2R N-C8, N-C3, N-C2 and the ricin domain

To generate PLA2R N-C3 (aa 20-663) the following primers were first used to amplify an N-terminal portion of the N-C8 by PCR using the following oligos:

5’CCCGCTAGCCGAGGGAGTGGCCGCTGCCC3’ (Forward) and
5’CCCGCGGCCGCTCAATGGTGATGGTGATGGTGGTGATGATGGTGTCCGGACCCGGGTGAAATGGCCATCTTTTCTCGTACTCAGC3’ (Reverse). To generate PLA2R N-C2 (aa 20-515), the same N-C3 forward primer was used with the following reverse primer:

5’ATGGCGGCCGCTCAATGGTGATGGTGATGGTGGTGATGATGGTGTCCGGATCTCGCTCAGCGTCACTCAGCACGTGGCCGGC3’. The resulting PCR product were digested with Nheli and NotI enzymes and inserted into the same vector backbone as the original PLA2R N-C8. The constructs were then transfected into HEK 293-EBNA1 cells (293c18 ATCC® CRL-10852™) using LipofectAMINE reagent (Life Technologies). Transfected cells were cultured in Dulbecco’s modified Eagle medium (Sigma) containing 10% foetal calf serum and antibiotic added after 24h. Positive clones were pooled and a stable line was expanded. For harvesting, stable cell lines were cultured in serum free medium for 7 days. The conditioned medium was collected and centrifuged at 1,600 rpm for 4 minutes. To generate the ricin expression vector, the N-terminal end of the N-C8 (aa 20-173) was amplified using the following primers:

5’ AATGGATCCGCCGAGGGAGTGGCCGCTGCCC 3’ (Forward) and 5’ GGGGAATTCTCATGTGTTGCCCTTAATGGTATGCA 3’ (Reverse). The PCR amplicon was digested with BamHI and EcoRI and cloned into a modified pET16b vector with N-terminal His tag. The plasmid was transformed into JM109 DE3 cells and plated onto LB ampicillin plates (100 ug ml⁻¹). A single colony was selected and grown to saturation in LB media with antibiotic selection. The seed culture was used to inoculate a 500 ml flask of LB broth, cells grown to OD600, chilled on ice and then IPTG added to 0.2 mM IPTG and left overnight at 16°C (220 rpm). Culture was pelleted and lysed in ice cold lysis buffer (150 mM NaCl, 25 mM Tris H-Cl pH7.5, 1% Triton X-100 with protease inhibitors (Sigma-EDTA free). Clarified
lysate was bound to nickel beads (Qiagen) and washed with an Imidazole gradient (60 mM). Bound protein was eluted with 250 mM Imidazole in binding buffer and then de-salted into PBS using a PD-10 column (GE). In addition, the transformed plasmid was grown in autoinduction broth and grown overnight at 37°C. The protein was found in the inclusion body pellet and so was purified using 8 M urea lysis buffer and re-folded into PBS buffer by rapid dilution.

Production of rabbit anti-PLA2R (N-C8)
Polyclonal anti-PLA2R (N-C8) was produced in rabbits using a standard immunization protocol (Eurogentec) and the antiserum was adsorbed with glutaraldehyde polymerized human serum¹ before use. Our rabbit anti-PLA2R (N-C8) was compared with a rabbit anti-PLA2R peptide antiserum (Sigma Aldrich, Poole, UK) by immunostaining on normal kidney tissue and membranous nephropathy biopsy tissue (Supplemental Figure 1). The polyclonal anti-N-C8 antibody was also used for western blotting and affinity measurement as shown in Figure 1A and Supplemental Figure 1.

pH dependent conformational change studied by dual polarisation interferometry
An initial procedure to calibrate the sensor chip was performed as follows. Running buffer 10 mM BisTris 150 mM NaCl pH 7.2 was passed through both channels at a flow rate of 50 μl min⁻¹ per channel. This was followed by a calibration solution (8:2 ethanol:water mixture by weight) at the same flow rate for 2 min before returning to BisTris followed by a 2 min water injection. This short procedure enabled the refractive index response of the chip to be calibrated and the bulk refractive index for the running buffer to be measured. The values obtained were used by the analysis software of the AnaLight® Bio200 instrument in all subsequent calculations.

Purification of anti-PLA2R antibodies from patient serum
Serum samples from patients with biopsy proven IMN were collected and used under ethics authority 06/Q1401/5 and 12/SW/0289. Patient serum was first precipitated with ammonium bicarbonate to remove the majority of the albumin. A HiTrap NHS-activated HP column was used to affinity purify the anti-PLA2R antibody. 1.8 mg of antigen N-C3 in 0.2 M NaHCO₃, 0.5 M NaCl pH 8.3 was coupled to the pre-washed column. The remaining NHS groups were deactivated using a succession of washes with 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 and 0.1 M sodium acetate, 0.5 M NaCl pH 4. The serum (in PBS) was applied to the affinity column, which was then washed with 10 column volumes of PBS. The bound antibody was eluted with 5 mL of 100 mM glycine pH 2.2 and 0.5 mL fractions were collected. The pH was neutralized using 50 µl of 1 M TrisHCl pH 8. The fractions containing the purified antibody were dialyzed against PBS. The concentration was determined using the spectrophotometer reading at 280 nm and the extinction coefficient of human IgG of 1.36.

**Determination of anti-PLA2R antibody concentration in patient sera**

Briefly, the N-C3 protein was immobilized at high concentration (4,000 units) and purified human anti-PLA2R was injected at concentrations of 1, 2, 5, 10, 15 and 20 nM ensuring that the initial rates are mass-transport limited and therefore relative to concentration. The initial binding rates (RU/sec) are calculated and correlated to concentration using the 5-parameter logistic equation:

\[
y = A_{\text{min}} + \frac{(A_{\text{max}}-A_{\text{min}})}{1+(\frac{X_0}{h})^s}
\]

Where \( A_{\text{min}} \) and \( A_{\text{max}} \) are the upper and lower asymmetry, \( X_0 \) is the concentration at half \( A_{\text{max}} \) and \( h \) is the slope factor at \( X_0 \) and \( s \) is the asymmetry factor.

**OFFGEL fractionation and Mass spectrometry analysis**

N-C3 purified proteins (300 µg) were digested with 15 µg proteomics grade trypsin (Sigma) overnight at 37°C. Samples were directly precipitated with 2 mL of cold acetone at -20°C for
at least 2 hours then centrifuged for 10 minutes at 10,000 x g. Dry pellets were resuspended in 1.8 mL IEF-sample buffer (7 M urea, 2 M thiourea, 10% glycerol and 1% ampholytes pH 4-7 containing no DTT). The assembly of the IPG-strip, 12 wells frame and electrodes was done as recommended by the manufacturer (Agilent). 150 µl of sample was then loaded into each well on the pre-hydrated IPG-strip pH 4-7. The focusing was accomplished until 25,000 Vh were reached (initial voltage was 500V for 1 hour, then increased using a gradient up to 1,000 V over 1 hour and held for 2 hours, finally a maximum of 8,000 V was applied until the end of the focusing; for all steps 50 µA was used as limit). Fractions were precipitated with cold acetone, centrifuged and then dried in a fume hood. Dry pellets were resolubilized in 24 µl dH₂O and 6 µl 5x SDS sample buffer. 20 µl were used for gel staining using Instant Blue (Expedeon) and 10 µl for Western blotting. Briefly fractionated N-C3 tryptic peptides were electrophoresed under non-reducing conditions on a 12% Bis-Tris PAGE gel in a MOPS running buffer system. Peptides were transferred to nitrocellulose membranes and the recombinant N-C3 fragments were blotted using patient sera as primary antibody (1:50 dilution) with biotinylated goat anti-human (1:25,000 dilution; Jackson Lab) as a secondary antibody. Detecting antibody was Alexa streptavidin 680 (dilution 1:5,000; Life Technologies) and peptides bands were visualized using the Odyssey system (Biorad). Bands of interest corresponding to the reactive bands on the Western blot were excised and in-gel tryptic digestion was performed. Dried gel pieces were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Gel pieces were then washed alternately with 25 mM ammonium bicarbonate followed by acetonitrile. This was repeated, and the gel pieces dried by vacuum centrifugation. Samples were digested with trypsin overnight at 37°C. Digested samples were analysed by LC-MS/MS using an UltiMate® 3,000 Rapid Separation LC (RSLC, Dionex Corporation) coupled to a LTQ Velos Pro (Thermo Fisher Scientific) mass spectrometer. Peptide mixtures were separated using a gradient from 92% A (0.1% FA in water) and 8% B (0.1% FA in acetonitrile) to 33% B, in 44 min at 300 nL min⁻¹, using a 250 mm x 75 µm i.d. 1.7 µM BEH C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data dependant analysis. Data produced
were searched using Mascot (Matrix Science UK), against the Uniprot_human database version 2011_05_03. Data were validated using Scaffold (Proteome Software).

**Gel filtration of the long peptide**

The lyophilized peptide was resuspended in 40% acetonitrile and the concentration was taken using Direct Detect system (Millipore). 5 µg of peptide diluted in PBS was then reduced and alkylated by incubation with 10 mM DTT for 1 h then 25 mM iodoacetamide for 20 min in the dark. The reduced sample along with unreduced was injected on a Superdex Peptide column using the Ettan system (GE Healthcare).

**Immunostaining of normal and MN kidney biopsy using rabbit α-PLA2R**

MN kidney and normal human kidney blocks were sectioned at 3-4 µm and baked for 20 minutes at 65°C. The automated Ventana BenchMark XT IHC / ISH Staining Module (Ventana Co., Tucson, AZ, USA) was used together with the XT Ultraview 3, 3' diaminobenzidine (DAB) version 3 detection system (Ventana Co.). Sections were firstly deparaffinised and incubated in EZPrep Volume Adjust (Ventana Co.). At intervals between steps the slides were washed with a TRIS-based reaction buffer, pH 7.6. A heat-induced antigen retrieval protocol (30 min) was carried out using a TRIS– ethylenediamine tetracetic acid (EDTA)–boric acid pH 8 buffer (Cell Conditioner 1). The sections were incubated with ultraviolet inhibitor blocking solution for 4 min, then with Rabbit anti-PLA2R (Sigma Aldrich, Poole) at 1:1,000 dilution or Rabbit anti-PLA2R (R38) at 1:2,700 dilution for 32 minutes at room temperature. This was followed by incubation with horseradish peroxidase-linked secondary antibody (8 min.), then DAB chromogen (8 min.), and copper for 4 min. Counterstain (haematoxylin II) was applied for 4 min before an incubation of 4 min with bluing reagent. Sections were mounted using PERTEX mounting media (Leica Biosystems, Nussloch, Germany) and imaged using the Nikon Eclipse E600 and Nikon Coolpix 995 (Nikon Instruments Inc, Melville, USA).
Immunofluorescent staining of human podocyte using rabbit α-PLA2R

Conditionally immortalized human podocytes\(^2\) were transiently transfected with full length PLA2R using Lipofectamine 2000 (Life Technologies). Podocytes were cultured at 33°C in RPMI-1640 medium with glutamine (R-8758; Sigma-Aldrich, St. Louis, MO) supplemented with 10% (vol/vol) FCS (Life Technologies) and 5% (vol/vol) insulin, transferrin, and selenium (1 ml/100 ml; I-1184; Sigma-Aldrich). Two days post transfection the cells were trypsinized then grown on coverslips. The cells were washed with PBS, fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.5% (vol/vol) Triton X-100 and blocked with 1% (wt/vol) BSA in PBS before incubation with primary antibodies (rabbit anti-PLA2R and mouse anti-Flag both used at dilution 1:200). Coverslips were mounted using ProLong Gold antifade reagent (Life Technologies). Images were acquired on a Delta Vision (Applied Precision) restoration microscope using a 60× objective. Raw images were deconvolved using the Softworx software.

Reference List


Supplemental Figure 1: Characterization of polyclonal rabbit antibody raised against the extracellular part of PLA2R (N-C8)*

Concentration analysis using SPR of rabbit serum in triplicate and three concentrations of 1/2,000, 1/1,000 and 1/500: 18 μM equivalent to 3 mg ml⁻¹

Affinity constant of the rabbit PLA2R antibody determined by SPR: $7.54 \times 10^{-11}$ M

Immunostaining of MN and normal kidney tissue by anti-PLA2R antibodies (Sigma and in house R38). Both anti-PLA2R antibodies specifically stain podocytes in normal kidney which is significantly increased in MN glomeruli with strong staining of the glomerular capillary loops

Immunofluorescent staining of human podocytes over-expressing Flag tagged full length PLA2R showing positivity on the membrane and around the nucleus

* The polyclonal rabbit antibody to PLA2R (N-C8) was produced using standard immunization procedure (Eurogentec)
Supplemental Figure 2: Inhibition ELISA (PLA2R-specific antibody binding to N-C8)

**Top panel**, Using an inhibition ELISA based on a variation of the standard ELISA for quantitating anti-PLA2R, anti-PLA2R positive sera from four patients are preincubated for two hours with a range of concentrations of NC-3 and then added to an ELISA plate coated with NC-8 and incubated for a further 2 hours. If all epitopes are shared by NC-3 and NC-8, then minimal antibody binding to the plate should be detected. Increasing concentrations of N-C3 in this assay resulted in increasing inhibition reaching ~90% inhibition to N-C8 using 9 µg ml⁻¹ of N-C3.

**Bottom panel**, Competitive ELISA screen of 43 PLA2R-positive patient sera on captured N-C8 inhibited by N-C3.
Purified N-C3 was trypsin digested under non-reducing conditions. The resulting peptides were separated out by charge (OFFGEL fractionation) and molecular weight. The fractions were resolved on a 12% BisTris SDS-PAGE gel in MOPS running buffer. Bands of interest corresponding to the reactive bands on the Western blot were excised (black boxes; white = control) and in-gel tryptic digestion was performed. The peptides were identified by mass spectrometry analysis.
Supplemental Figure 4: Affinity purification of IMN autoantibody

**Top panel,** elution profile of affinity purified human antibody to PLA2R. The purity of the eluted antibody was assessed by SDS-Page analysis and the concentration measured.

**Bottom panel,** single particle/negative staining TEM of the purified human PLA2R-specific antibody (peak 2).
Initial association rates of anti-PLA2R binding to immobilized N-C3 plotted as a standard curve used for calculation of the autoantibody concentrations in patient sera (Table 2).
Supplemental Figure 6: Estimation of the resolution of the 3D reconstructions of PLA2R N-C8

Fourier Shell Correlation (FSC) plot corresponding to the EM density map obtained for the N-C8 protein and showing a 20 Angstrom resolution.
Supplemental Figure 7: Transmission Electron Microscopy of N-C8 protein in complex with its autoantibody and a low resolution 3D structure of the complex

**Left panel,** Representative area micrographs of N-C8/antibody to PLA2R with highlighted images of single complex within white squares and two-dimensional projection averages of the images within the corresponding class. *Scale bar = 1,000 Å.*

**Right panel,** Best fit 3D model of the antibody-N-C8 complex. *Scale bar = 50 Å.*
Supplemental Figure 8: Western blot analysis of denatured N-C8 and N-C3 fragments under non-reducing and reducing conditions

Western blots (denatured condition)

Western blotting analysis of denatured N-C8 and N-C3 proteins under non-reducing and reducing conditions using a pool of five human sera. This confirms that the epitope to anti-PLA2R is sensitive to reduction.