Epitope Spreading of Autoantibody Response to PLA2R Associates with Poor Prognosis in Membranous Nephropathy

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ABSTRACT

The phospholipase A2 receptor (PLA2R1) is the major autoantigen in idiopathic membranous nephropathy. However, the value of anti-PLA2R1 antibody titers in predicting patient outcomes is unknown. Here, we screened serum samples from 50 patients positive for PLA2R1 for immunoreactivity against a series of PLA2R1 deletion mutants covering the extracellular domains. We identified reactive epitopes in the cysteine-rich (CysR), C-type lectin domain 1 (CTLD1), and C-type lectin domain 7 (CTLD7) domains and confirmed the reactivity with soluble forms of each domain. We then used ELISAs to stratify 69 patients positive for PLA2R1 by serum reactivity to one or more of these domains: CysR (n=23), CysRC1 (n=14), and CysRC1C7 (n=32). Median ELISA titers measured using the full-length PLA2R1 antigens were not statistically different between subgroups. Patients with anti-CysR–restricted activity were younger (P=0.008), had less nephrotic range proteinuria (P=0.02), and exhibited a higher rate of spontaneous remission (P=0.03) and lower rates of renal failure progression (P=0.002) and ESRD (P=0.01) during follow-up. Overall, 31 of 69 patients had poor renal prognosis (urinary protein/creatinine ratio $>$4 g/g or eGFR$<$45 ml/min per 1.73 m^2 at end of follow-up). High anti-PLA2R1 activity and epitope spreading beyond the CysR epitope were independent risk factors of poor renal prognosis in multivariable Cox regression analysis. Epitope spreading during follow-up associated with disease worsening (n=3), whereas reverse spreading from a CysRC1C7 profile back to a CysR profile associated with favorable outcome (n=1). We conclude that analysis of the PLA2R1 epitope profile and spreading is a powerful tool for monitoring disease severity and stratifying patients by renal prognosis.

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Idiopathic membranous nephropathy (iMN) is an autoimmune disease and a common cause of nephrotic syndrome in adults.[1] Disease evolution is highly variable with spontaneous remission, persistent proteinuria or ESRD. In 2009, Beck *et al.* identified the M-type phospholipase A2 receptor (PLA2R1) as the major podocyte antigen in iMN.[2] The presence of anti-PLA2R1 autoantibodies has been widely confirmed in subsequent studies in 53%–80% of patients with iMN.[3–6] The pathogenic role of these autoantibodies is not yet proven, but anti-PLA2R1 antibody titers appear to correlate with disease activity in most study populations. However, the individual outcome prediction from anti-PLA2R1 titers is unclear. Indeed, while autoantibodies disappear in most patients during remission, either under symptomatic or immunosuppressive treatments,[4,7–9] they may persist during apparent clinical remission under renin-angiotensin system (RAS) blockade.[10] Furthermore, high anti-PLA2R1 antibody titers at presentation appear to correlate with subsequent poor renal outcome in most cases, but some patients with high autoantibody titers at onset exhibit a sharp decrease of anti-PLA2R1 activity and disease remission.[11,12]

The treatment of iMN is controversial.[13,14] Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend a supportive symptomatic treatment with blockers of the RAS and diuretics in all patients with iMN, and immunosuppressive therapy only in the case of renal function deterioration or persistent nephrotic syndrome.[15] Therefore, immunosuppressive treatments are often started only after significant and potentially irreversible complications. On the other hand, an unnecessarily early start of immunosuppression can be futile in patients who might develop remission with symptomatic treatments. Therefore, there is a need for better predictors of renal outcome in iMN.

PLA2R1 is a 180-kDa membrane receptor with a large extracellular region comprising 10 distinct globular domains of 7–17 kDa, namely a cysteine-rich domain (CysR), a fibronectin type II domain (FNII) and eight distinct C-type lectin domains (CTLD1–8).[16] Each domain is separated by a small linker sequence of about 10 amino acids.

Evidence for several epitopes in PLA2R1 targeted by anti-PLA2R1 antibodies was recently obtained from two studies.[17] Kao *et al.* first identified an immunodominant epitope in PLA2R1 which seems to be intertwined between the CysR, FNII, and CTLD1 domains of PLA2R1.[18] Going further, Fresquet *et al.* identified the CysR domain alone as a dominant epitope.[19] However, these studies were performed on a limited number of patients or pooled sera and none of them analyzed the link between the identified epitopes and disease activity.

Here, we first screened a cohort of 50 patients with iMN for their reactivity against 9 PLA2R1 mutants, successively deleting each of the 10 extracellular domains of the receptor. We identified epitopes in three distinct domains: CysR, CTLD1, and CTLD7. We then confirmed the independent reactivity of each domain by using numerous soluble forms of these domains by both Western blot and ELISA. We then set up epitope-specific ELISAs and stratified a cohort of 69 patients into three epitope-specific subgroups, and finally analyzed the relationships between their epitope profiles and disease activity.

RESULTS

Identification of Three Epitope Profiles

We first generated by site-directed mutagenesis a series of nine deletion mutants of PLA2R1, in which we successively deleted each domain from the N-terminal sequence, thus leaving the receptor membrane-bound (Figure 1A). We introduced in the cytoplasmic tail a small HA tag which allowed us to validate the expression of the recombinant proteins in HEK293 cells (Figure 1B). All PLA2R1 constructs were readily expressed except for Δ6, which was poorly detected with anti-HA antibodies, but was clearly detected by some patients (Figure 1E). We then screened sera from 50 patients with iMN with anti-PLA2R1 antibodies for their reactivity against the wild-type protein versus deletion mutants (Figure 1, C–E and Supplemental Figure 1). We chose to screen patients for the IgG4 anti-PLA2R1 subclass because many studies have shown that IgG4 is the predominant IgG subclass in iMN, correlating the most with disease activity.[8,20,21] Successive deletion of CysR, CTLD1, and CTLD7 led to the progressive loss of PLA2R1 recognition for 12, then 11 and 27 more patients, identifying three epitope profiles that likely correspond to three distinct epitopes in each of these domains (Figure 1, C–E).

CysR and CTLD1 Domains Contain Distinct Epitopes Recognized By Two Different Anti-PLA2R1 Autoantibodies

Fresquet *et al.* recently described that the CysR domain alone contains an anti-PLA2R1 epitope, while Kao *et al.* suggested that one or several epitopes are intertwined between CysR and CTLD1 domains.[18,19] Our above data rather suggest the presence of two independent epitopes in the CysR and CTLD1 domains. To confirm this hypothesis, we designed four constructs driving the expression of HA-tagged soluble forms of CysR and CTLD1 domains alone, in either HEK293 cells or *Escherichia coli* (Figure 2A). We also prepared the two constructs described by Kao *et al.* in which the three domains CysR, FNII, and CTLD1 are expressed together (triple domain), with insertion of a thrombin protease site either at the linker region between CysR and FNII or between FNII and CTLD1[18] (Figure 2A). Expression of CTLD1 alone from an expression plasmid coding for the genuine human cDNA sequence in HEK293 cells was very low (not shown), consistent with a previous observation from Kao *et al.*[18] However, expression of CTLD1 could be obtained from a human codon–optimized expression vector coding for CTLD1 with a 3xFlag tag at the N-terminus. On the other hand, the CysR domain alone was expressed in HEK293 cells at low but significant levels (Figure 2B), consistent with data from Fresquet *et al.*[19]

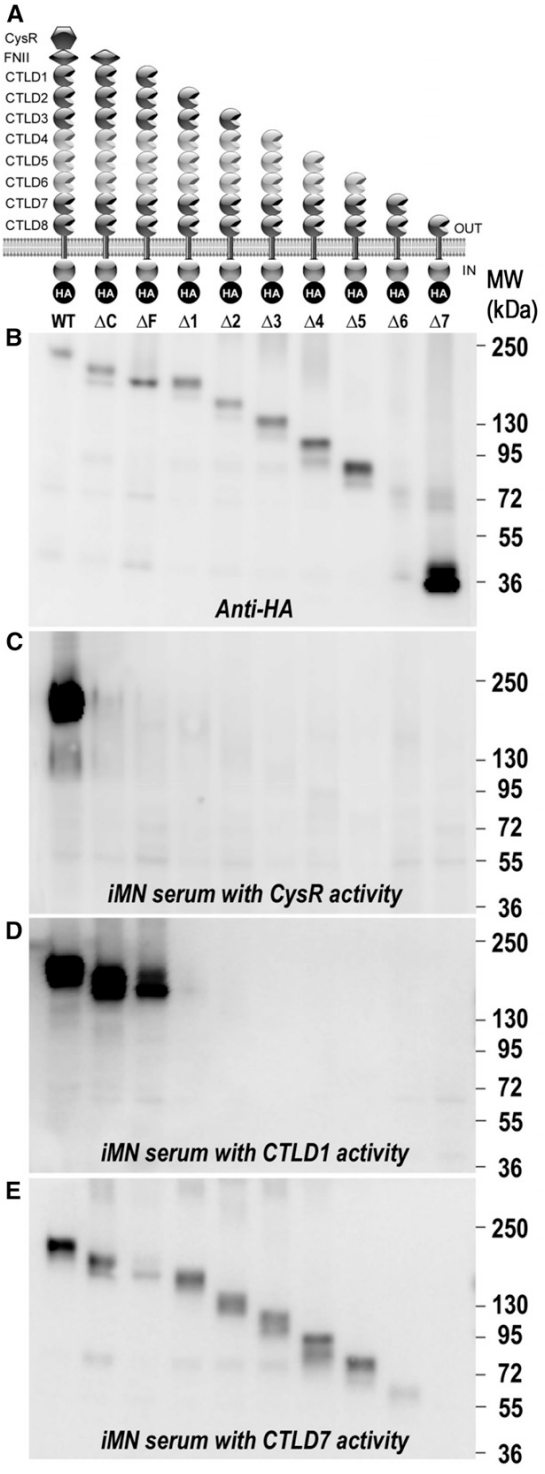

Figure 1. Design and expression of a series of 9 PLA2R1 deletion mutants and representative epitope profiles of sera from a cohort of 50 iMN patients with anti-PLA2R1 antibodies. (A) Schematic diagram of C-terminally HA-tagged membrane-bound PLA2R1 deletion mutants. See Concise Methods for the precise location of deletions. (B) Validation of expression of HA-tagged human PLA2R1 deletion mutants in HEK293 cells using anti-HA antibody. (C–E) Representative results from the screening of 50 patients with iMN, showing 3 patients exhibiting distinct epitope profiles: 12 patients with iMN did not recognize the ΔC mutant missing the N-terminal CysR domain of PLA2R1, indicating the presence of antibodies targeting CysR (C); 11 patients did not recognize the Δ1 mutant missing CTLD1, indicating the presence of at least antibodies targeting CTLD1 (D), 27 patients did not recognize the Δ7 mutant missing CTLD7 (E), indicating the presence of antibodies at least targeting CTLD7.

To circumvent the weak expression of the two single domains in HEK293 cells, we produced them in *E. coli* as HA-tagged DsbC fusion proteins (Figure 2A) and purified soluble and folded CysR and CTLD1 proteins (see Concise Methods). We could then easily detect the proteins by Western blot under both reducing and nonreducing conditions with anti-HA antibodies (Figure 2B). As for the two constructs from Kao *et al.*, only the first construct with a thrombin site between CysR and FNII was well expressed in HEK293 cells and could be detected with anti-HA before and after cleavage with thrombin (Figure 2B). Of importance, the band labeled with anti-HA after cleavage corresponds to the cleaved FNII-CTLD1-HA domain and not the cleaved CysR domain, which has no HA tag. In summary, five constructs could be obtained and validated by Western blot with anti-HA antibodies: the CTLD1 and CysR domains alone and the CysR-FNII-CTLD1 triple domain expressed in HEK293 cells, as well as the CysR and CTLD1 domains expressed as single domains in *E. coli* (Figure 2B). We then tested sera from patient 1 with a CysR profile and from patients 2 and 3 with a CTLD1 profile (Figure 1) against these constructs, with the triple domain cleaved or not by thrombin. Serum from patient 1 could only recognize constructs containing the CysR domain, and more strongly when expressed as a DsbC fusion protein or as a triple domain than after cleavage with thrombin (lane 4 of Figure 2B) or when the CysR-HA domain is expressed alone (lane 1 of Figure 2B). In fact, the CysR-HA domain could be detected with the anti-HA antibody and patient's serum under nonreducing conditions only after optimization of the protein transfer conditions (see Concise Methods), likely because the folded CysR domain is not hydrophobic enough by itself to properly transfer to Western blot polyvinylidene difluoride membranes in standard Western blot conditions. On the other hand, serum from patients 2 and 3 could recognize constructs containing both the CTLD1 domain and the CysR domains expressed alone as DsbC fusion proteins or as a triple domain, and cleaved or not with thrombin. Interestingly, the relative signals against the two domains were different, likely because of different titers for each anti-CysR and anti-CTLD1 autoantibodies in the two patients' sera.

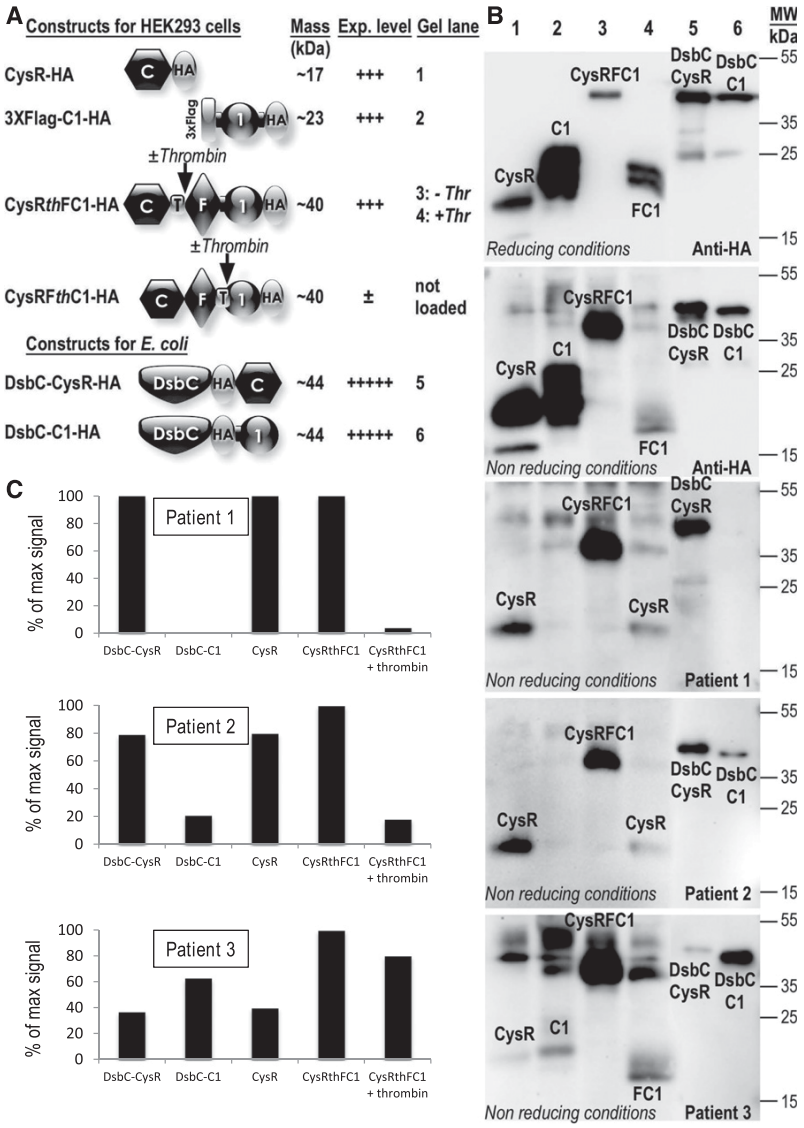

Figure 2. Expression of various soluble forms of CysR and CTLD1 domains and reactivity of iMN sera from three patients against two distinct and independent epitopes. (A) Schematic diagram of HA-tagged soluble constructs designed for expression in HEK293 cells or *E. coli*: single CysR and CTLD1 domains, CysR-FNII-CTLD1 triple domains with a thrombin cleavage site inserted either between CysR and FNII or FNII and CTLD1, CysR and CTLD1-HA-DsbC fusion proteins. (B) Validation of expression of HA-tagged domains expressed in HEK293 cells (cleaved or not with thrombin for the triple domain CysRthFC1-HA) and *E. coli* using anti-HA antibody (reducing and nonreducing conditions) and reactivity of the above constructs by patients with a CysR profile (patient 1) or a CTLD1 profile (patients 2 and 3) in Western blot (nonreducing conditions). The construct CysRFthC1-HA was poorly expressed and is not shown. (C) Reactivity of patients 1, 2 and 3 against the CysR and CTLD1 constructs in ELISA with affinity-captured soluble forms of PLA2R1 on immobilized anti-HA antibodies. Data correlated well with the epitope profiling shown in Figure 1, C and D for these patients.

To confirm these results, we set up an ELISA in which we took advantage of the HA-tag present in all antigens to affinity capture them into wells precoated with anti-HA antibody (Figure 2C). No signal was obtained when anti-HA or antigens were omitted in all conditions (not shown). The ELISA data were in perfect accordance with the Western blots and were much more sensitive and quantitative. As expected, patient 1 recognized the CysR and the CysR-FNII-CTLD1 triple domain not cleaved with thrombin, but neither the CTLD1 domain expressed alone nor the CysR-FNII-CTLD1 triple domain cleaved by thrombin, which generates a free CysR domain that cannot bind to the anti-HA–coated well and is washed away while the corresponding FNII-CTLD1-HA domain will bind to the well. We inferred from the Western blots that sera from patients 2 and 3 have two different autoantibodies directed against different epitopes in the CysR and CTLD1 domains, with likely higher titer to CysR for patient 2 and a higher titer to CTLD1 for patient 3. The ELISA data clearly confirmed this view, with higher signal on CysR than CTLD1 and no signal on the triple domain after thrombin cleavage for patient 2, and results in mirror for patient 3.

To further demonstrate the presence of two different autoantibodies targeting CysR or CTLD1, we performed (*1*) depletion experiments in which we preabsorbed the serum of patient 2 (containing CysR and CTLD1 autoantibodies) onto anti-HA beads loaded with either CysR or CTLD1 domains and analyzed the flow-through fraction for reactivity in western blot loaded with CysR and CTLD1 domains; and (*2*) competition experiments in which we preincubated the serum of patient 2 with an excess of CysR or CTLD1 domains from *E. coli* and then probed the serum in a Western blot loaded with CysR and CTLD1 domains. The results of these experiments are shown in Supplemental Figure 2. Finally, we performed competition experiments with the CysR, CTLD1 and CTLD6–7 domains expressed in *E. coli* against full-length PLA2R1 by ELISA and observed data in full accordance with the above results (Supplemental Figure 3).

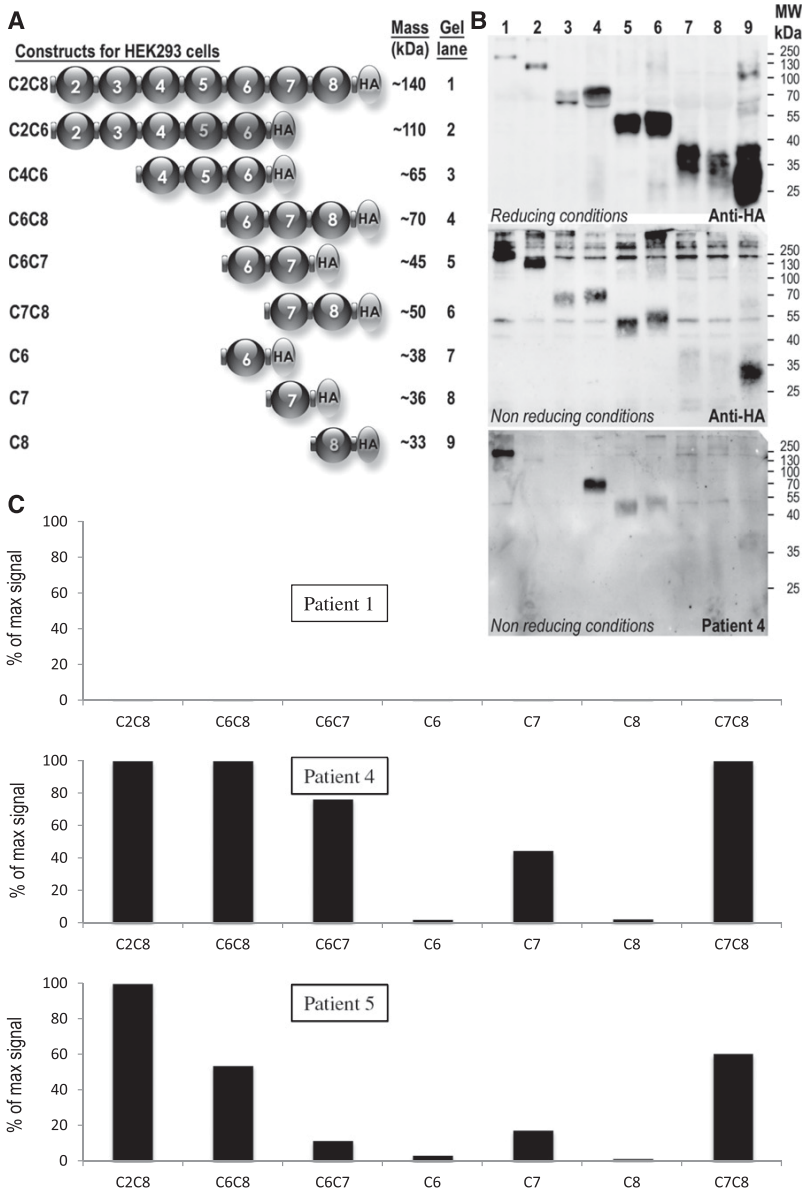

Figure 3. Expression of various soluble forms of PLA2R1 from the distal extracellular region and recognition of CTLD7 by two patients with iMN. (A) Schematic diagram of various soluble HA-tagged constructs designed for expression in HEK293 cells. (B) Validation of expression of HA-tagged soluble forms in HEK293 cells using anti-HA antibody under reducing and nonreducing conditions (top two panels), and reactivity of these mutants in Western blot by a patient with a CTLD7 profile under nonreducing conditions (bottom panel). (C) Reactivity of patient 1 with a CysR profile (Figure 1C) and of patients 4 and 5 with a CTLD7 profile (Figure 1, D and E) in ELISA with affinity-captured soluble forms of PLA2R1 on immobilized anti-HA antibodies. All constructs containing CTLD7 were recognized by patients 4 and 5 with a CTLD7 profile but not patient 1 with a CysR profile.

The CysR antigen could fully block the ELISA signal for sera of two patients with a CysR profile but only partially for sera from two other patients with a CTLD1 profile. In line with this, competition with the CTLD1 antigen did not inhibit the signal for the CysR sera but partially inhibited that of the CTLD1 sera, while the combination of the two antigens fully inhibited the PLA2R1 signal. Finally, addition of CTLD6–7 had no effect by itself, demonstrating that this antigen cannot interfere with the interaction of anti-PLA2R1 antibodies targeting the CysR and CTLD1 domains.

Together, this series of experiments clearly demonstrates the presence of two autoantibodies, with one recognizing CysR and the other recognizing CTLD1. The experiments also very clearly show that CysR and CTLD1 domains exhibit distinct epitopes which can be recognized independently by the two different autoantibodies, the latter being present at different titers in different patients' sera. We also produced the DsbC-HA-CTLD1 domains with the four possible combinations of single nucleotide polymorphisms (SNPs) described previously[22] and did not observe a difference in signal with sera from two patients with either CTLD1 or CTLD7 profiles (Supplemental Figure 4). We also performed Western blots under reducing versus nonreducing conditions and demonstrate that the reactivity of anti-PLA2R1 antibodies toward CysR and CTLD1 is dependent on the disulfide bonds present in these domains (Supplemental Figure 4).

CTLD7 Contains a Third Epitope Recognized By Anti-PLA2R1 Antibodies

To confirm the results from Figure 1 suggesting a third, more distal epitope in the PLA2R1 extracellular region, and to also demonstrate that this epitope is independent of the other domains of PLA2R1, we designed a series of soluble forms of PLA2R1 deleted from the CysR, FNII, and CTLD1 domains. We prepared a total of nine constructs converging toward CTLD7 which was hypothesized to contain the third epitope: CTLD2 to CTLD8 (C2C8), CTLD2 to CTLD6 (C2C6), CTLD4 to CTLD6 (C4C6), CTLD6 to CTLD8 (C6C8), CTLD6 to CTLD7 (C6C7), CTLD7 to CTLD8 (C7C8), CTLD6 (C6), CTLD7 (C7), and CTLD8 (C8). All these constructs had a HA-tag (Figure 3A) and we could confirm their expression in HEK293 cells by western blot with anti-HA

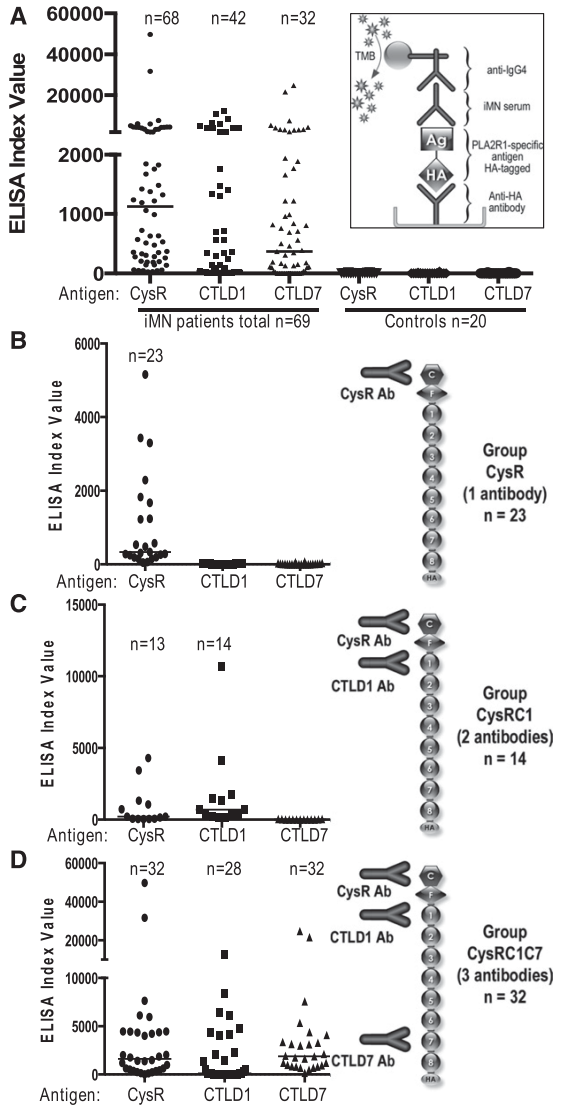

Figure 4. Validation of three epitope-specific ELISAs using CysR, CTLD1 and CTLD7 domains and reactivity of PLA2R1-positive patients with iMN. (A) ELISAs were validated with sera from 69 PLA2R1-positive patients with iMN versus 20 PLA2R1-negative controls (healthy donors and other diseases). CysR, CTLD1 and CTLD7 antigens were adsorbed by affinity capture on ELISA plates coated with anti-HA antibody (see inset). Titers are expressed as ELISA Index values (see Concise Methods). The bars indicate median values. Among the 69 patients with iMN, 68 were positive for IgG4 anti-PLA2R1 on the CysR domain, 42 on CTLD1, and 32 on CTLD7 domain. All 20 PLA2R1-negative sera were negative. (B–D) Definition of three epitope groups based on reactivity. (B) CysR group (n=23): sera from these patients contained antibodies targeting only the CysR domain. (C) CysRC1 group (n=14): 13 patients had two antibodies targeting CysR and CTLD1 domains but one patient had antibodies targeting only CTLD1 (see also Supplemental Figure 5). (D) CysRC1C7 group (n=32): 28 patients had three antibodies targeting the three domains but 4 patients had two antibodies targeting CysR and CTLD7 (see also Supplemental Figure 5).

antibody under reducing conditions (Figure 3B). However, as discussed above for the CysR and CTLD1 domains, the detection with anti-HA under nonreducing conditions was more difficult for the short soluble forms narrowing down to CTLD7, likely because the CTLD7 and its neighboring domains are highly polar (probably due to the high glycosylation as evidenced from the migration of the soluble forms) and do not transfer well under nonreducing conditions, even under optimized protein transfer conditions (see Concise Methods). This view likely explains the fact that patient 4, who had a CTLD7 profile (Figure 1), could recognize clearly the C2C8 and C6C8 soluble forms, less efficiently the C6C7 and C7C8 soluble forms, but not the single construct C7 (Figure 3B). The epitope was not present in CTLD6 because the constructs C2C6 and C4C6 were not labeled, nor in CTLD8, because this construct could be labeled with anti-HA but not serum from patient 4. Furthermore, the fact that this patient recognizes C2C8 and C6C8 which are devoid of CysR and CTLD1 domains clearly demonstrates that the C6C8, C6C7 or C7C8 constructs contain a third anti-PLA2R1 epitope independent of CysR and CTLD1, which is likely in the CTLD7 domain. To further demonstrate the presence of an epitope within CTLD7, we probed the different soluble constructs using the anti-HA ELISA assay as performed above for CysR and CTLD1. We observed that all the constructs containing a CTLD7 domain and even CTLD7 alone were well recognized by the sera from patients 4 and 5 with a CTLD7 profile (Figure 3C). As a negative control, we used serum from patient 1 that as expected did not recognize any of the constructs (Figure 3C). Together, these data demonstrate the presence of a third independent epitope in CTLD7 with a corresponding autoantibody only present in sera from patients with a CTLD7 profile (Figure 1). As above for CysR and CTLD1, we performed Western blots under reducing versus nonreducing conditions and demonstrated that the reactivity of anti-PLA2R1 antibodies toward CTLD7 is dependent on the disulfide bonds present in this domain (Supplemental Figure 4).

Set-up of Three Specific ELISAs with CysR, CTLD1 and CTLD7 as Antigens

Based on the above results, we set up three ELISAs to specifically measure autoantibodies targeting the CysR, CTLD1, and CTLD7 domains. We used as antigens the CysR domain produced in HEK293 cells, the CTLD1 domain produced in *E. coli* and the CTLD6–7 construct produced in HEK293 cells, as this latter was easier to produce in large scale than CTLD7 alone. As above, the two antigenic domains produced in HEK293 cells were HA-affinity captured on ELISA plates precoated with anti-HA antibody while the CTLD1 domain from *E. coli* could be directly coated or captured with anti-HA.

Table 1. Clinical characteristics of all patients enrolled in this study (*n*=69)

Clinical Characteristics	Value
Age at diagnosis (years)	55±15
Gender (male/female)	54/15
Proteinuria at diagnosis (g/g)	5.0 (1.4–24.0)
Serum creatinine at diagnosis (μmol/l)	99 (43–385)
Months between diagnosis and PLA2R1 assay	0 (0–100)
Anti-PLA2R1 titer (ELISA Index value)	3241 (210–50,817)
Proteinuria at PLA2R1 assay (g/g)	4.0 (0.3–24.0)
Serum creatinine at PLA2R1 assay (μmol/l)	94 (43–600)
Patients treated before PLA2R1 assay[a]	6 (9%)
Patients treated during follow-up[a]	41 (59%)
LOCF[b] (months)	36 (12–216)
Proteinuria at LOCF (g/g)	2.0 (0.0–20.0)
Serum creatinine at LOCF (μmol/l)	119 (48–926)
Spontaneous remission[c]	18 (26%)
Poor prognosis at LOCF[d]	31 (45%)
Hemodialysis[d]	12 (17%)

Normal values are mean±SD; non-normal values are median (ranges); qualitative values are number (%).
[a]Treated with immunosuppressive treatment. All patients received symptomatic treatment.
[b]LOCF, last observation carried forward.
[c]Spontaneous remission is defined by remission induced by symptomatic treatment (RAS blockers and diuretics) without immunosuppressive treatment.
[d]Poor renal prognosis at LOCF is defined by proteinuria >4 g/g and/or serum creatinine increased over 30% and/or eGFR<45 ml/min per 1.73 m^2. Patients with ESRD on hemodialysis.

Adsorbed antigens were then incubated with patients' sera followed by detection with a secondary anti-IgG4 antibody coupled to peroxidase (Figure 4A, inset). We validated the three ELISAs and measured the epitope-specific titers using a cohort of 69 patients with iMN positive for PLA2R1 (Table 1) versus 20 healthy donors. The cohort of 69 patients with iMN comprised the 50 sera screened in Figure 1 and 19 additional cases. All patients were clinically well characterized and positive for anti-PLA2R1 by ELISA using full-length PLA2R1 as antigen (Table 1). Most patients had nephrotic range proteinuria (urinary protein/creatinine ratio (UPCR over 3.5 g/g) at diagnosis (74%) and were naive of immunosuppressive treatment (91%) at the time of PLA2R1 assays by ELISA and Western blot. Patients had a mean follow-up of 36 months from PLA2R1 assays. All patients received symptomatic treatment. Eighteen patients entered into remission under RAS blockade. Thirty-six patients received an immunosuppressive treatment and 21 entered into remission. We analyzed their renal outcome and defined a poor renal prognosis according to KDIGO as persistence of UPCR >4.0 g/g and/or serum creatinine increase over 30% and/or eGFR (using the simplified modified diet in renal disease formula) <45 ml/min per 1.73 m^2 at last observation carried forward (LOCF). Thirty-one patients had a poor renal outcome at the end of the follow-up including 12 patients with ESRD. We also analyzed renal survival 24 months after anti-PLA2R1 assay and defined an event by an increase over 30% of serum creatinine from baseline (Table 1).

All but one sera (*n*=68, see below) recognized the CysR domain by ELISA and detection with IgG4, indicating that CysR is the dominant and common epitope in most patients (Figure 4A). Forty-two sera recognized the CTLD1 domain and 32 sera recognized the CTLD7 domain (Figure 4A). We then classified patients into three groups based on their ELISA

Table 2. Comparison of clinical characteristics and follow-up of patients stratified according to their epitope profiles[a]

Patients[b]	Group CysR (*n*=23)	Group CysRC1 (*n*=14)	Group CysRC1C7 (*n*=32)	*P* Value[c]
Age at diagnosis (years)	48±12	54±16	61±15	0.008
Gender (male/female)	19/4	10/4	25/7	ns
Proteinuria at diagnosis (g/g)	3.7 (2.0–10.8)	4.7 (1.4–15.0)	6.0 (2.5–24.0)	ns
Serum creatinine at diagnosis (μmol/l)	93 (54–134)	96 (43–150)	103 (59–385)	ns
Months between diagnosis and PLA2R1 assay	0 (0–36)	1 (0–84)	0 (0–100)	ns
Anti-PLA2R1 assay (ELISA Index value)[a]	3175 (394–8043)	1625 (367–5947)	4288 (210–50,817)	ns
Proteinuria at PLA2R1 assay (g/g)	3.0 (0.3–5.1)	3.0 (0.8–10.6)	5.0 (0.3–24.0)	0.02
Serum creatinine at PLA2R1 assay (μmol/l)	92 (45–149)	96 (43–280)	100 (59–926)	ns
Patients treated before PLA2R1 assay[d]	3/23	2/14	1/32	ns
Patients treated during follow-up[d]	12/23	8/14	21/32	ns
LOCF[e] (months)	36 (12–201)	44 (12–133)	33 (12–216)	ns
Proteinuria at LOCF (g/g)	0.6 (0.0–5.0)	2.0 (0.0–10.6)	5.0 (1.0–20.0)	0.01
Serum creatinine at LOCF (μmol/l)	89 (55–181)	130 (57–297)	157 (48–600)	ns
Spontaneous remission[f]	10/23 43%	4/14 29%	4/32 12%	0.03
Hemodialysis[g]	0/23 (0%)	2/14 (14%)	10/32 (31%)	0.01

[a]Epitope profile and anti-PLA2R1 ELISA assays were performed on the same serum sample.
[b]Normal values are mean ±SD; non normal values are median (ranges); qualitative values are number (%).
[c]ANOVA or Kruskal–Wallis tests for continuous (for Gaussian or non-Gaussian distribution, respectively) variables and chi-squared or Fisher's exact tests for categorical variables. NS, not significant: *P* value >0.05.
[d]Treated with immunosuppressors. All patients received symptomatic treatment.
[e]LOCF, last observation carried forward.
[f]Spontaneous remission is defined by remission induced by symptomatic treatment (RAS blockers and diuretics) without immunosuppressive treatment.
[g]Patients with ESRD on hemodialysis.

pattern. Twenty-three patients were classified in the CysR group as they had only antibodies targeting the CysR domain (Figure 4B). Fourteen patients were classified in the CysRC1 group, with 13 having activities against both the CysR and CTLD1 domains, and one having antibodies only against CTLD1 (Figure 4C). However, the serum from this latter patient was found to contain IgG1 but not IgG4 anti-CysR antibodies (Supplemental Figure 5), further indicating that CysR is the dominant epitope. Finally, thirty-two patients were classified into the CysRC1C7 group, with 28 having antibodies targeting the three domains CysR, CTLD1 and CTLD7 and 4 patients targeting CysR, CTLD7 but not CTLD1 (Figure 4D). We screened three of these four latter patients (serum was no longer available for one patient) for IgG subclasses of anti-CysR and anti-CTLD1 and found one patient positive for IgG3 anti-CTLD1 (Supplemental Figure 5). When considering the 50 patients initially screened by Western blot (Figure 1), we observed a perfect concordance between the ELISA data and their Western blot epitope profiles (Supplemental Figure 1). Finally, we observed that the CysR titer was significantly higher in the CysRC1 and CysRC1C7 groups than in the CysR group (Supplemental Figure 6). On the other hand, no difference was observed for the CTLD1 titer between CysRC1 and CysRC1C7 groups (Supplemental Figure 6). Together, the above findings using ELISA and IgG4 detection stratify our cohort of 69 patients into three major groups: CysR, CysRC1, and CysRC1C7. Five patients showed intermediate epitope patterns that can be reminiscent of one of the above groups, as shown by the presence of epitope-specific autoantibodies from other IgG subclasses. Nonetheless, an important result of this study is that all patients are positive for the CysR epitope but not for CTLD1 and CTLD7, showing that CysR is a primary dominant epitope while CTLD1 and CTLD7 are likely the result of epitope spreading in PLA2R1 for some patients.

Clinical Characteristics of Patients with the Three Epitope Profiles

We compared the clinical characteristics of patients from the three groups: CysR, CysRC1 and CysRC1C7 (Table 2). There was no statistically significant difference between the three groups (including the five patients with intermediate epitope patterns) for sex, number of patients with immunosuppressive treatment and titers of anti-PLA2R1 antibodies measured by ELISA using full-length PLA2R1. In contrast, we observed a significant difference for age: patients from the CysR group were significantly younger than patients from CysRC1 and CysRC1C7 groups (P=0.008; using the ordinary one-way ANOVA test). The mean age at presentation was 48 years for the CysR group, 54 years for the CysRC1 group, and 61 years for the CysRC1C7 group (Figure 5).

Proteinuria at the time of PLA2R1 testing was significantly lower in the CysR group (Table 2, P=0.02 using the Kruskal–Wallis test). We then classified patients based on their UPCR at serum sampling into three subgroups: below 3 g/g (n=22), between 3 and 5 g/g (n=22) and over 5 g/g (n=25), and compared their ELISA titers measured with full-length PLA2R1, CysR, CTLD1, and CTLD7 antigens (Figure 6). Only anti-CTLD7 titers were significantly higher in the group with UPCR over 5 g/g compared with the two other groups (P=0.006 using the Kruskal–Wallis test). Furthermore, we observed a progressive increase of CTLD7 titer with increasing proteinuria (Figure 6).

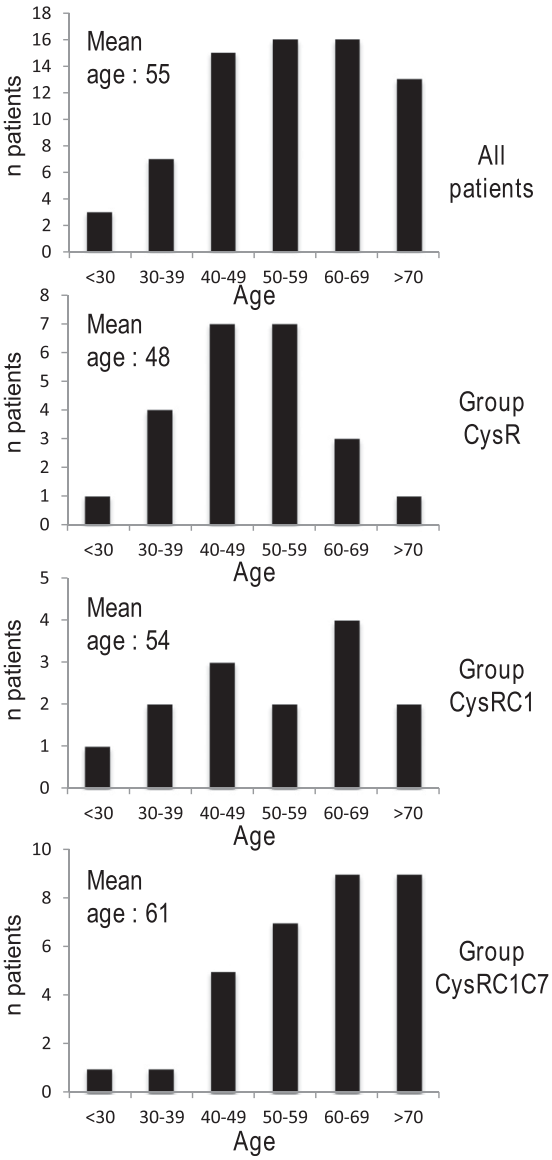

Figure 5. Age distribution at presentation in the whole cohort and in the different epitope groups. The mean age of the CysR group (48 years) is lower than those of CysRC1 (57 years) and CysRC1C7 (61 years) groups. The mean age of the full cohort is 55 years.

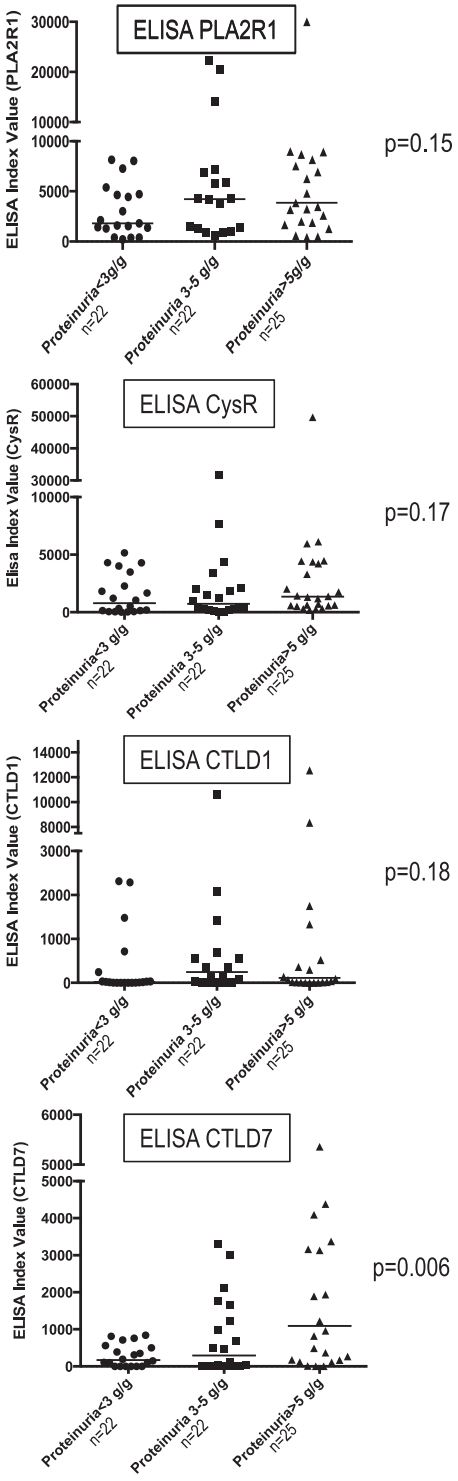

Figure 6. Levels of anti-PLA2R1 antibodies targeting CysR, CTLD1 and CTLD7 domains in patients with different proteinuria. Only anti-CTLD7 titers were significantly different between the three groups of patients with different proteinuria. Titers are expressed as ELISA index values (see methods). The bars indicate median values. $P<0.05$ was considered to be significant.

Evolution of Patients in the Three Epitope Groups

In our cohort, all patients received a symptomatic treatment with RAS blockade and/or diuretics, and 41 patients received an immunosuppressive treatment (59%). The use of immunosuppressive treatments and the median follow-up were not different between the three epitope groups (P=0.6 using the chi-squared test and P=0.24 using the Kruskal–Wallis test, respectively).

In the CysR group (n=23), among the 12 patients who received an immunosuppressive treatment, 10 achieved remission defined by a UPCR lower than 4 g/g with an eGFR over 45 ml/min per 1.73 m^2 and only two had a poor renal prognosis at the end of follow-up (UPCR >4 g/g or eGFR<45 ml/min per 1.73 m^2). Among the 11 patients who did not receive any immunosuppressive treatment, 10 entered into remission and one patient who already had a significant renal failure at presentation had poor prognosis at LOCF (Figure 7A). In this group, only 13% of patients had a poor renal prognosis (Figure 7A).

In the CysRC1 group (n=14), among the eight patients treated with immunosuppressive therapy, three entered into remission and five presented a poor renal prognosis (one patient developed ESRD). Among the six patients who did not receive any immunosuppressive treatment, four reached remission and two had a poor renal prognosis (one patient entered into ESRD). In this group, 50% of patients had a poor renal prognosis (Figure 7A).

In the CysRC1C7 group (n=32), among the 21 patients who received immunosuppressive treatment, eight achieved remission (38%) and 13 had a poor renal prognosis at LOCF including seven patients with ESRD. Among the 11 patients who did not receive immunosuppressive treatment, seven already had renal failure at presentation and four entered spontaneous remission (Figure 7A). In this group, 62% of patients had a poor renal prognosis (Figure 7A).

In the CysR group, we observed more spontaneous remission (Table 2, P=0.03 using the chi-squared test). ESRD developed in two and 10 patients, respectively, in CysRC1 and CysRC1C7 groups, but in none of the patients from the CysR group (Table 2, P=0.01 using the chi-squared test). Moreover, proteinuria at LOCF was significantly and progressively higher in the three different groups from CysR to CysRC1C7 (Table 2).

Finally, a survival curve analysis without serum creatinine increase over 30% from baseline after 2 years of follow-up showed a striking increase of renal failure progression for patients in the CysRC1 and CysRC1C7 groups compared with the CysR group (Figure 7B) (P=0.0025 using the log-rank test). In the subgroup of 33 patients who received an immunosuppressive treatment (P=0.014 using the log-rank test), renal failure progression was also significantly slower in patients from the CysR group (Figure 7B).

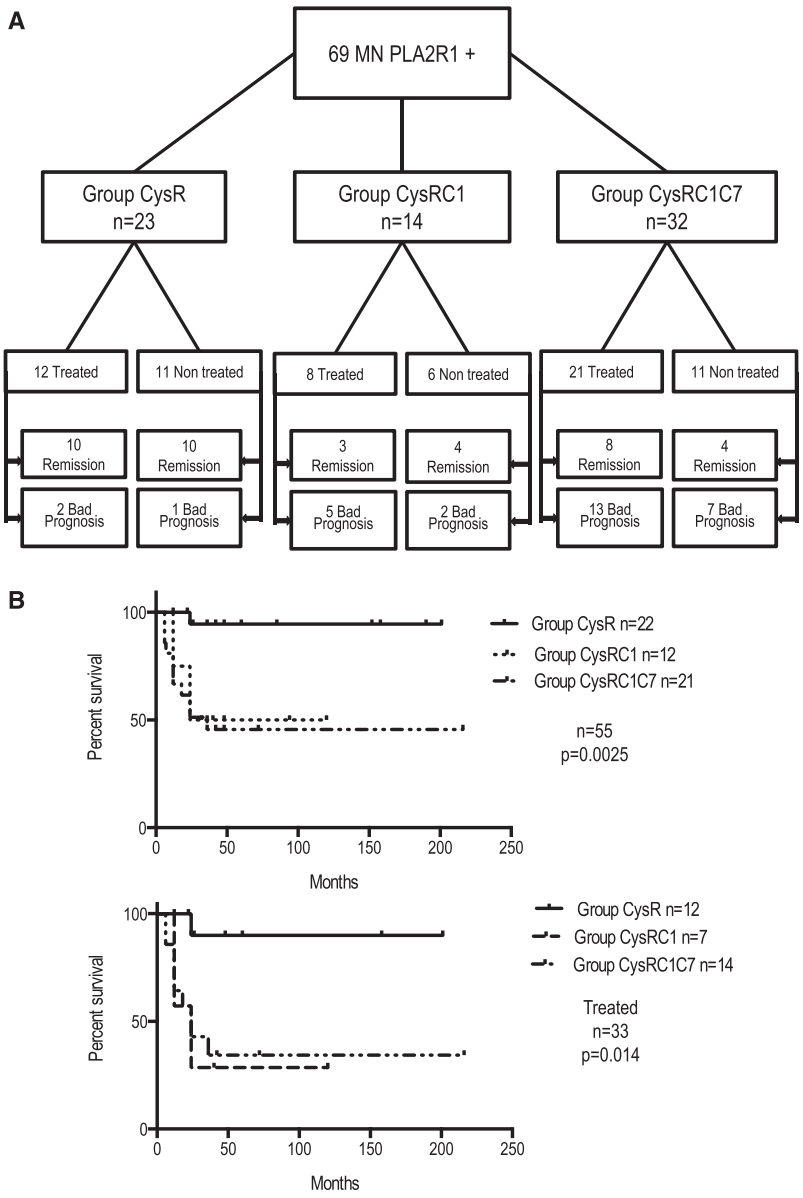

Figure 7. (A) Flow chart of patients included in this study and their follow-up in each epitope groups. Of the 23 patients in the CysR group, 12 received immunosuppressive treatment: 10 reached remission and two had a bad prognosis; and 11 were treated with supportive care only: 10 reached remission and one had a bad prognosis. In the CysRC1 group, eight received immunosuppressive treatment: three reached remission and five had a bad prognosis; and six were treated with supportive care only: four reached remission and two had a bad prognosis. In the CysRC1C7 group, 21 received immunosuppressive treatment: eight reached remission and 13 had a bad prognosis; and 11 were treated with supportive care only: four reached remission and seven had a bad prognosis. (B) Renal event is defined by serum creatinine increase over 30% 2 years after anti-PLA2R1 epitope-specific ELISA. Patients with a CysR profile had a lower incidence of renal events (p=0.0025) when analyzing only the group of patients with iMN without events at the time of serum sampling (n=55). Renal survival of patients with a CysR profile was also better in the group of patients who received immunosuppressive therapy (n=33, p=0.014).

Identification of Prognostic Factors

As summarized in Table 3, patients were divided into a remission group (n=38) and a poor renal prognosis group (n=31) at the end of follow-up according to KDIGO guidelines (UPCR>4 g/g or eGFR<45 ml/min per 1.73 m^2). There were no significant differences in age, proteinuria at diagnosis, mean follow-up and percentage of patients receiving immunosuppressive therapy between the two groups at the time of inclusion. As described in other studies,[23,24] age and serum creatinine levels at diagnosis were higher in the group who did not reach remission (p<0.0001 and P=0.0002 using the unpaired t-test and Mann–Whitney test, respectively). The ELISA titers for full-length PLA2R1, CysR, CTLD1, and CTLD7 specific assays were all significantly higher in the group with a poor renal prognosis, and more particularly for CTLD1 and CTLD7 specific assays (P=0.04, P=0.03, P=0.003, and p<0.0001, respectively; Mann–Whitney test; Figure 8). A multiple Cox regression analysis identified CysRC1 and CysRC1C7 groups and a high titer with full-length PLA2R1 as independent risk factors for a poor renal prognosis (Figure 9). The epitope profiling is thus a prognostic factor for the onset of subsequent renal events.

Epitope Spreading During Follow-up

We also had 16 patients with sera available during follow-up. We searched for switches in PLA2R1 epitope profile during the course of the disease (Figure 10, Supplemental Figure 7). Nine patients had stable epitope profiles during follow-up with stable disease activity. Five had stable or decreasing anti-CysR restricted activity after immunosuppressive treatment or symptomatic treatment and entered into remission (Patients 6 and 7 in Figure 10, A and B and patients 8, 9 and 10 in Supplemental Figure 7A). The anti-CysR restricted activity was also seen by Western blot for patient 7 (Supplemental Figure7E). Patient 6 remained in remission for 14 years after kidney transplantation and under cyclosporine treatment while he had high ELISA titers measured with the full-length PLA2R1. This patient had strong activity against CysR with no intramolecular spreading. Two biopsies in May 1997 and May 2011 confirmed the presence of immune deposits at the time of recurrence and 14

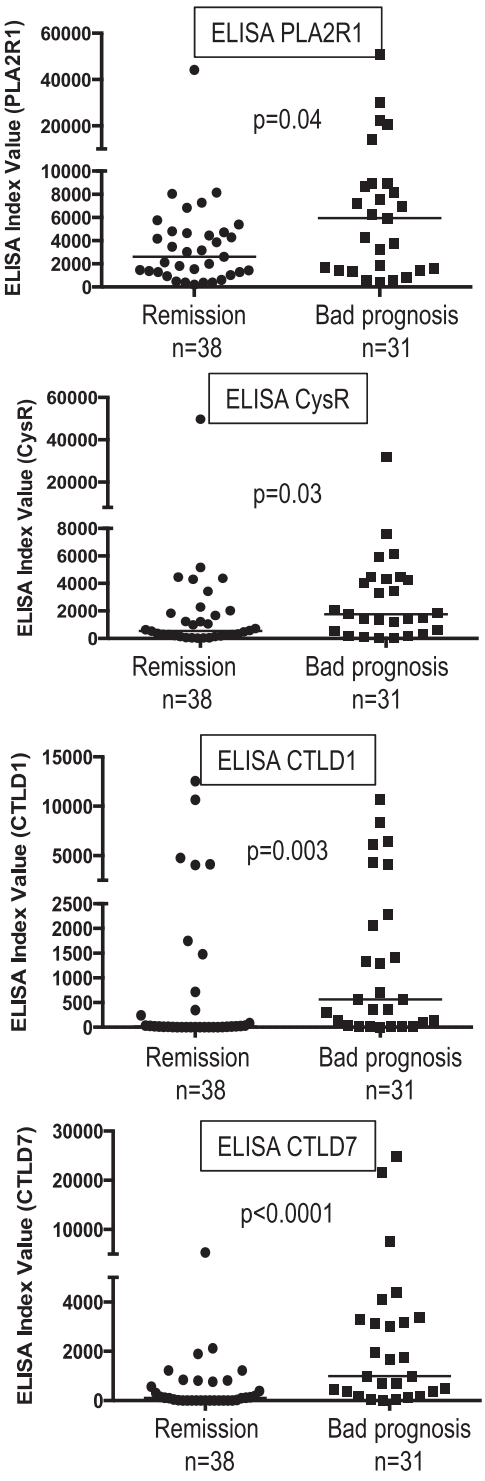

Figure 8. ELISA titers with PLA2R1, CysR, CTLD1, and CTLD7 antigens in patients with remission versus bad prognosis. Patients who reached remission at LOCF have significantly lower titers against the full-length receptor and against each domain recognized. However, *P* values were much more significant for CTLD1 and CTLD7-specific ELISAs. Titers are expressed as ELISA Index values (see Concise Methods). The bars show the median values. $P<0.05$ was considered to be significant.

years later (not shown). Patients 11 and 12 had a stable CysRC1 profile with active disease (Supplemental Figure 7B). Patients 13 and 14 had a stable CysRC1C7 epitope profile with active disease (Supplemental Figure 7C and Western blot in Supplemental Figure 7E for patient 13).

Four patients switched their epitope profile with a modification of disease activity. Patients 15 and 16, respectively, switched from CysR to CysRC1 and CysRC1C7 groups and relapsed with nephrotic proteinuria (Figure 10, C and D and Western blot in Supplemental Figure 7E for patient 16). Patient 17 had positivity against CysR and CTLD7 with stable active disease and became positive also against CTLD1 after 2 years (Figure 10E). Inversely, patient 18 from the CysRC1C7 group switched to the CysR group and entered into remission under immunosuppressive treatment (Figure 10F and Western blot in Supplemental Figure 7E). Finally, three patients with active disease presented a progressive decrease of all three antibodies against each domain under immunosuppressive treatment and entered into remission (Patients 19, 20 and 21 in Supplemental Figure 7D).

IgG Subclasses

All of the above epitope studies were designed to measure IgG4 anti-PLA2R1 antibodies, as there is a large body of evidence that IgG4 and anti-PLA2R1 IgG4 play a more prominent role in iMN pathogenesis.[8,20,21] To confirm this view, we compared the reactivity of sera from patients selected from the three epitope groups by Western blot on the deletion mutants of PLA2R1 (Figure 1) and by ELISA on CysR, CTLD1 and CTLD7 antigens with detection for the different IgG subclasses (1, 2, 3 and 4) and total IgGs (Supplemental Figure 8). To ascertain our results, we validated the specificity of IgG subclass detection with purified IgGs (Supplemental Figure 8A) and then performed Western blot assays and ELISAs with simultaneous detection for all IgGs (Supplemental Figure 8, B–F). In addition to anti-PLA2R1 IgG4 antibodies, we observed in some patients the presence of IgG1, IgG2 and IgG3 anti-CysR and anti-CTLD1 autoantibodies. However, we never observed anti-CTLD7 autoantibodies different from IgG4. Finally, the pattern observed with total IgG by Western blot was quite similar to that of IgG4. All together, these results suggest that our choice to detect anti-PLA2R1 IgG4 was likely the most powerful, and with most likely no change in the patients' epitope classification if we were choosing detection with total IgG.

DISCUSSION

We screened 69 patients with iMN disease and identified epitopes in three domains of PLA2R1: CysR, CTLD1, and

Table 3. Clinical baseline characteristics, epitope profile, and PLA2R1 antibody level of patients reaching remission and bad prognosis at last observation carried forward

	Remission (*n*=38)	Bad prognosis (*n*=31)	*P* Value univariate
Sex	9F/29M	6F/25M	ns
Age	50±2.2	62±2.6	<0.0001
Proteinuria at diagnosis (g/g)	4.45 (1.4–15.0)	5.2 (2.5–24.0)	ns
Creatinemia at diagnosis (μmol/l)	88 (43–187)	113 (60–385)	0.0002
Follow-up (months)	36 (12–216)	30 (12–158)	ns
Patients on immunosuppressive treatment	21 (55%)	20 (64%)	ns
Group CysR	20 (52%)	3 (10%)	0.0002
Group CysRC1	7 (18%)	7 (23%)	ns
Group CysRC1C7	11 (30%)	21 (67%)	0.001
Anti-PLA2R1 titer	2594 (210–44,194)	5947 (424–50,817)	0.04

Normal values are mean±SD; non-normal values are median (ranges); qualitative values are numbers (%).
At the end of the study, 38 patients reached remission and 31 had a bad prognosis (proteinuria >4 g/g and/or serum creatinine increased over 30% and/or eGFR<45 ml/min per 1.73 m^2). Patients with a bad prognosis at the end of the follow-up were significantly older, had higher creatinine level at diagnosis, were not in the group CysR but more in the group CysRC1C7 and had higher anti-PLA2R1 antibody levels. There was no difference in sex ratio, proteinuria at diagnosis, time of follow-up, percentage of patients who received immunosuppressive therapy and the number of patients in the CysRC1 group between patients with good (remission) and bad prognosis.

CTLD7. Interestingly, several common genetic variants associated with iMN have been localized in these domains.[25] CysR clearly appears as the primary dominant epitope with evidence for epitope spreading toward CTLD1 and CTLD7. Our results thus further extend those of Kao *et al.* and Fresquet *et al.* who identified epitopes in the CysR-CTLD1 region and CysR alone, respectively.[18,19] Our data clearly show that CysR and CTLD1 are two independent domains recognized by distinct anti-PLA2R1 antibodies. Our ability to identify CTLD7 as an additional domain and more clearly identify CysR and CTLD1 as two separate epitopes is likely due to the facts that we generated a different and full set of PLA2R1 deletion mutants and soluble forms, and also screened a large number of patients with IgG4 detection which is more sensitive than total IgG. Indeed, the two previous studies only used a very limited number of patients or used a pool of five patients' sera, and then performed competition assays with more patients. Furthermore, it was unclear whether sera were collected from patients with active disease or in remission.

We then observed that anti-PLA2R1 reactivity against CysR at serum sampling was associated with favorable outcome while reactivity against CTLD1 and CTLD7 was associated with active disease and poor renal prognosis. Furthermore, we showed that the epitope profiles could change during follow-up. Anti-CTLD1 and anti-CTLD7 antibodies disappeared with disease remission and reappeared with disease relapse, while anti-CysR restricted activity was associated with stable and mild disease activity. Importantly, all patients still had anti-PLA2R1 activity when measured by ELISA with full-length PLA2R1 (Table 1). These findings may explain the discrepancies observed between the level of disease activity and the anti-PLA2R1 titers.[10]

Our findings led us to hypothesize that anti-PLA2R1 antibodies may be initially raised against the N-terminal CysR domain with pauci-symptomatic iMN disease: patients in the CysR group are younger than those in the other groups, suggesting that these patients are probably at the beginning of the disease. A second immune challenge (allergy, infection, etc.) might then induce intramolecular spreading in PLA2R1 toward the C-terminal end (CTLD1 or CTLD7) leading to more active disease. Epitope spreading is a phenomenon in which new epitopes, within the same or a different molecule, are recognized over time by T or B cells from an original noncross-reactive antigenic site. As described in many autoimmune diseases such as human antiglomerular basement membrane disease,[26] Pemphigus vulgaris,[27] rheumatoid arthritis,[28] experimental autoimmune encephalomyelitis,[29] multiple sclerosis,[30] type 1 diabetes,[31] and Heymann nephritis,[32] intramolecular epitope spreading is associated with worsening of the disease. Moreover, intramolecular epitope spreading may also modulate remission and relapse of iMN. As described in experimental autoimmune encephalomyelitis, epitope spreading plays a major role in disease progression in mice, and short-term costimulation blockade can specifically inhibit initiation of the T cell response, preventing epitope spreading and worsening of the disease.[33,34]

In conclusion, our results show that three PLA2R1 domains are involved in anti-PLA2R1 binding, with two of them more closely associated with active iMN and linked by a mechanism of epitope spreading. These findings should now be confirmed in independent prospective cohorts and prospective randomized trials.

CONCISE METHODS

Patients

Sera of patients with biopsy-proven iMN were collected from five French nephrology centers. iMN was defined by the absence of secondary MN features such as positivity for anti-nuclear antibodies, history of hepatitis B or C, cancer or other immune pathologies (cryoglobulinemia, sarcoidosis, graft versus host disease, etc.). Sera

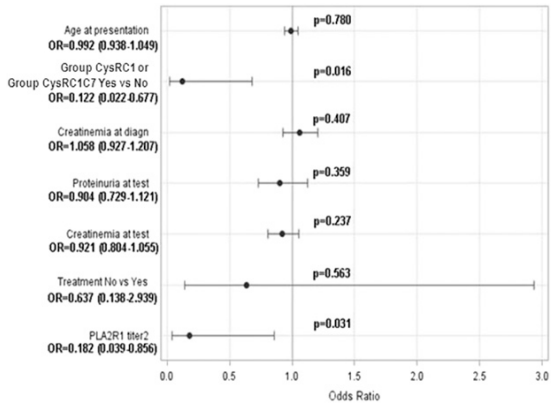

Figure 9. Multivariate Cox regression analysis. Anti-PLA2R1 antibody titers measured by ELISA (full-length PLA2R1) and epitope spreading (group CysRC1 and CysRC1C7 defined by the presence of at least two different antibodies against CysR and CTLD1 and/or CTLD7) are identified as risk factors for not achieving remission at LOCF. Hazard ratios for achieving remission are expressed per natural logarithm unit of anti-PLA2R1 levels measured by ELISA and dichotomized for sex, epitope group, and treatment.

from a total of 69 patients were collected, with sequential serum samplings for 16 patients. A poor renal prognosis was defined by proteinuria >4 g/g and/or serum creatinine increase over 30% and/or eGFR<45 ml/min per 1.73 m^2, as defined by the KDIGO recommendations. Active disease was defined by nephrotic proteinuria>3.5 g/g. Renal failure progression was analyzed after 2 years of follow-up and was defined by an increase over 30% of serum creatinine from baseline.

Generation and Expression of Membrane-bound PLA2R1 Deletion Mutants

We generated the series of human PLA2R1 deletion mutants by PCR and cloned them into the pLPCX expression vector (Clontech), as previously described for wild-type human PLA2R1 (GenBank NM 007366).[35] Deletion mutants were generated using the Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). All mutants comprised the PLA2R1 signal peptide (Met-1 to Ala-20) and the N-terminal linker sequence (Ala-21 to Trp-35) followed by the PLA2R1 sequence with the following domains deleted at the linker regions (Figure 1): deletion of the N-terminal domain CysR (Δ C, deletion from Gln-36 to Asp-165), CysR and FNII domains (Δ F, Gln-36 to Thr-223), CysR to CTLD1 domains (Δ 1, Gln-36 to Tyr-357), CysR to CTLD2 domains (Δ 2, Gln-36 to Ala-504), CysR to CTLD3 domains (Δ 3, Gln-36 to Pro-660), CysR to CTLD4 domains (Δ 4, Gln-36 to Lys-805), CysR to CTLD5 domains (Δ 5, Gln-36 to Lys-947), CysR to CTLD6 domains (Δ 6, Gln-36 to His-1105), and CysR to CTLD7 domains (Δ 7, Gln-36 to Pro-1235). All mutants were generated from a C-terminal HA-tagged (HA:YPYDVPDYA) version of the human full-length PLA2R1 cDNA cloned into the pGEMTeasy vector (Promega) (Figure 1). After full sequencing, all deletion constructs were subcloned into the pLPCX retroviral vector and transfected into HEK293 cells using a Ca/PO_4 transfection kit (InvitroGen) or Exgen (Biomol GmbH). Expression was confirmed by Western blot from transiently transfected HEK293 cell lysates using anti-HA antibodies (see below).

Generation and Expression of Soluble Domains of PLA2R1

Soluble PLA2R1 mutants were generated by PCR as above and cloned into the pcDNA3.1Z-expression vector (Life Technologies). They all comprised the PLA2R1 signal peptide followed by the human PLA2R1 sequence coding for the different PLA2R1 domains as follows: CysR domain (CysR, from Ala-21 to Lys-164), CTLD1 domain (CTLD1 or C1, Thr-223 to Asn-359), CTLD2 to CTLD8 (C2C8, 357-Tyr to 1397-Ser), CTLD6 to CTLD8 (C6C8, 947-Lys to 1397-Ser), CTLD6 to CTLD7 (C6C7, 947-Lys to 1246-Leu), and CTLD7 to CTLD8 (C7C8, Glu-1097–1397-Ser), CTLD6 (C6, 947-Lys to 1114-Pro), CTLD7 (C7, Glu-1097–1246-Leu) and CTLD8 (C8, Pro-1235–1397-Ser). All soluble mutants were C-terminally HA-tagged. The CysR-FNII-CTLD1 domains were produced and cleaved with thrombin (Sigma-Aldrich) as described by Kao *et al.*,[18] except that the domains were C-terminally HA-tagged. The human codon–optimized cDNA coding for soluble CTLD1 (Thr-223 to His-377) was synthesized (Genecust, Dudelange, Luxembourg) and added in frame after the signal peptide of the human secreted phospholipase A2 PLA2G2A gene followed by a 3xFlag tag sequence. It included a C-terminal HA-tag. All mutants were expressed in HEK293 cells as described above and cell medium containing the expressed proteins were collected. When proteins were expressed at low levels, medium was precipitated with trichloroacetic acid using standard procedure or affinity-purified with anti-HA beads.

DsbC-His-HA-PLA2R1 fusion proteins were produced in *E. coli* essentially as described previously.[36] The detailed procedure will be published elsewhere. PLA2R1 domains with optimized codons for *E. coli* expression were added in frame with the leaderless DsbC open reading frame followed by His and HA tags for purification and detection, respectively. Sequences of inserted PLA2R1 domains were as follows: CysR, Ala-26 to Lys-164; CTLD1, Thr-223 to Asn-359; CTLD7, Thr-1102 to Glu-1237; CTLD6–7, 947-Lys to Glu-1237. The four SNPs variants of CTLD1 were also designed:[22] SNP1, Val-292 and Asp-300; SNP2, Met-292/Asp-300; SNP3, Met-292/His-300; and SNP4, Val-292/His-300.

Western Blot Analysis

The different deletion mutants and soluble forms of PLA2R1 were analyzed by SDS-PAGE under nonreducing conditions unless indicated otherwise. Total proteins (10–50 μg/well to adjust for the different expression level of each deletion mutant) and cell medium (for transfected soluble forms) were run on 4%–15% precast TGX SDS-PAGE gels (Bio-Rad) and transferred to methanol-soaked polyvinylidene difluoride membranes (Bio-Rad) under semi-dry conditions using Trans-blot Turbo (Bio-Rad) at 25 V constant for 12 min. For the soluble forms difficult to detect by Western blot, we added 0.05% SDS in the transfer buffer. Membranes were blocked overnight at 4°C in 5% milk with PBS-Tween (PBS-T) 0.05% and then incubated with primary and secondary antibodies for 2 h at room

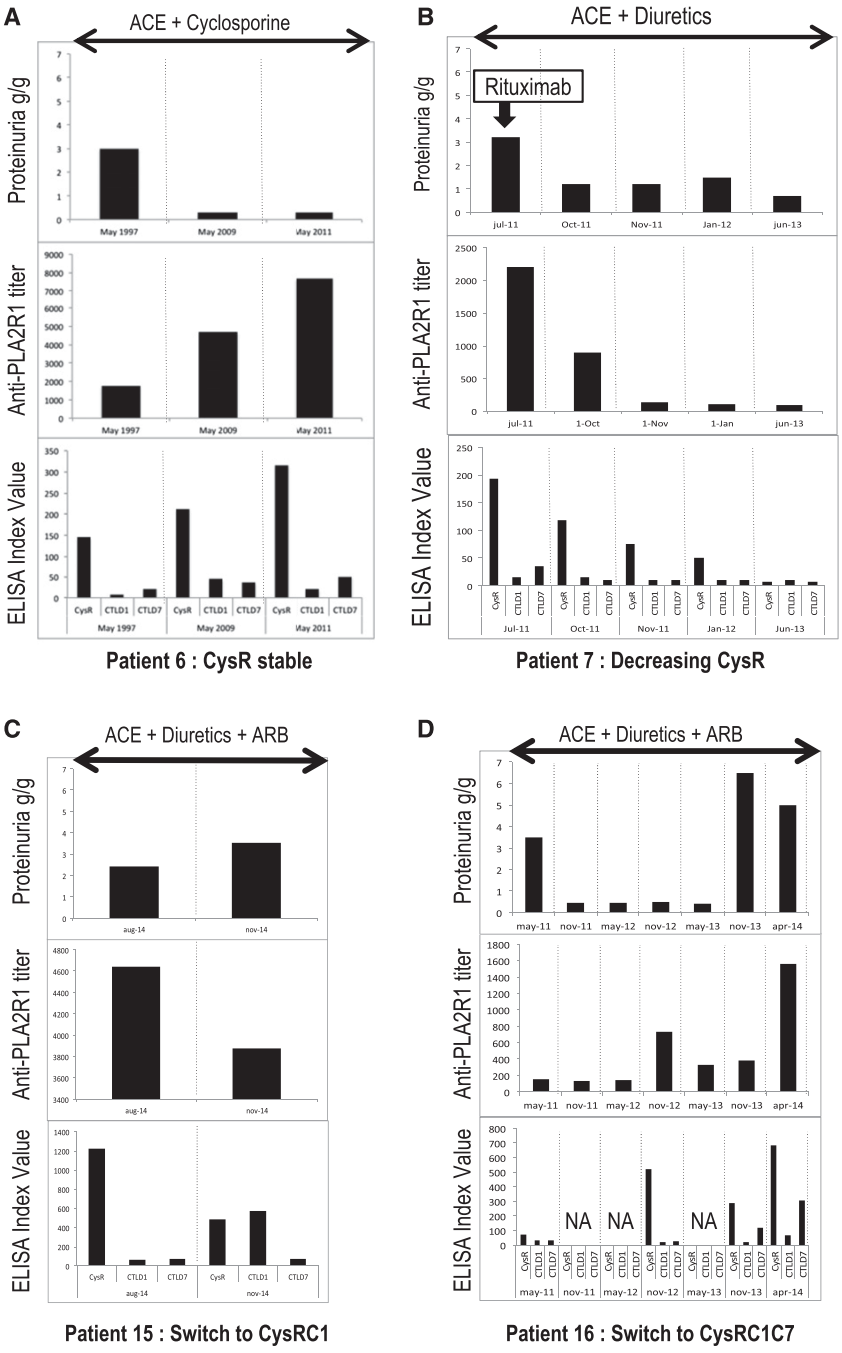

Figure 10. Evidence for a mechanism of epitope spreading and evolution of titers against CysR, CTLD1 and CTLD7 domains during follow-up in six patients. (A) Patient 6 had biopsy-proven iMN recurrence in May 1997 after transplantation and then entered into remission with symptomatic treatment, with still strong CysR activity during 14 years. Kidney biopsy in 2011 confirmed the persistence of immune deposits. (B) Patient 7 had weakly active disease and entered into remission under rituximab treatment, with decreasing anti-CysR activity. (C) Patient 15 was in remission under supportive care. Proteinuria increased after 3 months of follow-up while he became positive for CTLD1. (D) Patient 16 was in remission in November 2011 and relapsed 2 years later, while he became positive for CTLD7. (E) Patient 17 had active disease during 2 years without immunosuppressive treatment while being always positive for CTLD7 and increasing anti-CTLD1 titers. (F) Patient 18 entered into remission under immunosuppressive treatment with parallel decrease of several antibodies. After 6 months, he was in remission with only CysR antibodies. NA, no serum sample available.

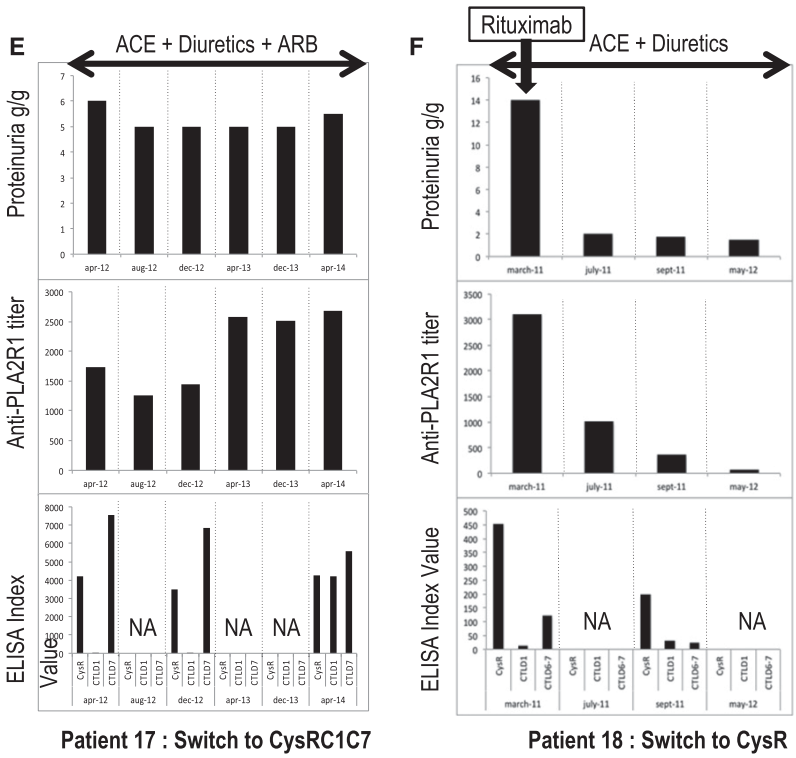

Figure 10. Continued.

temperature. Primary antibodies were diluted with 0.5% dry milk in PBS-T. Membranes were prepared in multiple replicates and probed with a mouse monoclonal anti-HA antibody (Sigma-Aldrich) at 1:5000 to validate expression or with the 50 different iMN sera to screen for the epitope profile at a working dilution of 1:25–1:500, depending on anti-PLA2R1 titers. The secondary antibody for anti-HA was a goat anti-mouse IgG (Southern Biotech #1030–05) diluted 1:20,000 in PBS-T. IgG secondary antibody for iMN sera was horse radish peroxidase–conjugated mouse anti-human IgG4 (Southern Biotech #9200–05) diluted 1:30,000 in PBS-T. For anti-PLA2R1 IgG subclass analysis (see Supplemental Material), we also used a panel of subclass specific anti-IgGs from Southern Biotech and two different anti-total IgGs (Southern Biotech and Santa Cruz Biotechnology). Purified IgGs for validation of IgG subclass specificity were from Fitzgerald. Membranes were washed three times for 5 min in PBS-T after incubation with primary and secondary antibodies. The detection of protein bands was performed with a chemiluminescent substrate (EMD Millipore) and a Fuji LAS3000 imager.

Anti-PLA2R1 ELISA

The anti-PLA2R1 assay was run essentially as described for the standardized and commercially available ELISA.[37] Pure recombinant human PLA2R1 protein corresponding to the entire extracellular domain was coated to ELISA plates in 20 mM Tris pH 8.0 (100 μl/well, 1 μg/ml) at 4°C/overnight. Plates were blocked for 2 h with SeramunBlock (Seramun Diagnostica). Patients' sera were diluted at 1:100 (or higher as needed) in PBS/0.1% dry milk and added in duplicate (100 μl per well) to the ELISA plates, which also contained a serial dilution of an iMN standard serum and a quality control calibrator (between plates). After 2 h incubation at room temperature on a plate shaker, plates were washed four times with PBS/0.02% Tween 20. Anti-human IgG4-horse radish peroxidase conjugate (Southern Biotech #9200–05) diluted 1:7500 in SeramunStab ST plus was added (100 μl per well; Seramun Diagnostica) and incubated for 1 h at room temperature on a plate shaker. After four washes, tetramethylbenzidine was added, and the reactions were developed for 15 min and then stopped with HCl 1.2N. Plates were read at 450 nm. Sixty-seven sera from healthy donors were used to define the normal range, using mean+3SD. The cut-off was optimized by receiver operating characteristics curve analysis. A highly positive index patient serum was used in each plate to generate a standard curve and a negative control.

ELISA Using Soluble Forms of PLA2R1

Plates were coated with anti-HA antibody (Sigma-Aldrich) diluted at 1:5000 in 20 mM Tris pH 8.0 (100 μl/well) at 4°C/overnight. Plates were then blocked for 2 h with SeramunBlock (Seramun Diagnostica). Cell medium from HEK293 cells transfected with the soluble forms of PLA2R1 (10–100 μl/well depending on protein expression) or purified *E. coli* DsbC-HA-CTLD1 fusion protein (50 ng/well) were then added and incubated for 1 h. Plates were washed and

patients' sera diluted at 1:100 (or higher as needed) in PBS/0.1% dry milk were added in duplicate (100 μl per well) to the ELISA plates, which also contained a serial dilution of an iMN standard serum and a quality control calibrator (between plates). After 2 h incubation at room temperature on a plate shaker, plates were washed four times with PBS/0.02% Tween 20. Anti-human IgG4-horse radish peroxidase conjugate (Southern Biotech #9200–05) diluted 1:7500 in SeramunStab ST plus was added (100 μl per well; Seramun Diagnostica) and incubated for 1 h at room temperature on a plate shaker. Subclass analyses were performed with specific anti-IgGs as indicated above. After four washes, tetramethylbenzidine was added, and the reactions were developed for 15 min and then stopped with HCl 1.2N. The plates were read at 450 nm. Twenty sera from healthy donors were used to define the normal range, using mean+3SD. The cut-off was optimized by receiver operating characteristics curve analysis. A highly positive index patient serum was used in each plate to generate a standard curve and a negative control.

An ELISA Index value for each antigen was obtained for patients or normal subjects as follows (mean test result–mean domain negative control)/(mean domain positive control–mean domain negative control)$\times$domain correction factor$\times$100. The domain correction factor was determined for each domain as the mean of all the positive controls for that domain on all plates minus the mean of the negative controls, divided by the cut-off for that domain assay as described by Warren *et al.*[38]

Statistical Analyses

For descriptive statistics, data are presented as mean$\pm$SD (for variables with Gaussian distribution) or median (ranges) (for variables with nonGaussian distribution). We used the ShapiroWilk test to determine if a variable has a Gaussian distribution. Qualitative criteria were compared using the chi-squared test or Fisher's exact test according to the terms of use. Quantitative variables were compared using the Student *t*-test or Wilcoxon–Mann–Whitney test (for variables with non-Gaussian distribution) and for multiple comparisons using the ordinary one-way ANOVA (for variables with Gaussian distribution), Kruskal–Wallis (for variables with non-Gaussian distribution) and Tukey's test (for comparison two by two). A poor prognosis was defined at LOCF as defined by KDIGO by a proteinuria $>$4 g/g and or serum creatinine increased over 30% and/or eGFR$<$45 ml/min per 1.73 m^2. Survival curves for renal survival were calculated using Kaplan–Meier estimates for survival distribution. The end point for renal survival analysis was the time where we measured an increase over 30% of serum creatinine from baseline. Differences between groups based on epitope profile were analyzed with the log-rank test. Multivariable cox regression analysis was performed. In the analysis, we included and adjusted for all clinical parameters that might influence the prognosis (age, sex, proteinuria, creatinemia, immunosuppressive treatment, and anti-PLA2R1 titer). Hazard ratios are expressed per natural logarithm unit of PLA2R1 antibody level measured by ELISA and are dichotomized for sex, treatment and different domains group. All statistics were performed using Prism6 and SAS 9.3 softwares. *P* values $<$0.05 were considered as statistically significant.

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DISCLOSURE

None.

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