Biochimie 118 (2015) 104–115

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Cross-reactivity of anti-PLA2R1 autoantibodies to rabbit and mouse PLA2R1 antigens and development of two novel ELISAs with different diagnostic performances in idiopathic membranous nephropathy

Barbara Seitz-Polski [a, b, c, **], Guillaume Dolla [a, 1], Christine Payré [a, 1], Nicola M. Tomas [a, d], Marine Lochouarn [a, b, c], Louise Jeammet [a], Christophe Mariat [e], Thierry Krummel [f], Stéphane Burtey [g], Cécile Courivaud [h], Wolfgang Schlumberger [i], Kévin Zorzi [j], Sylvia Benzaken [c], Ghislaine Bernard [c], Vincent L.M. Esnault [b, 2], Gérard Lambeau [a, *, 2]

[a] Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275 CNRS and Université de Nice-Sophia Antipolis, Valbonne Sophia-Antipolis, France
[b] Service de Néphrologie, Hôpital Pasteur, Université de Nice-Sophia Antipolis, France
[c] Laboratoire d'Immunologie, Hôpital l'Archet, Université de Nice-Sophia Antipolis, France
[d] University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[e] Service de Néphrologie Dialyse Transplantation Rénale, CHU Hôpital Nord, Saint-Etienne, and Université de Saint-Etienne PRES Université de Lyon, Saint-Etienne, France
[f] Service de Néphrologie, CHU de Strasbourg, Strasbourg, France
[g] Service de Néphrologie Dialyse Transplantation Rénale, Assistance Publique-Hôpitaux de Marseille, Hôpital de la Conception, UMR S1076, Université Aix-Marseille, Marseille, France
[h] Service de Néphrologie Dialyse Transplantation Rénale, CHU Saint-Jacques, Besançon, France
[i] EUROIMMUN AG, Lübeck, Germany
[j] Centre de Recherche Clinique, Hôpital l'Archet, Université de Nice-Sophia Antipolis, France

ARTICLE INFO

Article history:
Received 14 May 2015
Accepted 14 August 2015
Available online 19 August 2015

Keywords:
Autoimmunity
Conformational epitope
ELISA
Membranous nephropathy
PLA2R1 autoantibodies
Mouse PLA2R1
Rabbit PLA2R1
Orthologs

ABSTRACT

About 70% of patients with idiopathic membranous nephropathy (iMN) have autoantibodies to the phospholipase A2 receptor PLA2R1. We screened sera from iMN patients for their cross-reactivity to human (h), rabbit (rb) and mouse (m) PLA2R1 by western blot (WB) and antigen-specific ELISAs. All iMN patients recognized hPLA2R1 and rbPLA2R1 by WB, and a rbPLA2R1 ELISA was as sensitive as the standardized hPLA2R1 ELISA to monitor anti-PLA2R1 in patients with active disease or in drug-induced remission. In contrast, only 51% of patients were reactive to mPLA2R1 by WB, and a maximum of 78% were weakly to highly positive in the mPLA2R1 ELISA, suggesting that iMN patients exhibit different subsets of anti-PLA2R1 autoantibodies against epitopes that are shared or not among PLA2R1 orthologs. In a cohort of 41 patients with a mean follow-up of 42 months from anti-PLA2R1 assay, the detection of anti-mPLA2R1 autoantibodies was an independent predictor of clinical outcome in multivariate analysis ($p = 0.009$), and a ROC curve analysis identified a threshold of 605 RU/mL above which 100% of patients (12 patients) had a poor renal outcome ($p < 0.001$). A similar threshold could not be defined in hPLA2R1 and rbPLA2R1 ELISAs. We conclude that rbPLA2R1 is an alternative antigen to hPLA2R1 to measure anti-PLA2R1 in active disease while mPLA2R1 is a unique antigen that can detect a subset of anti-PLA2R1 autoantibodies present at high levels (>605 RU/mL) only in iMN patients at risk of poor prognosis, and is thus useful to predict iMN outcome.

Abbreviations: ELISA, enzyme-linked immunospecific assay; ESKD, end-stage kidney disease; IIFT, indirect immunofluorescence test; iMN, idiopathic MN; MN, membranous nephropathy; PLA2R1, M-type phospholipase A2 receptor 1; hPLA2R1, human PLA2R1; rbPLA2R1, rabbit PLA2R1; mPLA2R1, mouse PLA2R1; UPCR, urinary protein to creatinine ratio; WB, western blot.

* Corresponding author. Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275 CNRS et Université de Nice-Sophia Antipolis, Valbonne Sophia-Antipolis, France.
** Corresponding author. Service de Néphrologie, Hôpital Pasteur, Université de Nice Sophia Antipolis, 30 Voie Romaine, 06002 Nice, France.
E-mail addresses: seitz-polski.b@chu-nice.fr (B. Seitz-Polski), lambeau@ipmc.cnrs.fr (G. Lambeau).
[1] These authors contributed equally to this work.
[2] Co-last authors.

http://dx.doi.org/10.1016/j.biochi.2015.08.007
0300-9084/

1. Introduction

Idiopathic membranous nephropathy (iMN) is a common cause of nephrotic syndrome in adults [1–5]. Clinically, iMN is associated with high proteinuria, with about 80% of patients loosing more than 3.5 g of protein per day. During follow-up, spontaneous remission occurs in about one third of patients while end-stage kidney disease (ESKD) with persistent proteinuria develops in another third of cases. The treatment of iMN is controversial [3,6]. KDIGO guidelines recommend supportive symptomatic treatment with renin-angiotensin system-blockade and diuretics in all patients with iMN, but immunosuppressive therapy is only recommended in the case of renal function deterioration or a worsening nephrotic syndrome [7]. Therefore, immunosuppressive treatment is often started only after significant and potentially irreversible complications. On the other hand, an unnecessary early start of immunosuppressive therapy can be futile in patients who might develop spontaneous remission. For these reasons, there is a need for clinical and diagnostic parameters at disease onset and during follow-up that would allow the identification of patients at risk of poor clinical outcome and who should benefit from immunosuppressive versus supportive treatment.

In 2009, Beck et al. identified the M-type phospholipase A2 receptor PLA2R1 as the major podocyte antigen involved in adult iMN [8], with about 70% of iMN patients having circulating autoantibodies against PLA2R1. The presence of anti-PLA2R1 autoantibodies has been widely confirmed in subsequent studies in 60–80% of patients with iMN, as well as in a few cases of apparent secondary MN [9–17]. The pathogenic role of anti-PLA2R1 autoantibodies is not yet proven, but antibody titers, especially IgG4 anti-PLA2R1, have been associated with clinically active disease and response to immunosuppressive therapy [10,13,18]. High titers at early presentation of disease also appear to be predictive of poor clinical outcome [16,19]. However, anti-PLA2R1 activity seems to persist in some patients with apparent clinical remission or low proteinuria [11–13,20] or under renin-angiotensin system-blockade [21].

PLA2R1 is a large transmembrane receptor of 180 kDa with an extracellular region comprising 10 distinct globular domains, each harboring 2 to 3 disulfide bridges [22]. Anti-PLA2R1 autoantibodies in iMN patients were initially detected by western blot (WB) using recombinant human PLA2R1 (hPLA2R1) expressed in HEK293 cells [8]. The study also revealed that patients' autoantibodies only recognize hPLA2R1 under non reducing conditions, indicating that the autoantibodies bind exclusively to the folded protein on one or more conformational epitopes [8]. Anti-PLA2R1 autoantibodies were then measured by indirect immunofluorescence test (IIFT) using HEK293 cells overexpressing hPLA2R1 at the cell surface [10,23], a laser bead immunoassay [24] and ELISA using a recombinant soluble form of hPLA2R1 [13,14]. When compared, the different methods of detection were in good accordance, but with different sensitivities, which might be explained by the fact that the methods are either only semi-quantitative, or use preparations of hPLA2R1 antigen with various levels of folded and reactive protein.

Importantly, all the above methods have used hPLA2R1 as antigen. Hence, no study has evaluated the cross-reactivity of anti-PLA2R1 autoantibodies to other mammalian species of PLA2R1. In this study, we compared for the first time the cross-reactivity of anti-PLA2R1 autoantibodies from a cohort of iMN patients to human (h), rabbit (rb) and mouse (m) PLA2R1 orthologs and observed different cross-reactivities, especially for mPLA2R1. We then established species-specific ELISAs using recombinant, purified and folded PLA2R1 antigens and compared their diagnostic and prognostic performances in a cohort of iMN patients with clinical follow-up.

2. Materials and methods

2.1. Patients

Sera of patients with biopsy-proven iMN and secondary MN 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 (cryoglobulinaemia, sarcoidosis, graft versus host disease,...). Sera from a total of 153 patients were collected: 130 with iMN and 23 with secondary MN. In the latter group, two had lupus nephritis, one had cryoglobulinaemia, one had HBV infection, one had HCV infection, and one was co-infected with both HBV and HCV. We also collected 67 sera from disease controls: 34 patients without renal involvement including rheumatoid arthritis, psoriatic arthritis, systemic sclerosis, Sjoegren's syndrome and 33 patients with other glomerular diseases such as IgA nephropathy, ANCA-positive systemic vasculitis, lupus nephritis (type V), focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, minimal change disease and Henoch Schoenlein purpura. Finally, we collected 67 sera from healthy blood donors. Consents were obtained from all patients. Proteinuria levels were classified in three stages: nephrotic range proteinuria when UPCR was >3.5 g/g, residual non nephrotic proteinuria when UPCR was between 0.5 and 3.5 g/g, and low proteinuria when UPCR was <0.5 g/g. Active disease was defined as a UPCR >3.5 g/g or eGFR <60 mL/min/1.73 m^2 and remission was defined as a UPCR <3.5 g/g and eGFR >60 mL/min/1.73 m^2.

2.2. Expression of PLA2R1 orthologs as folded antigens

The cDNAs coding for full-length membrane-bound hPLA2R1, rbPLA2R1 and mPLA2R1 with an HA tag added to the C-terminal end were transiently transfected into HEK293 cells as described previously [25–27]. Three days after transfection, cells were lysed and centrifuged to prepare the cytosolic and microsomal fractions. The microsomal fraction was then solubilized with detergents and centrifuged to obtain detergent-soluble and detergent-insoluble fractions. The expression of PLA2R1 orthologs was validated in the 4 fractions by WB using specific antibodies (Figs. 1 and 2A). See Supplementary information online for more details.

2.3. Western blot analyses

The different forms of recombinant PLA2R1 were analyzed by SDS-PAGE gels under reducing and non reducing conditions and western blotting according to the detailed methods described in the online Supplementary information. The primary antibodies used to identify folded PLA2R1 in the four fractions of PLA2R1-transfected HEK293 cells were a mouse monoclonal anti-HA (Sigma, St. Louis, USA, working dilution 1:5000), a rabbit polyclonal anti-hPLA2R1 (Atlas Antibodies, working dilution 1:1000), a guinea-pig polyclonal anti-rbPLA2R1 (homemade as described [28], working dilution 1:5000) and a rabbit polyclonal anti-mPLA2R1 (homemade as described [27], working dilution 1:2000). HRP-conjugated goat anti-mouse or anti-rabbit IgG (SouthernBiotech, Birmingham, USA) and goat anti-guinea-pig IgG (Cappel, Organon Teknika) were used as secondary antibody at a dilution of 1:5000. For detection of cross-reacting anti-PLA2R1 antibodies in iMN sera, the three folded PLA2R1 orthologs were run on SDS-PAGE gels under non reducing conditions and mini WBs

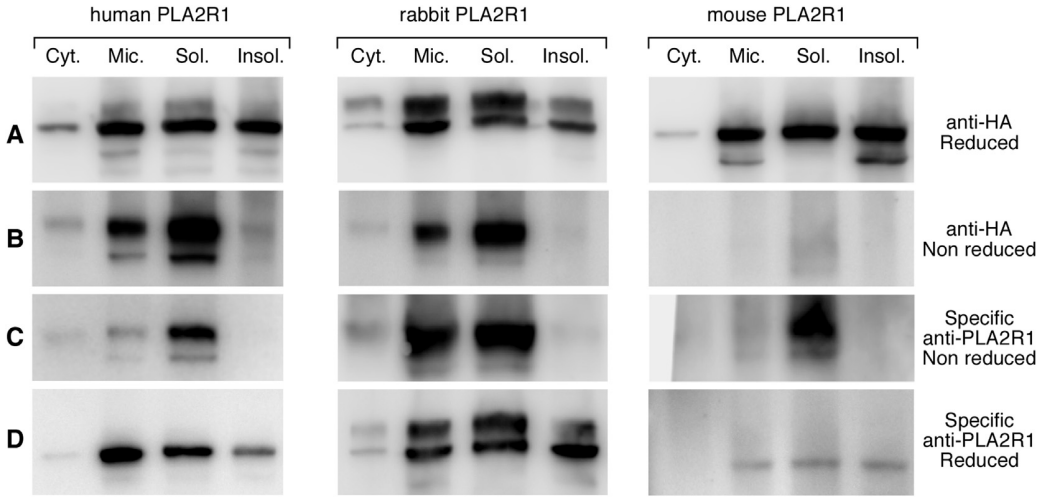

Fig. 1. Expression of membrane-bound human, rabbit and mouse PLA2R1-HA in cytosolic, microsomal, detergent-soluble and detergent-insoluble fractions of transiently transfected HEK293 cells. Proteins from each fraction (30 μg of total protein) were loaded on SDS-PAGE gels under reducing and non reducing conditions and WBs were prepared in parallel. Panel A, signal with anti-HA antibody under reducing conditions showing the total level of PLA2R1 expression (folded plus aggregated protein). Panel B, signal with anti-HA under non reducing conditions showing the level of folded PLA2R1 detectable with this antibody. Panels C and D, same as panels A and B but using specific anti-PLA2R1 antibodies (specific anti-hPLA2R1, rbPLA2R1 and mPLA2R1 antibodies were respectively used for each PLA2R1 orthologs in these panels, see methods and Fig. 2). Note the anti-hPLA2R1 recognizes better hPLA2R1 under reducing conditions while the anti-mPLA2R1 antibody is conformational and recognizes the antigen only under non reducing conditions. The detergent-soluble fraction is enriched in folded PLA2R1 protein for all three species. All mature glycosylated forms PLA2R1 orthologs have molecular masses at around 180–200 kDa. The lower bands may represent non glycosylated forms of PLA2R1 orthologs, which are more readily visible under reducing conditions in some cellular fractions.

were prepared in replicates. iMN sera were tested at a working dilution of 1:50 to 1:500 depending on anti-PLA2R1 titers. Secondary antibodies were HRP-conjugated mouse anti-human IgG4 (SouthernBiotech, Birmingham, USA) diluted at 1:30,000.

2.4. Preparation of recombinant soluble rbPLA2R1, hPLA2R1 and mPLA2R1

The recombinant soluble form of rbPLA2R1 comprising the full extracellular region (NP_001075803.1, amino acid residues 24–1393) [26] was produced in stably transfected HEK293 cells and purified from cell medium by sPLA2 affinity chromatography as described in the online Supplementary information. Large scale production in roller bottles produced about 3 mg of rbPLA2R1 per liter per week. The concentrated purified recombinant rbPLA2R1 protein was pure and folded as indicated by SDS-PAGE gel analysis and western blotting with anti-rbPLA2R1 antibodies and iMN sera (Fig. S1), as well as inhibition of sPLA2 enzymatic activity [27] (not shown). The recombinant soluble form of hPLA2R1 (NP_031392.3, amino acid residues 20–1397) and mPLA2R1 (NP_032893, amino acid residues 27–1392) comprising the full extracellular region was obtained from transfected HEK293 [14] and NS0 mouse myeloma cells (R&D systems #5367-PL, Minneapolis, USA), respectively. The two proteins were judged pure based on gel electrophoresis in both reducing and non reducing conditions (Fig. S1C), and the folded native forms were validated by WB and sPLA2 inhibition assays as previously described [27].

2.5. Competition assays between PLA2R1 orthologs and iMN serum by western blot

Recombinant human, rabbit and mouse soluble PLA2R1 (50–100 ng) were analyzed by SDS-PAGE gels under non reducing conditions and western blotting according to the detailed method described in the online Supplementary information. For antigen competition, the iMN serum MN2 (1:100) which is reactive to all three orthologs was pre-incubated for 1 h at room temperature with a >20-fold excess of each ortholog antigen (or no antigen) relative to the antigen amount loaded in WB. The mixture was then added to the above WB and proceed as for the regular WB analysis, with detection with HRP-conjugated anti-human IgG4 (Southern Biotech).

2.6. Competition assays between PLA2R1 orthologs and iMN serum by ELISA

ELISA plates were coated with mouse monoclonal anti-HA antibody (Sigma, St. Louis, USA, working dilution 1:5000) in a solution of 20 mM Tris pH 8.0 (100 μL/well) overnight at 4 °C. Plates were blocked for 2 h with SeramunBlock (Seramun Diagnostica, Germany). The detergent-soluble fraction of each ortholog tagged with HA was then added to the wells for 1 h at room temperature, and wells were washed with PBS. iMN serum at 1:100 in PBS-milk 0.1% were pre-incubated with no antigen or 1 μg of each of the three purified orthologs (mPLA2R1, rbPLA2R1 and hPLA2R1) and then added to wells. After 2 h of incubation at room temperature on a plate shaker, the plates were washed 4 times with PBS plus 0.02% Tween 20. Anti-human IgG4-horseradish peroxydase conjugate (Southern Biotech, 1:7500; 100 μl/well) diluted in SeramunStab ST plus (Seramun Diagnostica, Germany) was added and incubated for 1 h at room temperature on a plate shaker. After four washings, enzyme substrate tetramethylbenzidine (TMB) was added, developed for 15 min, and the reaction was stopped with HCl 1.2 N. The plates were read at 450 nm.

2.7. Detection of IgG4 and total IgG anti-PLA2R1 antibodies by ELISA

Pure recombinant PLA2R1 proteins containing the full extracellular domain (approx. 180 kDa, Fig. S1C) were used to coat ELISA plates with a solution of 20 mM Tris pH 8.0 (100 μL/well) at a concentration of 1 μg/mL (rbPLA2R1), 0.7 μg/mL (hPLA2R1) and 0.5 μg/mL (mPLA2R1) at 4° C/overnight. Plates were blocked for 2 h with SeramunBlock (Seramun Diagnostica, Germany). Plates were

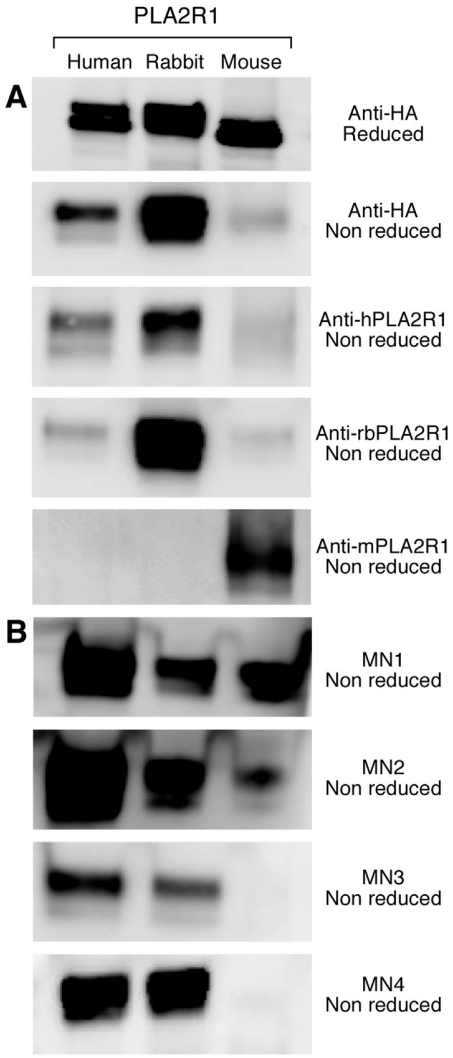

Fig. 2. Cross-reactivity of representative sera from iMN patients to human, rabbit and mouse PLA2R1-HA. Folded PLA2R1 orthologs from the detergent-soluble fraction (30 μg of total protein) were loaded on SDS-PAGE gels under reducing or non reducing conditions and WBs were prepared in parallel. Panel A, signals with anti-HA and cross-reactivity of the three different specific anti-PLA2R1 antibodies under reducing and non reducing conditions showing the loaded amount of folded PLA2R1 orthologs. Note that each of the three anti-PLA2R1 antibodies have different cross-reactivities. Panel B, signals obtained with 4 representative sera out of 53 sera from iMN patients illustrating the cross-reactivity. All 4 iMN sera cross-reacted to rbPLA2R1 (100% of iMN sera) while only iMN1 and iMN2 (about 50% of iMN sera) cross-reacted to mPLA2R1. All mature glycosylated forms PLA2R1 orthologs have molecular masses of around 180–200 kDa.

kept at 4 °C until use (within 5 days). For long-term storage, plates were lyophilized and stored dried at −80 °C. Patient sera were diluted at 1:100 in PBS/0.1% milk and added in duplicate 100-μL aliquots to the ELISA plate, which also contained in duplicate a iMN standard dilution series (to convert results in RU/mL) and a quality control calibrator (between plates). After 2 h of incubation at room temperature on a plate shaker, the plates were washed 4 times with PBS/0.02% Tween 20. Anti-human IgG4-horseradish peroxydase conjugate (Southern Biotech) diluted 1:7,500 for rabbit and mouse ELISA and 1:40,000 for human ELISA in SeramunStab ST plus was added (100 μl per well, (Seramun Diagnostica, Germany) and incubated for 1 h at room temperature on a plate shaker. After four washings, enzyme substrate tetramethylbenzidine (TMB) was added, developed for 15 min, and the reaction was stopped with HCl 1.2 N. The plates were read at 450 nm. Detection of total IgG anti-PLA2R1 were performed as above using a slightly different protocol as described in the online Supplementary information. Sixty-seven sera from healthy donors were used to define the normal range, using mean + 3 SD. The cut-off was optimized by receiver operating characteristics (ROC) curve analysis. A highly positive index patient serum was used in each plate to generate a standard curve consisting of five calibrators: 3,000; 1,000; 333; 111; 37 relative units per mL (RU/mL).

2.8. Detection of anti-PLA2R1 antibodies by IIFT

Anti-PLA2R1 antibodies were detected using the recombinant cell-based indirect immunofluorescence test (RC-IFA, Euroimmun, Germany) containing a BIOCHIP mosaic of formalin-fixed HEK 293 cell over-expressing PLA2R1 and mock-transfected HEK 293 cells as negative control as described[22]. See Supplementary information online for more details.

2.9. Statistical analyses

For descriptive statistics, data are presented as mean ± standard deviation (for variables with Gaussian distribution) or median (ranges) (for variables with non Gaussian distribution). We used the Shapiro–Wilk test to determine if a variable has a Gaussian distribution. Qualitative criteria were compared using Chi-square 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). Bad prognosis was defined at LOCF as defined by a proteinuria >3.5 g/g and/or eGFR <60 mL/min/1.73 m^2. Multivariable cox regression analysis was performed including all clinical parameters with p value < 0.2 in univariate analysis. Odds ratio and p values were calculated with multivariate logistic regression model. Survival curves for renal survival were calculated using Kaplan–Meier estimates for survival distribution. The endpoint for renal survival analysis was a doubling of serum creatinine from baseline. Differences between groups based on epitope profile were analyzed with the log-rank test. All statistics were performed using Prism6 and SAS 9.3 softwares. P-values < 0.05 were considered as statistically significant.

3. Results

3.1. Expression of human, rabbit and mouse PLA2R1 orthologs as folded proteins

The anti-PLA2R1 autoantibodies from iMN patients recognize conformational epitopes and only react against folded hPLA2R1 by western blot under non reducing conditions. It was thus essential to obtain preparations of recombinant PLA2R1 orthologs enriched in folded proteins. Toward this goal, we transiently transfected HEK293 cells with cDNAs coding for full-length, membrane-bound HA-tagged PLA2R1 from human (h), rabbit (rb) and mouse (m) species. After transfection, we lyzed cells and prepared by centrifugation a cytosolic fraction containing soluble proteins and a microsomal fraction containing membrane proteins and cellular aggregates. The microsomal fraction was then solubilized with detergent and centrifuged to obtain a detergent-soluble fraction (containing solubilized and folded membrane proteins) and a detergent-insoluble fraction (containing protein aggregates).

The presence of PLA2R1 orthologs was analyzed in the 4 fractions by WB using anti-HA and three different anti-PLA2R1 specific antibodies under both reducing and non reducing conditions (Fig. 1). All three PLA2R1 orthologs were clearly expressed as membrane proteins, as indicated by the strong signal detected with the anti-HA and the specific anti-PLA2R1 antibodies in the microsomal fraction before and after solubilization, compared to the weak signal in the cytosolic fraction. In general, detection with the anti-HA antibody was stronger under reducing conditions, and this was especially the case for the insoluble fraction, suggesting the presence of PLA2R1 aggregates in this fraction that required strong denaturation and reduction with SDS and beta-mercaptoethanol to migrate as monomers of about 180 kDa (Fig. 1A and B). In contrast, a strong HA signal was observed with the detergent-soluble fraction for human and rabbit PLA2R1 under non reducing conditions, showing that the PLA2R1 protein is properly folded and does not require strong denaturation to migrate as a monomer (Fig. 1B). For all three orthologs, the lower signal observed with the microsomal fraction compared to the detergent-soluble fraction is in line with the fact that the microsomal fraction contains a mixture of folded and misfolded/aggregated PLA2R1 (with a higher ratio of misfolded/aggregated protein for mPLA2R1 and hPLA2R1 as compared to rbPLA2R1). Unexpectedly, we observed that the anti-HA antibody does not react against mPLA2R1 under non reducing conditions in both microsomal and detergent-soluble fractions while it does react under reducing conditions (Fig. 1A and B). However, under the same non reducing conditions, the mPLA2R1 antigen was clearly reactive with the rabbit polyclonal antibody raised against a folded recombinant form of mPLA2R1 (see below and methods), indicating that mPLA2R1 was well transferred to the PVDF membrane in our WB conditions. The fact that the anti-HA antibody can detect mPLA2R1 under reducing conditions indicates that the HA tag is present but likely not exposed under non reducing conditions, possibly because of steric hindrance or a preferred orientation towards the PVDF membrane. Because the HA tag was added to the C-terminal cytoplasmic tail of PLA2R1 orthologs and because mPLA2R1 has a longer and less conserved cytoplasmic tail with a free intracellular cysteine (Cys 1484), which is next to the HA tag and may interfere with HA detection, we prepared and expressed a second mPLA2R1 construct in which the cysteine was mutated into a glycine (Cys1484Gly). Unfortunately, we obtained the same results as for the WT protein, indicating that this cysteine is not responsible for the absence of anti-HA detection under non reducing conditions (not shown). Nonetheless, we could confirm that the mPLA2R1 antigen is properly folded and that the HA tag is in fact exposed in solution by using an ELISA in which mPLA2R1 is bound to the anti-HA antibody precoated to the plastic of the ELISA plate and then reacted with anti-PLA2R1 autoantibodies from iMN serum (see below and Fig. 3).

We also validated that all three PLA2R1 orthologs are present as folded proteins in the detergent-soluble fraction by using a set of three specific anti-PLA2R1 antibodies and comparing the signals measured in the different cell fractions under both reducing and non reducing conditions. We observed that the specific anti-PLA2R1 antibodies have different reactivities against the three orthologs under reducing and non reducing conditions (Fig. 1C and D and Fig. 2A). The commercially available anti-hPLA2R1 rabbit polyclonal antibody (Atlas Antibodies) reacts against hPLA2R1 under both reducing and non reducing conditions (Fig. 1C and D, left panels). This is in line with the fact that this antibody was raised against a long unfolded peptide overlapping the CTLD2 and CTLD3 domains. This antibody cross-reacts weakly with rbPLA2R1 and mPLA2R1 under non reducing conditions (Fig. 2A). The guinea-pig polyclonal antibody was raised against the folded and purified full-length membrane-bound rbPLA2R1 and reacts against both non reduced and reduced rbPLA2R1 (Fig. 1C and D, middle panels). It cross-reacts weakly with hPLA2R1 and mPLA2R1 under non reducing conditions (Fig. 2A). Finally, the rabbit polyclonal antibody that was raised against the full extracellular domain of mPLA2R1 reacts only against non reduced mPLA2R1 (Fig. 1C and D, left panels), in a way that appears similar to autoantibodies from iMN patients. The antibody did not cross-react against hPLA2R1 and rbPLA2R1 under non reducing conditions (Fig. 2A). All together, a careful comparison of the signals obtained with the microsomal fraction before and after detergent solubilization and under reducing and non reducing conditions clearly indicated that the detergent-soluble fraction was enriched in folded PLA2R1 orthologs while the microsomal and detergent-insoluble fractions contained folded PLA2R1 mixed with different amounts of PLA2R1 aggregates (Fig. 1). This was true for all three PLA2R1 proteins in which the detergent-soluble fraction provided the strongest signal under both reducing and non reducing conditions, while the two other fractions showed stronger signals under reducing conditions. Hence, hPLA2R1 and mPLA2R1 were expressed in HEK293 cells as a mixture of a folded membrane-bound receptor that can be readily solubilized with detergent and misfolded proteins that form aggregates and cannot be solubilized with detergents like Triton X-100 or SDS, but require strong solubilization with SDS in the presence of reducing agents (like beta-mercaptoethanol). In line with these results, we found that the insoluble fraction could not be dissolved with urea, strong detergents or a combination of both, but could be dissolved in the presence of reducing agents (not shown). Interestingly, rbPLA2R1 was essentially expressed as a folded receptor with relatively small amounts of misfolded protein (Fig. 1B and C).

3.2. Cross-reactivity of sera from 53 iMN patients to hPLA2R1, rbPLA2R1 and mPLA2R1

We thus used the above detergent-soluble fraction containing folded hPLA2R1, rbPLA2R1 and mPLA2R1 to test the cross-reactivity of 53 sera from iMN patients already known to be positive to hPLA2R1 (selected from the patients listed in Table S1 and with sufficient serum available). To rule out false negative results, we set up WB conditions in which sufficient amounts of folded PLA2R1 orthologs were loaded. The amounts of PLA2R1 antigens from the detergent-soluble fractions were calibrated using the anti-HA antibody and the PLA2R1 specific antibodies (Fig. 2A). Under non reducing conditions, anti-HA or at least one PLA2R1 specific antibody was giving a strong signal, validating the WB conditions. Western blots performed in the same conditions revealed that all 53 sera reacting with hPLA2R1 also recognized rbPLA2R1, with similar or weaker intensities (Fig. 2B). Conversely, only 27 (51%) sera recognized mPLA2R1, of which 14 (26%) showed the same signal intensity as for hPLA2R1 (illustrated by MN1, Fig. 2B) while 13 (25%) exhibited a reduced reactivity with mPLA2R1 (illustrated by MN2, Fig. 2B). The other 26 sera (49%) with high anti-hPLA2R1 activity failed to cross-react to mPLA2R1 (illustrated by MN3 and MN4, Fig. 2B).

3.3. Indirect evidence for multiple epitopes in hPLA2R1 and corresponding autoantibodies in iMN sera by competition assays between PLA2R1 orthologs

The above data raised the possibility that sera from iMN patients are different and contain one or several autoantibodies, with each of these latters targeting distinct epitopes in PLA2R1 that may be conserved or not between human, rabbit and mouse orthologs, thereby leading to different levels of cross-reactivities. This hypothesis is supported by two recent studies showing the presence

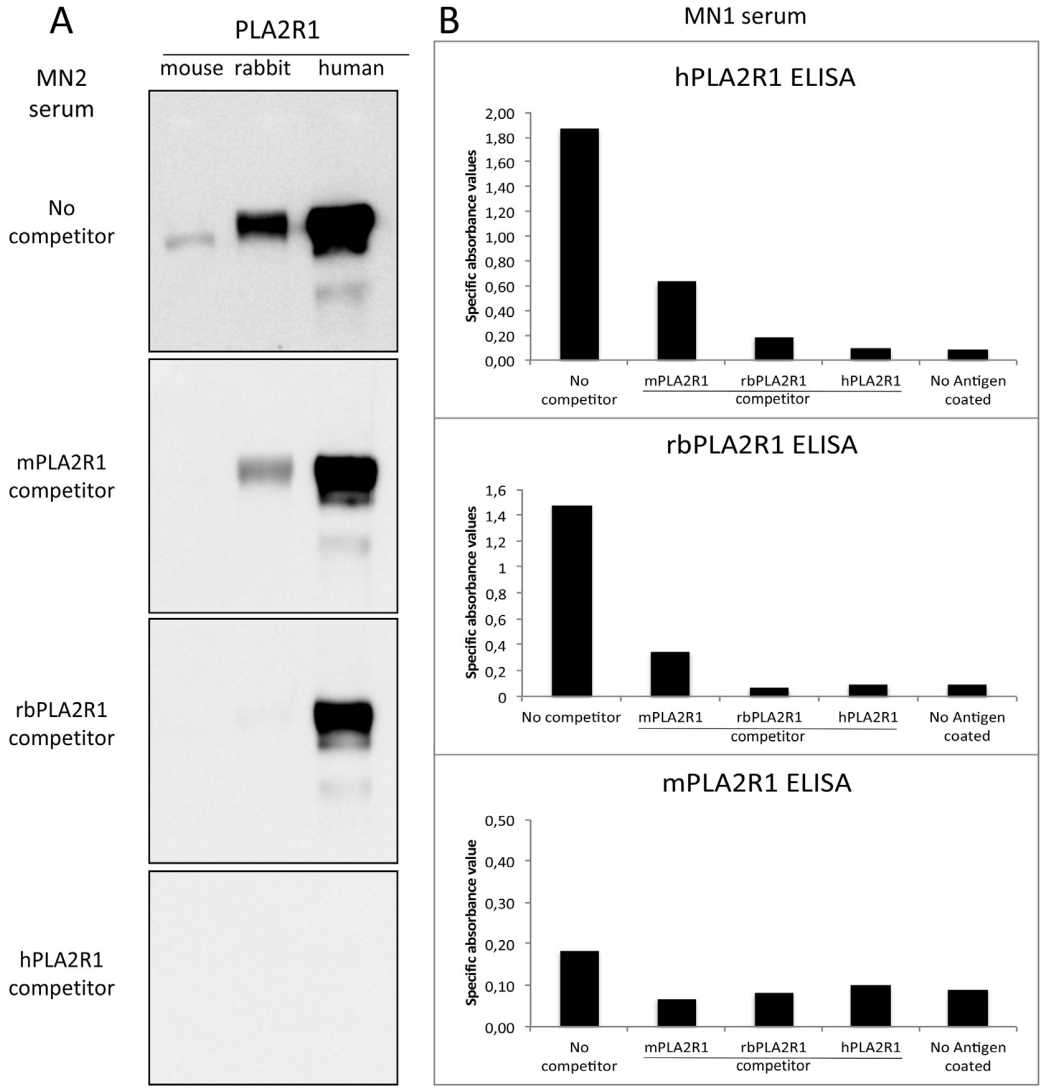

Fig. 3. Competition assays between orthologs of PLA2R1. Panel A, competition by western blot for patient MN2 shown in Fig. 2. Panel B, competition by ELISA for patient MN1 shown in Fig. 2. MN1 and MN2 sera were preincubated or not with an excess of each purified ortholog and then incubated with the antigens loaded on WB or in ELISA plates (see methods for more details).

of at least 2 distinct epitopes in hPLA2R1, yet the reactivity of a full cohort of iMN sera towards these epitopes was not fully investigated [29,30]. To lend credence to our hypothesis of different PLA2R1 epitopes differentially conserved among orthologs, we performed competition assays with two iMN sera that recognize all three receptors by western blot and ELISA in which we bound each orthologs to the PVDF membrane or the well of an ELISA plate and added iMN sera preincubated or not with an excess of soluble orthologs. Fig. 3A illustrates the results obtained by WB. As expected, homologous competition with an excess of each ortholog fully inhibited the signal observed on human, rabbit and mouse PLA2R1. However, heterologous competition with soluble mPLA2R1 and rbPLA2R1 on hPLA2R1 did not fully erase the hPLA2R1 signal, yet rbPLA2R1 was more effective than mPLA2R1. This suggests that hPLA2R1 exhibits several epitopes that are gradually less conserved or lost in rbPLA2R1 and then in mPLA2R1. Similarly, heterologous competition with soluble mPLA2R1 on rbPLA2R1 did not fully erase the rbPLA2R1 signal, suggesting that rbPLA2R1 exhibits more epitopes than mPLA2R1. In agreement with this view, an excess of soluble rbPLA2R1 fully erased the mPLA2R1 signal, but incompletely inhibited the hPLA2R1 signal. These results were confirmed by ELISA competition assays using another iMN serum that recognize all three orthologs (Fig. 3B). For these ELISAs, the antigens, especially mPLA2R1, were adsorbed to the ELISA plates via their HA tag on wells precoated with the anti-HA antibody. We observed that mPLA2R1-HA could be readily bound to the ELISA plate via its HA tag and was recognized by the MN1 serum that was positive in WB (Fig. 2), validating the fact that the antigen was folded and exposed its HA tag in solution, but not after transfer under non reducing conditions (please refer to the above paragraph). As expected, we observed that the signal of the iMN serum on hPLA2R1 antigen is fully inhibited by soluble hPLA2R1, almost completely by soluble rbPLA2R1 but only partially by soluble mPLA2R1. When the iMN serum was reacted with rbPLA2R1, the inhibition was full with both soluble hPLA2R1 and rbPLA2R1, but not with soluble mPLA2R1. Finally, when the iMN serum was reacted with mPLA2R1, the inhibition was complete with all three orthologs. Together, these results suggest again the presence of several epitopes that are gradually less conserved from human to rabbit and mouse species.

3.4. Development of a rbPLA2R1 ELISA and comparison with the standardized hPLA2R1 ELISA

Since rbPLA2R1 was recognized by all patients' sera positive to hPLA2R1, we set up a rbPLA2R1 ELISA as an alternative of the recently described hPLA2R1 ELISAs [13,14]. For this purpose, we produced a soluble form of rbPLA2R1 comprising the full-length extracellular domain of the receptor in HEK293 cells (see Supplementary information and Fig. S1). The purified recombinant rbPLA2R1 was highly reactive against PLA2R1 specific antibody and iMN sera (Fig. S1) and was used to develop a rbPLA2R1 ELISA for detection of both total IgG and IgG4 anti-PLA2R1 (see methods).

We validated the rbPLA2R1 ELISA using a cohort of 130 patients with iMN (93 males and 37 females) and different levels of disease activity (62 in active disease, 38 in partial remission and 23 in complete remission), 23 patients with secondary MN (11 males and 12 females), 67 patients with other diseases and 67 healthy donors. The baseline characteristics are presented in Table S1. IgG4 anti-rbPLA2R1 antibodies were found in 78 out of 130 (60%) patients with iMN and in 6 out of 23 (26%) patients with secondary MN (Fig. 4A). No reactivity was seen in sera from patients with other kidney diseases or healthy donors (Fig. 4A and C).

We then compared the results obtained with the rbPLA2R1 ELISA with the established standardized hPLA2R1 ELISA [14]. Within our cohort, sufficient serum was available from 84 patients with iMN, 18 with secondary MN, 33 controls with other diseases and 18 healthy subjects to compare the performance of the anti-rbPLA2R1 with that of the anti-hPLA2R1 ELISA (for both IgG4 and total IgG) and IIFT (Table S2). There was a good correlation between titers of anti-rbPLA2R1 and anti-hPLA2R1 for both IgG4 anti-PLA2R1 ($r^2 = 0.72$) (Fig. 4B) and total IgG anti-PLA2R1 ($r^2 = 0.82$) (Fig. 4D). IgG4 anti-hPLA2R1 ELISA was positive in 50/84 (60%) sera from iMN patients, all of which were also positive in the IgG4 anti-rbPLA2R1 ELISA (Table S2). The IgG4 anti-rbPLA2R1 ELISA detected 4 more positive patients (54/84, 64%), which were also weakly positive by IIFT (Table S2). RbPLA2R1 and hPLA2R1 IgG4 ELISAs were as sensitive ($p = 0.99$). When measuring total IgG, both assays were identical but less sensitive than the corresponding IgG4 assays. They were both in high concordance with IIFT detecting also total IgG (Table S2). An excellent concordance was also found between all assays when detecting anti-PLA2R1 in patients diagnosed with secondary MN (Table S2). Finally, all PLA2R1 ELISA assays were highly specific (100%) with no positivity in patients with other diseases and healthy controls.

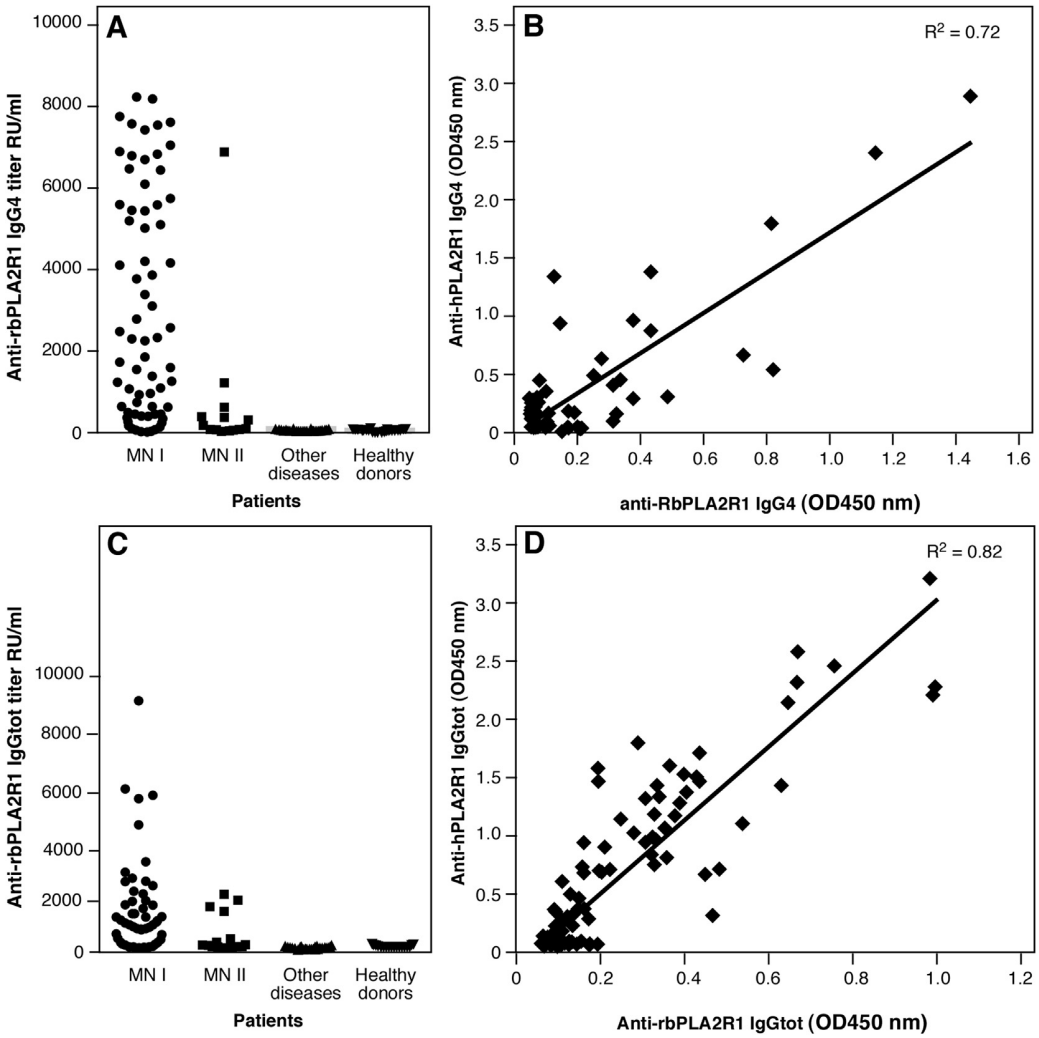

Fig. 4. Specificity of the rbPLA2R1 ELISA for iMN patients and comparison with the standardized hPLA2R1 ELISA. Panel A, IgG4 anti-rbPLA2R1 titers for the cohort of 130 iMN patients, 23 secondary MN, 67 other diseases and 67 healthy controls. Panel B, correlation between IgG4 anti-rbPLA2R1 titers and IgG4 anti-hPLA2R1 titers measured by ELISA. Panel C and D, same as panels A and B, but for total IgG anti-PLA2R1 titers.

3.5. Association of anti-PLA2R1 antibody levels with disease activity and immunosuppressive treatment measured with the rbPLA2R1 ELISA

Several studies have shown an association between anti-PLA2R1 antibody levels and clinically active iMN disease, first by comparing the levels of anti-PLA2R1 in active disease versus spontaneous remission and relapse, second by comparing the levels of anti-PLA2R1 before and after immunosuppressive treatment with rituximab or tacrolimus [8–10,12,13,15,18–20,23,31,32].

In accordance with those studies, we found high levels of anti-rbPLA2R1 autoantibodies in iMN patients with active disease and significantly lower levels in patients in partial or complete remission, particularly when measuring IgG4 anti-PLA2R1 (Fig. 5). Among iMN patients, 7 of them were on dialysis at the time of anti-PLA2R1 assay (Table S1), and 5 had high levels of anti-rbPLA2R1 autoantibodies, indicating that these patients still had a persistent autoimmune disease activity while in ESKD.

We also tested 6 iMN patients for anti-PLA2R1 serum levels during follow-up and treatment with rituximab (Fig. S2). In all cases, rituximab induced a decline or disappearance of anti-PLA2R1 autoantibodies. Changes in antibody levels preceded changes in proteinuria, confirming that a decrease of anti-PLA2R1 antibodies predicted response to immunosuppressive treatment with a better clinical outcome. Two subjects who relapsed during follow-up had a concomitant return of anti-PLA2R1, which was overcome by a second treatment with rituximab. These results are in line with previous data [18,20,32,33] and confirm that measuring anti-PLA2R1 levels helps to follow and predict response to treatment with rituximab in iMN patients.

3.6. High levels of anti-PLA2R1 antibody measured with mPLA2R1, but not hPLA2R1 and rbPLA2R1 ELISAs can predict clinical outcome

Another important area of interest is the evaluation of anti-PLA2R1 levels at the time of first serum sampling in iMN patients to predict clinical outcome. Several studies have evaluated the association between levels of anti-PLA2R1 and patient outcome over several months of follow-up, but different conclusions have been drawn [13,15,16,19,20]. Of note, these studies were performed by IIFT or ELISA using hPLA2R1 as antigen.

Since sera from iMN patients exhibited different cross-reactivities to hPLA2R1, rbPLA2R1 and mPLA2R1 antigens, we explored the prognostic values of anti-PLA2R1 activity measured with each of the three antigens in a retrospective cohort of 41 iMN patients with a mean follow-up of 42 months from anti-PLA2R1 assay (Table 1). Most patients were male (68%), with a mean (±SD) age of 56 years. Most patients did not receive immunosuppressive treatment before serum sampling (34/41, 83%) and presented with severe nephrotic syndrome with a median proteinuria of 4.5 g/g (2.2–15.5) and a median serum creatinine level of 92 μmol/L (54–385). Patients were categorized into two groups at the end of follow-up (Table 2). The first group included those with bad prognosis throughout follow-up, defined as a urinary protein to creatinine ratio (UPCR) > 3.5 g/g or an estimated GFR (eGFR) < 60 mL/min/173 m^2, and the second group included patients who entered into remission, defined as a UPCR <3.5 g/g and eGFR >60 mL/min/1.73 m^2. After follow-up, 21 patients had bad prognosis (14 had progressed to ESKD) and 20 patients were in remission (7 had remitted spontaneously and 13 after various immunosuppressive treatments).

We tested the cross-reactivity of all 41 iMN sera at the time of first serum sampling towards the three PLA2R1 orthologs first by WB (Table 1), then using antigen-specific ELISAs (Fig. 6 and Table 2). All sera that recognized hPLA2R1 in WB analysis also recognized rbPLA2R1, but only 26 out of 41 patients reacted to mPLA2R1 (Table 1). To confirm these results and quantitatively determine the levels of anti-PLA2R1 autoantibodies, we measured the same sera with ortholog-specific ELISAs. Toward this goal, we set up another ELISA for mPLA2R1 using a recombinant soluble form of mPLA2R1 comprising the full-length extracellular domain, as for rbPLA2R1 and hPLA2R1 (Fig. S1C). We validated this recombinant form of mPLA2R1 by WB and sPLA2 binding assays as previously described [27]. As for hPLA2R1 and rbPLA2R1, the mPLA2R1 ELISA was specific for iMN patients and did not recognize any other disease or healthy controls (Fig. 6A). All 41 iMN sera reacted in hPLA2R1 and rbPLA2R1 ELISAs, but only 32 of them (78%) had activity in the mPLA2R1 ELISA (Fig. 6A). Furthermore, in contrast to hPLA2R1 and rbPLA2R1 ELISAs, only a few iMN sera gave a high titer in the mPLA2R1 ELISA. We then analyzed the association between age, gender, proteinuria, serum creatinine, immunosuppressive treatment as well as human, rabbit and mouse PLA2R1 antibody titers at

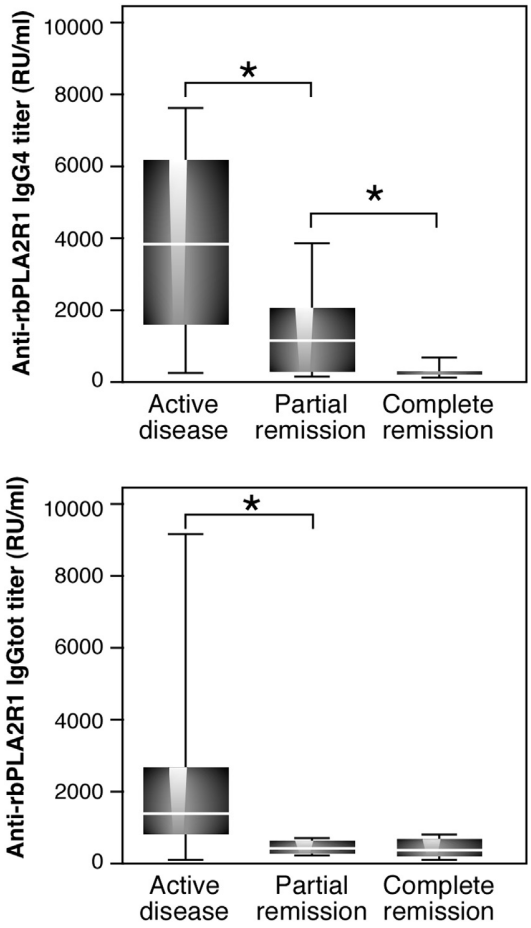

Fig. 5. Anti-rbPLA2R1 titers for IgG4 and total IgG correlate with clinical status in iMN patients. Anti-rbPLA2R1 titers for IgG4 and total IgG were measured in sera from iMN patients with active disease (62 nephrotic patients with a mean proteinuria of 8.05 g/g), in partial remission (38 non-nephrotic patients with a mean proteinuria of 1.93 g/g) or in complete remission (23 patients with a mean proteinuria of 0.16 g/g). The prevalence of anti-rbPLA2R1 antibodies was higher in patients with nephrotic proteinuria (71% with IgG4 anti-rb-PLA2R1 and 60% with total IgG anti-rbPLA2R1) than in patients with proteinuria between 0.5 and 3.5 g/g (52% with IgG4 anti-rb-PLA2R1 and 48% with total IgG anti-rbPLA2R1) and without proteinuria (39% with IgG4 anti-rb-PLA2R1 and total IgG anti-rbPLA2R1), when measuring IgG4 and total IgG anti-rbPLA2R1. The anti-rbPLA2R1 titer between these three groups was significantly different using Kruskal–Wallis test when measuring IgG4 and total IgG anti-rbPLA2R1 ($p = 0.001$, $p = 0.002$, respectively).

Table 1
Characteristics of the 41 iMN patients with 42 months of follow-up from anti-PLA2R1 assay and their WB cross-reactivity to PLA2R1 orthologs.

Characteristic	Result[a]
No. of cases	41
Gender: Female/Male	13F/28M (32/68%)
Age at onset (year)	56 (23–87)
Total follow-up (months)	72 (36–264)
Patients with nephrotic proteinuria at onset	38 (86%)
Proteinuria at onset (g/g)	4.5 (2.0–15.5)
Creatinine at onset (μmol/L)	92 (54–385)
Anti-PLA2R1 assay from onset (months)	30 (0–240)
Follow-up from anti-PLA2R1 assay (months)	42 (12–216)
Patients with WB hPLA2R1 cross-reactivity	41 (100%)
Patients with WB rbPLA2R1 cross-reactivity	41 (100%)
Patients with WB mPLA2R1 cross-reactivity	26 (63%)
Treatment naïve at anti-PLA2R1 assay	34 (83%)
Patients treated after anti-PLA2R1 assay	24 (59%)
Patients with bad prognosis at the end of follow-up: Proteinuria >3.5 g/g or eGFR <60 mL/min/1.73 m^2	21
ESKD at the end of follow-up	14 (67%)
Patients in remission (R) at the end of follow-up: Proteinuria <3.5 g/g and eGFR >60 mL/min/1.73 m^2	20
ESKD at the end of follow-up	0

[a] Normal values are mean ± standard deviation; non normal values are median (ranges); qualitative values are number (%).

first serum sampling and the clinical outcome of the patients, ie those with bad prognosis versus those in remission. Only the anti-mPLA2R1 activity was significantly associated with persistent active disease in both univariate ($p = 0.007$, Fig. 6B) and multivariate analyses ($p = 0.009$) (Table S3). A ROC curve analysis of the mPLA2R1 ELISA titers defined a threshold of 605 RU/mL above which 100% of patients (12 patients) had a poor prognosis (100% specificity, 57% sensitivity, AUC 0.76, $p = 0.004$, 95% IC 0.60–0.91). Accordingly, analysis of renal outcome by the Kaplan–Meier method showed that patients with high titers of anti-mPLA2R1 antibodies had poor renal outcome (Fig. 6C). Indeed, patients with anti-mPLA2R1 antibody titers above 605 RU/mL ($n = 12$) had a higher rate of doubling of serum creatinine ($p < 0.001$ using the log-rank test) than patients with anti-mPLA2R1 antibody titer below 605 RU/mL ($n = 29$) (Fig. 6C). ROC curves could not identify such a threshold for hPLA2R1 and rbPLA2R1 antibody titers and the corresponding subgroups of 12 patients with highest anti-hPLA2R1 or anti-rbPLA2R1 titers (Fig. 6A) did not have a statistically significant poor renal outcome compared to the remaining patients ($p = 0.88$ and $p = 0.21$, respectively) (Fig. 6C).

4. Discussion

The identification of circulating autoantibodies directed towards PLA2R1 has been a major advance in the serological diagnosis of iMN. Anti-PLA2R1 autoantibodies were successively measured by WB, IIFT, ELISA and laser bead immunoassay to monitor iMN disease activity and predict clinical outcome [10,13,14,23,24]. All of these assays used hPLA2R1 as antigen. Here, we tested for the first time the cross-reactivity of anti-PLA2R1 autoantibodies to rbPLA2R1 and mPLA2R1 orthologs and developed two corresponding ELISAs which turned out to have different performances in diagnosis and prognosis of iMN. As discussed below, we also provide indirect evidence for the presence of several autoantibodies in iMN patients, each of them targeting different PLA2R1 epitopes which are conserved or not among human, rabbit and mouse PLA2R1 orthologs.

We first tested the cross-reactivity by WB using a large series of serum samples from 53 iMN patients positive to hPLA2R1. All patients positive to hPLA2R1 were also positive to rbPLA2R1 while only about 50% were positive to mPLA2R1 in the same WB conditions (Fig. 2). To rule out false-negative results due to poor antigen quality, we carefully prepared folded antigens for the three PLA2R1 orthologs and validated the preparations with a panel of specific antibodies in WBs run under reducing and non reducing conditions (Fig. 1). As for mPLA2R1, we also used a commercially available preparation of recombinant receptor and obtained similar results (not shown). When over-expressed in HEK293 cells, we observed that the expression level of rbPLA2R1 is higher than that of hPLA2R1 and mPLA2R1. Furthermore, the ratio of folded versus unfolded protein is higher for rbPLA2R1. Finally, we could establish HEK293 cells stably expressing rbPLA2R1, constitutively and at high level (giving a yield of about 1 mg of folded and purified protein per liter of cell medium) while we could not do it for hPLA2R1 (unpublished data), and hardly for mPLA2R1 [27]. These observations might be reminiscent of the pro-senescent/apoptotic role of hPLA2R1 and mPLA2R1 observed in various normal and cancer cells [34,35]. Together, our data indicate that rbPLA2R1 is a valuable antigen that can be produced at high level as a folded protein and that can replace hPLA2R1 to detect anti-PLA2R1 autoantibodies. On the other hand, the mPLA2R1 antigen is expressed at lower levels, but might be useful in iMN as it detects only a subgroup of patients.

We then developed, validated and tested the diagnostic performances of two distinct ELISAs respectively using purified rbPLA2R1 and mPLA2R1 as antigens. The rbPLA2R1 ELISA was as

Table 2
Characteristics of patients with bad prognosis or in remission at the end of follow-up.

Characteristic[a]	Bad prognosis	Remission	p value[b] (univariate)
Cases	21	20	
Age	61 ± 17	52 ± 12	0.06
Gender: F/M	8F/13M	5F/15M	0.81
Total follow-up (months)	48 (36–264)	48 (36–216)	0.18
Proteinuria at onset (g/g)	4.2 (2.5–15.5)	4.5 (2.0–15.0)	0.30
Creatinine level at onset (μmol/L)	100 (56–385)	84 (54–187)	0.73
Immunosuppressive treatment[c]	11 treated 10 untreated	13 treated 7 untreated	0.41
Follow-up from anti-PLA2R1 assay (months)	36 (12–138)	31 (12–216)	0.54
Proteinuria at anti-PLA2R1 assay (g/g)	4.2 (0.8–20.0)	3.7 (0–11.5)	0.29
Creatinine level at anti-PLA2R1 assay (μmol/L)	90 (57–400)	89 (45–187)	0.73
Anti-hPLA2R1 titer (RU/ml)	4706 (182–5896	2398 (130–5835)	0.66
Anti-rbPLA2R1 titer (RU/ml)	5611 (261–8237)	2366 (134–7766)	0.07
Anti-mPLA2R1 titer (RU/ml)	879 (42–11542)	243 (0–605)	0.007

[a] Normal values are mean ± standard deviation; non normal values are median (ranges); qualitative values are number (%).
[b] t student tests for continuous or Mann-Whitney for non continuous variables (for Gaussian or non Gaussian distribution, respectively) and Chi-square or Fisher's exact tests for categorical variables. NS: not significant, p value > 0.05.
[c] Treated with immunosuppressors. All patients received symptomatic treatment.

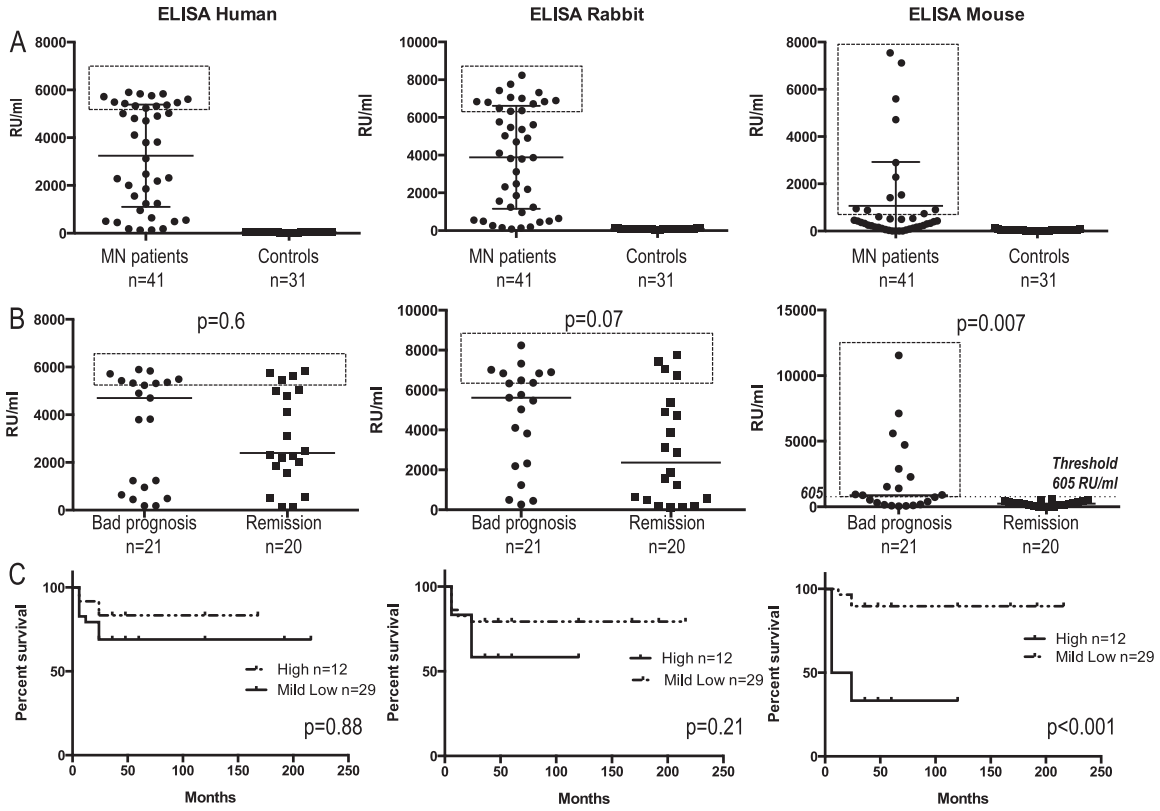

Fig. 6. The specific detection of a subset of anti-PLA2R1 autoantibodies using the mPLA2R1 ELISA can predict long-term clinical outcome in a cohort of 41 iMN patients. Panel A, IgG4 anti-PLA2R1 titers measured in the 41 iMN patients at the time of first serum sample analysis versus controls with the hPLA2R1, rbPLA2R1 and mPLA2R1 ELISAs. None of the 31 other disease control patients showed activity in the three ELISAs. All 41 iMN patients showed anti-PLA2R1 activity in the hPLA2R1 and rbPLA2R1 ELISAs (titers >126 and > 120 RU/mL, respectively), but only 32 of them (78%) in the mPLA2R1 ELISA (titers >126 RU/mL). Panel B, comparison of IgG4 anti-PLA2R1 titers between iMN patients in remission and bad prognosis at the end of the clinical follow-up (mean follow-up of 42 months from anti-PLA2R1 serum sample analysis, see Table 1). Patients in remission (n = 20) had preserved renal function (MDRD >60 mL/min) and remission of nephrotic syndrome (UPCR <3.5 g/g). Patients with bad prognosis (n = 21) had active disease (nephrotic syndrome with urinary protein creatinine ratio (UPCR) > 3.5 g/g) and/or impaired kidney function (MDRD <60 mL/min). There was a large overlap between anti-PLA2R1 activity in the bad prognosis and remission groups in the hPLA2R1 and rbPLA2R1 ELISAs, but not in the mPLA2R1 ELISA. All patients (n = 12) with an mPLA2R1 ELISA titer >605 RU/mL had active disease. Univariate analyses showed that only the anti-mPLA2R1 titer is associated with bad prognosis (p = 0.007). Panel C, survival analysis for doubling of serum creatinine in relation to mild/low versus high (>605 RU/mL; values within the hatched rectangle) mPLA2R1 ELISA titers at serum sampling showed a striking increase in the risk of progression in patients with high titers of anti-mPLA2R1 antibodies (p < 0.001 using the log-rank test, Table 2). No significant difference was observed between the subgroups of 12 patients with highest anti-hPLA2R1 or anti-rbPLA2R1 titers (values within the hatched rectangles) versus patients with mild/low titers for their clinical outcome (p = 0.88 and p = 0.21, respectively).

sensitive as the standardized hPLA2R1 ELISA and appears to be useful to monitor anti-PLA2R1 in iMN diagnosis and clinical follow-up. For instance, titers of anti-rbPLA2R1 antibodies showed a good correlation with disease activity (Fig. 5), and with clinical response to treatment with rituximab (Fig. S2), as previously described for the hPLA2R1 ELISA [10,13,16]. High titers of anti-rbPLA2R1 autoantibodies were also measured in ESKD patients, and the monitoring of anti-PLA2R1 in these patients would help to predict the risk of recurrence after kidney graft [21]. In contrast, the mPLA2R1 ELISA would not be useful for similar clinical investigations as it detects only a subset of iMN patients. However, this latter property likely explains why the mPLA2R1 ELISA turned out to perform better than hPLA2R1 and rbPLA2R1 ELISAs at identifying iMN patients at risk of poor clinical outcome. Former studies using two similar hPLA2R1 ELISAs have suggested that high levels of anti-PLA2R1 antibodies are linked with a higher risk of renal function decline during follow-up [16,19]. Using our retrospective cohort of 41 iMN patients with serum samples available at disease onset and a subsequent clinical follow-up of 42 months, we did not find a significant association between high anti-PLA2R1 titers and clinical outcome using hPLA2R1 and rbPLA2R1 ELISAs (Fig. 6B and C). This might be because of the small size of the cohort. However, we observed that the mPLA2R1 ELISA performed much better than hPLA2R1 and rbPLA2R1 ELISAs at identifying patients at risk of poor clinical outcome. Indeed, patients with high titers of anti-mPLA2R1 antibodies (>605 U/mL) always have a poor renal outcome with a specificity of 100%. If confirmed in independent cohorts, this test may prove useful to identify a subgroup of iMN patients that should benefit from early immunosuppressive treatments.

All our tests were equal or more sensitive with IgG4 detection as compared to total IgG, and none of the sera had total IgG anti-PLA2R1 antibodies without IgG4 antibodies. Since IgG4 anti-PLA2R1 antibodies are more specifically associated with disease activity [8,13], we suggest that IgG4 specific assays may be used to monitor anti-PLA2R1 activity.

We also described in our cohort several cases of secondary MN with anti-PLA2R1 antibodies (6/26, Table S1), especially with HBV or HCV infections. As discussed in previous studies [5,12,36,37], the presence of anti-PLA2R1 antibodies in patients with secondary MN might in fact result from the co-incidental development of iMN with another unrelated systemic disease rather than true cases of secondary MN positive for anti-PLA2R1. For all cases, we did not

find clear clinical evidence for an impact of the other disease on MN activity, supporting iMN cases with a co-incidental other disease. There is more and more evidence that the presence of anti-PLA2R1 is in fact specific for iMN, and used to differentiate iMN from secondary forms [5,12,36,37].

Cross-reactivity and competition studies indicate the presence of several epitopes in PLA2R1 which are differentially conserved between human, rabbit and mouse orthologs (Figs. 2 and 3). In turn, this indicates that iMN sera should contain several corresponding autoantibodies targeting each of these epitopes, with different titers between patients. The two recent studies by Kao et al. and Fresquet et al. who collectively described one or two epitopes in the CysR domain and/or CTLD1 domain as well as a possible other epitope in the CTLD4-CTLD8 region of PLA2R1 are in line with our hypothesis [29,30]. Thus, sera that recognize human, rabbit and mouse PLA2R1 should have one or more autoantibodies directed against one or more conserved epitopes between the three species. On the other hand, sera that recognize human and rabbit but not mouse PLA2R1 should have one or more autoantibodies directed against one or more additional epitopes conserved between the two species but lost in the mouse species. We found that all of the cross-reacting autoantibodies recognize only conformation-dependent epitopes. Indeed, as previously observed for hPLA2R1 [8], iMN sera recognized rbPLA2R1 and mPLA2R1 only under non reducing conditions (not shown). In addition, pretreatment of the 3 PLA2R1 orthologs with chaotropes such as urea (8 M) or high temperature (95° C/10 min) had no effect on detection by several iMN sera (not shown). This indicates that all of the conformational epitopes present in the three orthologs are thermostable and that their three-dimensional structure is maintained by disulfide bonding. All patients positive to hPLA2R1 were positive to rbPLA2R1, but the signal ratio measured on rbPLA2R1 versus hPLA2R1 was different among iMN patients, as illustrated by WBs (Figs. 2 and 3) and the imperfect match in the correlation between rbPLA2R1 and hPLA2R1 titers (Fig. 4). This suggests that iMN patients always share at least one autoantibody targeting a conserved epitope between hPLA2R1 and rbPLA2R1 but may contain other autoantibodies targeting identical, similar or different epitopes. The different titers of the corresponding antibodies among patients will further explain the different signal ratios. As for mPLA2R1, only about 50% of patients positive to hPLA2R1 recognized mPLA2R1 by WB, and this value was maximally increased to 78% when using the more sensitive mPLA2R1 ELISA. The absence of cross-reactivity of the remaining 22% of patients was not due to low sensitivities of the assays because of misfolding of mPLA2R1. Indeed, this was ruled out by using two independent preparations of recombinant mPLA2R1, one from HEK293 cells and another from a commercially available source (see methods). In both cases, we validated that the preparations were folded using specific antibodies in WBs as well as using sPLA2 inhibition assays with the purified mPLA2R1 (Fig. 1 and [27]). Compared to rbPLA2R1, the absence of cross-reactivity for some patients to mPLA2R1 suggests that this latter receptor has a lower number of shared epitopes with hPLA2R1 and that patients' sera have different titers of the corresponding autoantibodies.

The exact nature and location of the PLA2R1 epitopes recognized by the patients' autoantibodies on the different orthologs are currently unknown. Besides the above studies by Kao et al. and Fresquet et al. [29,30], one more study has suggested the presence of several epitopes in PLA2R1, but the identification of these molecular epitopes should be considered with caution, as the conformational property of the epitopes was not considered in this work and none of the epitopes were validated when introduced in the context of the tertiary structure of folded PLA2R1 [38]. The level of protein sequence identity between hPLA2R1 and rbPLA2R1 is 85%, and falls to 75% with mPLA2R1. This fits with the better cross-reactivity of anti-PLA2R1 autoantibodies to rbPLA2R1 than mPLA2R1. However, among the 10 domains of PLA2R1 constituting its large extracellular region, we could not identify any obvious domain or region more highly conserved than another between the orthologs, thereby precluding easy information on the location of the possible epitopes. The identification of the different epitopes targeted by the different anti-PLA2R1 autoantibodies in the three receptors should thus await future studies.

Regardless of the aforementioned scenarios on diversity and titers of autoantibodies for each patient and the corresponding PLA2R1 epitopes, the clinically important finding inferred from our study is that iMN patients exhibit different subsets of anti-PLA2R1 antibodies, with some of these latter antibodies more preferentially associated with clinically active disease and likely pathogenicity. This view would explain some of the discrepancies between anti-PLA2R1 titers measured with the hPLA2R1 antigen and proteinuria [11–13,20]. Based on their cross-reactivity and ELISA titers towards the three orthologs, patients can be classified into subgroups, with an impact on their clinical outcome and treatment. More specifically, the subgroup of patients having high titers in the mPLA2R1 ELISA would have a bad renal prognosis and should be treated with immunosuppressors. This would also suggest that a few epitopes conserved from humans to mouse are more centrally involved in pathogenicity. Finally, it will be also important to consider our results on the cross-reactivity of anti-PLA2R1 autoantibodies when developing animal models of iMN disease.

5. Conclusion

We conclude and summarize our findings as follows: rbPLA2R1 likely shares with hPLA2R1 many of the PLA2R1 epitopes and thus rbPLA2R1 is a suitable alternative antigen to develop a general anti-PLA2R1 ELISA. On the other hand, mPLA2R1 likely shares with hPLA2R1 only a few epitopes and mPLA2R1 then becomes a unique antigen to develop a more specific anti-PLA2R1 ELISA that would notably help to identify patients at risk of poor renal prognosis. We infer that these patients would specifically exhibit one or more autoantibodies directed against one or more epitope(s) conserved between the three orthologs. These autoantibodies might be the pathogenic ones. The prognostic and diagnostic performance of the mPLA2R1 epitope specific assays should now be confirmed in larger cohorts. This would also allow designing a prospective randomized trial to test whether patients with specific anti-mPLA2R1 autoantibodies detected prospectively should receive an early immunosuppressive treatment.

Disclosure

The authors declare no conflicts of interest.

Acknowledgments

We are grateful to Joel Polidori and Franck Aguila for expert technical assistance. Supported by grants from CNRS (including a contrat d'interface CNRS-CHU de Nice to BSP), the French Government (National Research Agency, ANR) through the “Investments for the Future” LABEX SIGNALIFE: programme reference #ANR-11-LABX-0028-01, the Fondation pour la Recherche Médicale (FRM ING20140129210), and the Centre Hospitalier Universitaire de Nice and the Direction générale de l'offre de soins of the French Ministry of Health (PHRC 2011-A01302-39, NCT01897961).

Appendix A. Supplementary information

Supplementary information related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.08.007.

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