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'CCCGCGGCCGCTCAATGGTGATGGTGATGGTGGTGATGATGGTGTCCGGACCCGGGGTGAAATGGCCATCTTTCCTCGTACTCAGC3' (Reverse). To generate PLA2R N-C2 (aa 20-515), the same N-C3 forward primer was used with the following reverse primer: 5'ATGGCGGCCGCTCAATGGTGATGGTGATGGTGGTGATGATGGTGTCCGGATCCTGACTCAGCGTCACTCAGCACGTGGCCGGC3'. The resulting PCR product were digested with NheI 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 serum[1] 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 $NaHCO_3$, 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:

$$y = Amin + \frac{(Amax - Amin)}{(1+\left(\frac{X}{x0}\right)^{h})^{s}}$$

Where *Amin* and *Amax* are the upper and lower asymmetry, X_0 is the concentration at half *Amax* 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 4-7containing 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 focussing 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 focussing; 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 dH_2O 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 Instrumnents 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.

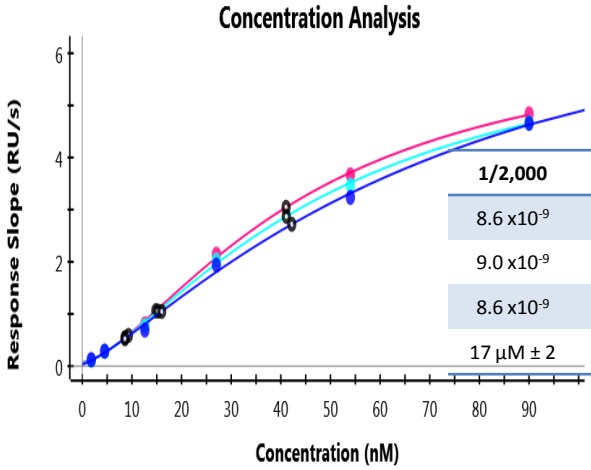
Reference List

1. Avrameas S, Ternynck T: The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry* 6:53-66, 1969

2. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P: A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 13:630-638, 2002

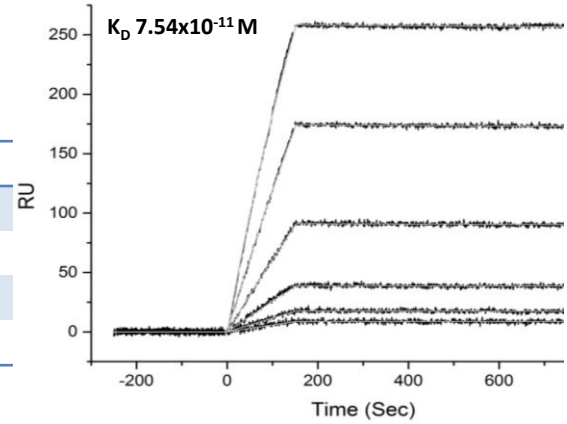
Supplemental Figure 1: Characterization of polyclonal rabbit antibody raised against the extracellular part of PLA2R (N-C8)*

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}

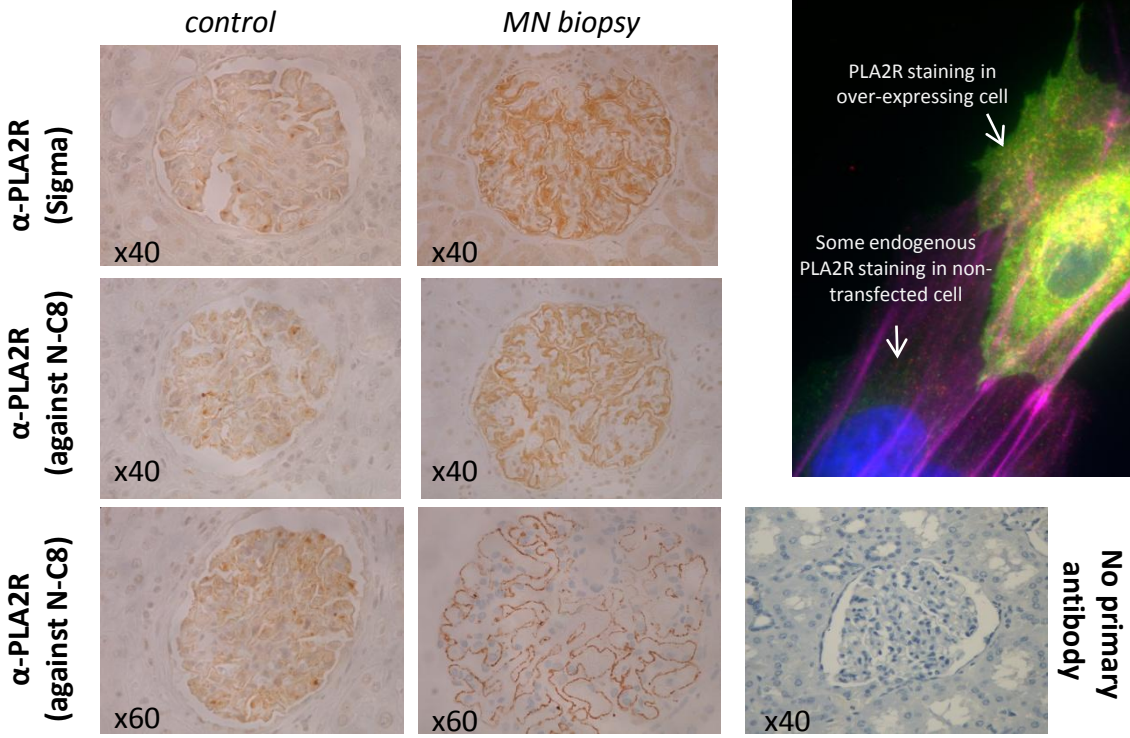


1/2,000	1/1,000	1/500
8.6 x10^{-9}	15 x10^{-9}	40 x10^{-9}
9.0 x10^{-9}	15 x10^{-9}	41 x10^{-9}
8.6 x10^{-9}	16 x10^{-9}	42 x10^{-9}
17 µM ± 2	18 µM ± 3	18 µM ± 3

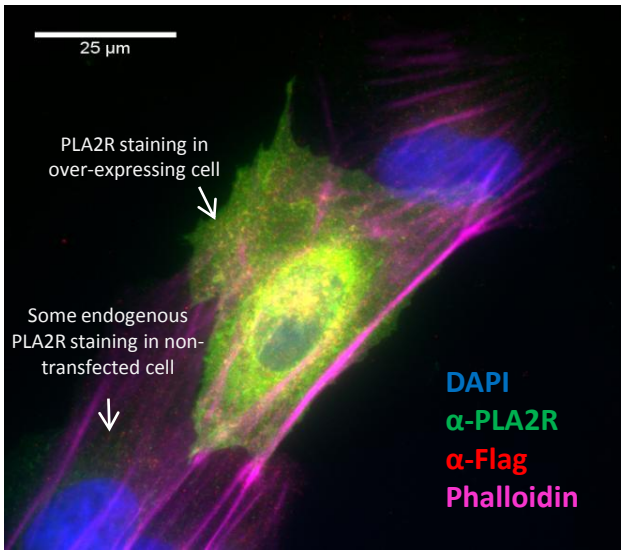
Affinity constant of the rabbit PLA2R antibody determined by SPR: **7.54x10^{-11} M**



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

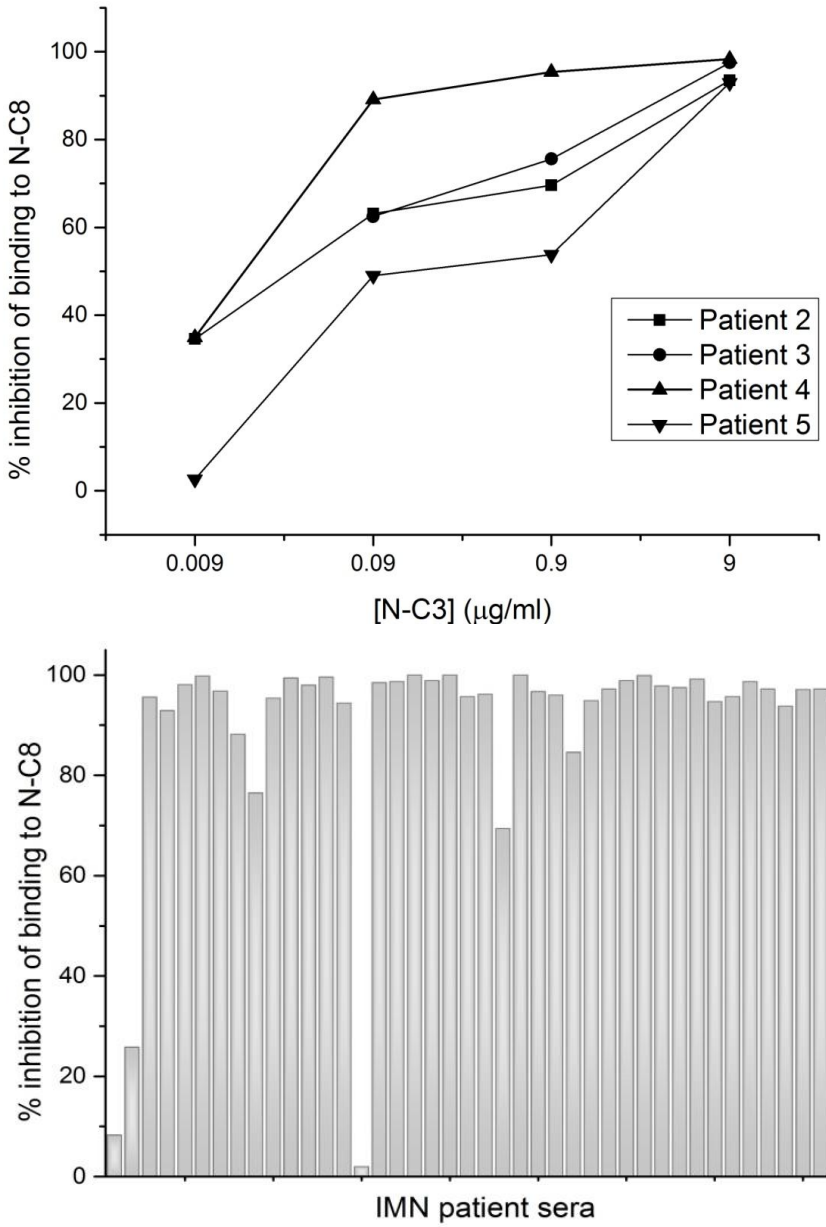


Immunofluorescent staining of human podocytes over-expressing Flag tagged full length PLA2R showing positivity on the membrane and around the nucleus



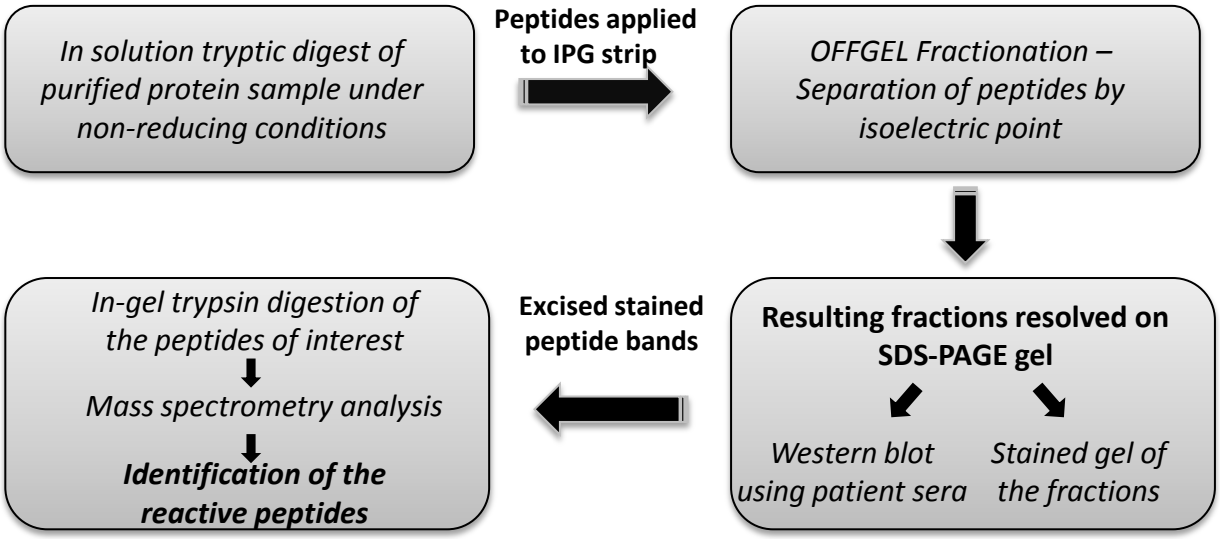
* The polyclonal rabbit antibody to PLA2R (N-C8) was produced using standard immunization procedure (Eurogentec)

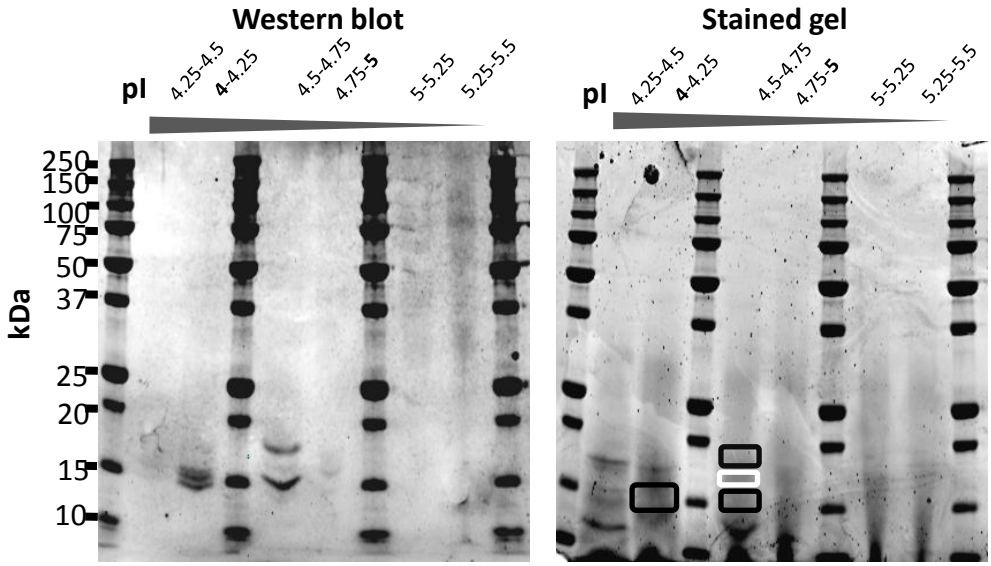
Supplemental Figure 2: Inhibition ELISA (PLA2R-specific antibody binding to N-C8)



Top panel, Using an inhibition ELISA based on a variation of the standard ELISA for quantitating anti-PLA2R[6], 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.

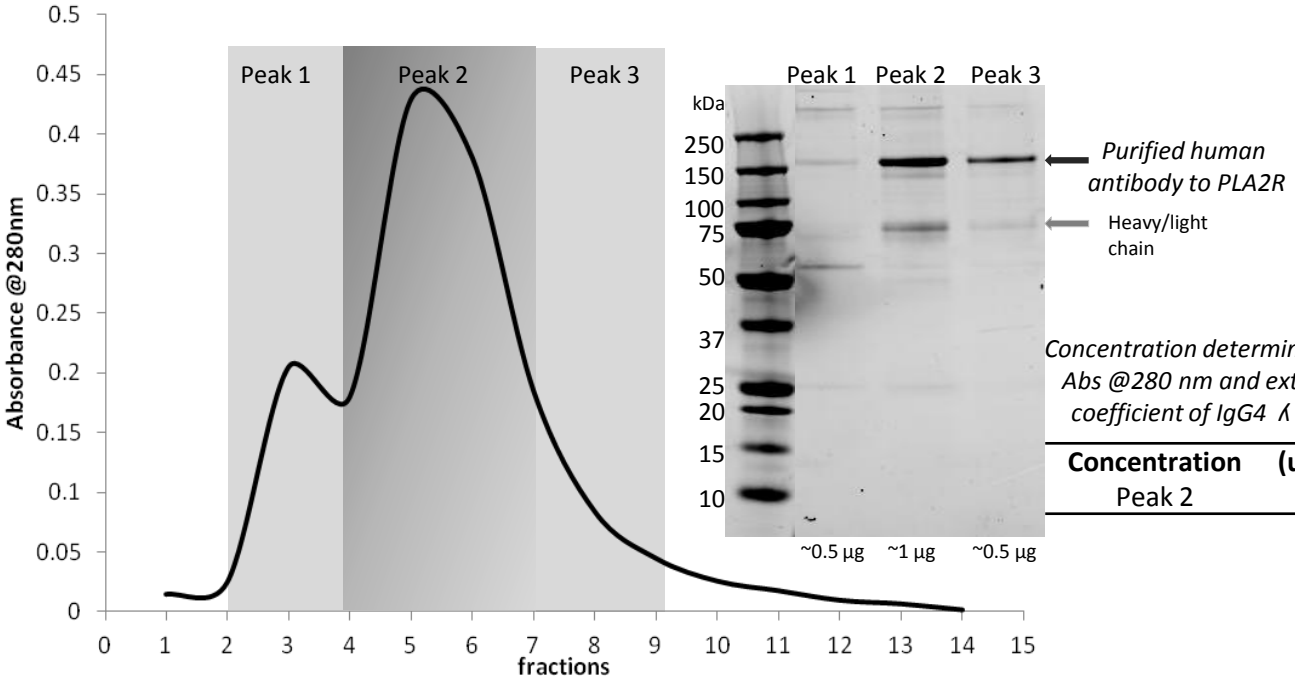
Supplemental Figure 3: Flow diagram showing experimental details and data analysis





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.

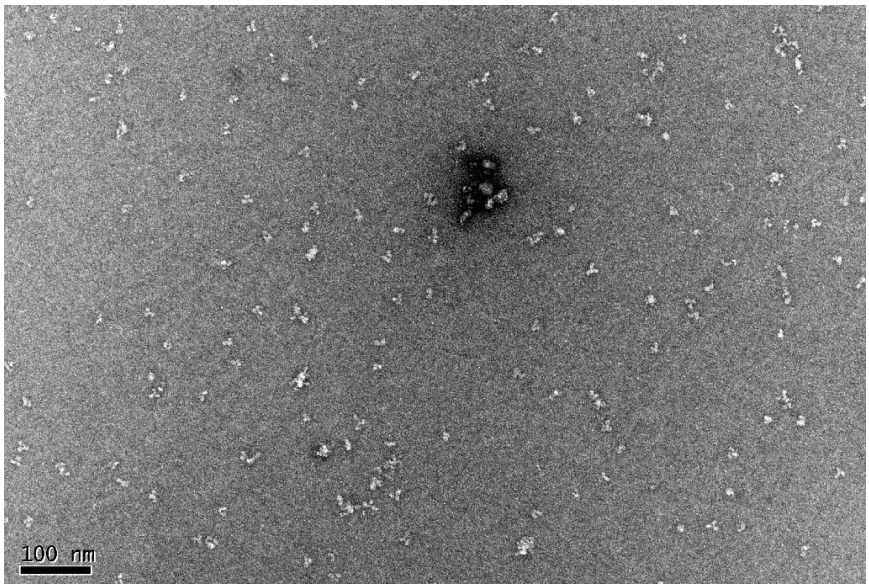
Supplemental Figure 4: Affinity purification of IMN autoantibody



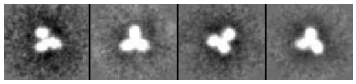
Concentration determined using Abs @280 nm and extinction coefficient of IgG4 ʎ = 1.36

Concentration	(ug ml^{-1})
Peak 2	212

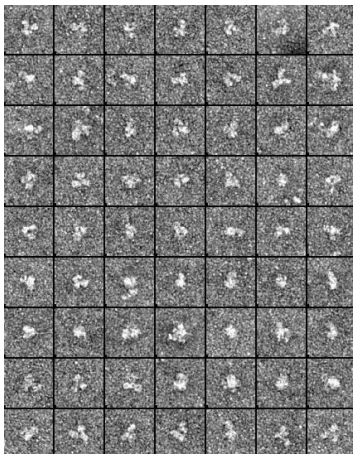
TEM field of particles



MSA reference free averages

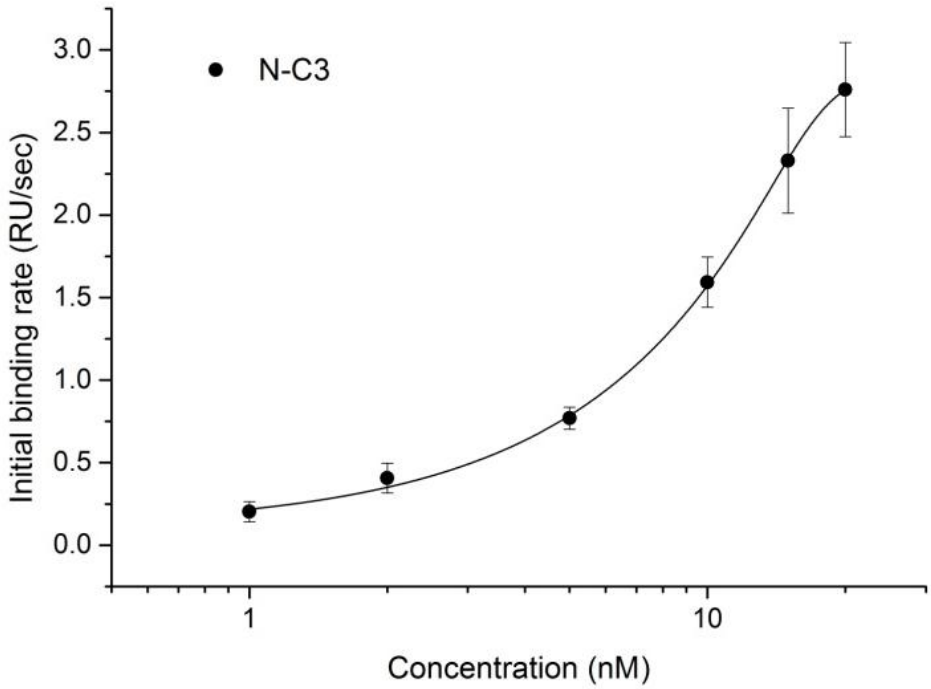


Selected particles



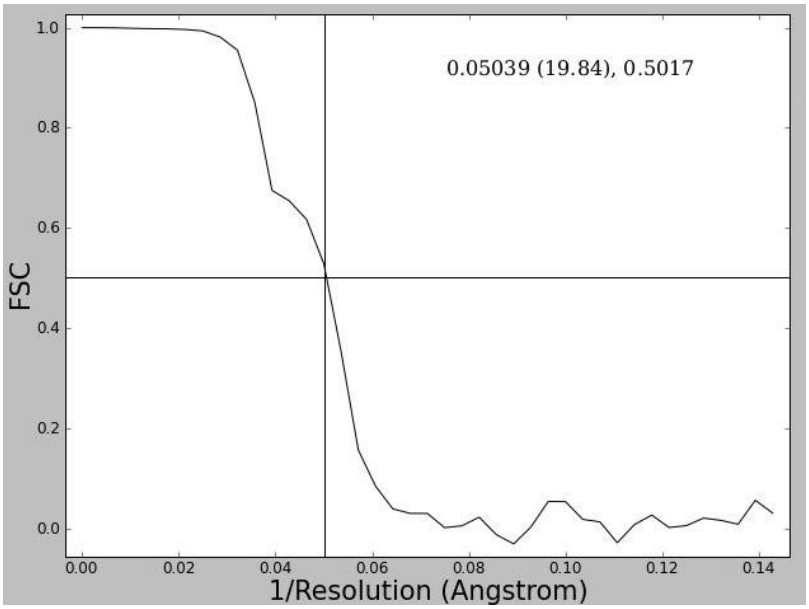
Top panel, elution profile of affinity purified human antibody to PLA2R. The purity of the eluted antibody was assessed by SDS-Page analysis and the concentration measured.
Bottom panel, single particle/negative staining TEM of the purified human PLA2R-specific antibody (peak 2).

Supplemental Figure 5: Serum anti-PLA2R concentration analysis



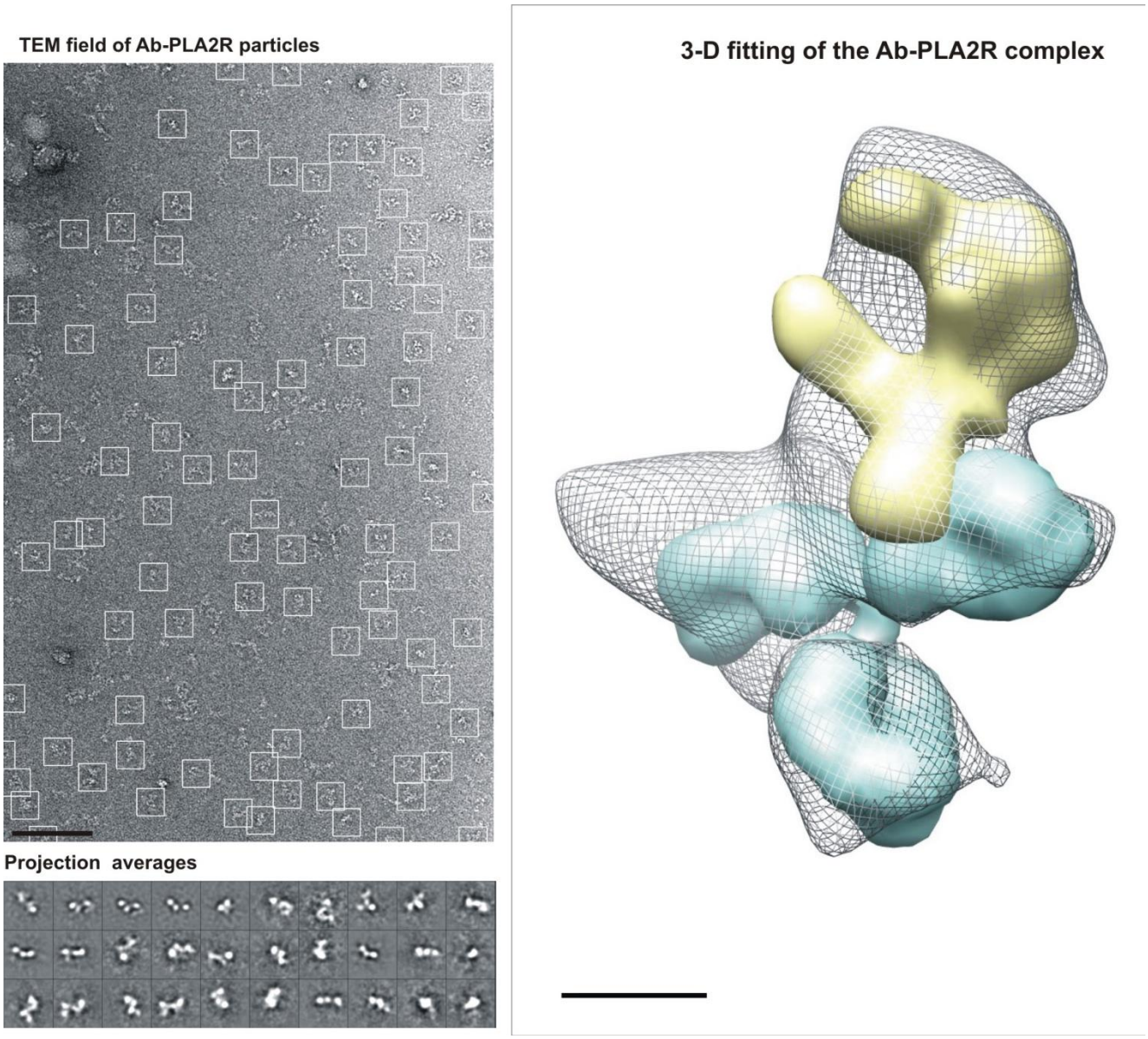
Initial association rates of anti-PLA2R binding to immobilized N-C3 plotted as a standard curve used for calculation of the autoantibody concentrations in patient sera (Table 2).

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.

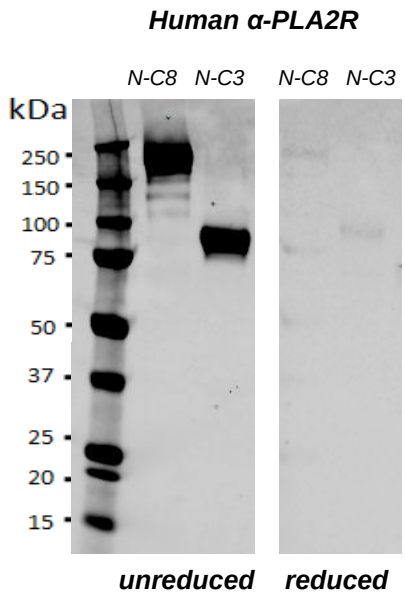
Supplemental Figure 7: Transmission Electron Microscopy of N-C8 protein in complex with its autoantibody and a low resolution 3D structure of the complex



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.