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Unmet Challenges in Membranous Nephropathy

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Abstract

Purpose of review: Despite major advances in since the discovery of the phospholipase A2 receptor (PLA2R) as the major autoantigen on podocytes in primary membranous nephropathy (MN), there are still several unanswered questions as highlighted here.

Recent findings: A substantial body of literature, included in more than 680 papers since 2009, has documented genetic susceptibility to primary MN involving *PLA2R1* and class II MHC alleles, the clinical value of anti-PLA2R assays, the significance of epitope spreading of the anti-PLA2R response, discovery of thrombospondin type I domain containing 7A (THSD7A) as a minor antigen in primary MN, and the ability to transfer disease into mice by infusion of anti-THSD7A sera. However, the normal physiology and pathophysiology of PLA2R and THSD7A in podocytes is still unknown and the genetic influence on disease susceptibility is unexplained. We still don't know what causes loss of self-tolerance to PLA2R and THSD7A or how the autoantibodies, which are predominantly of the IgG4 subclass, cause podocyte injury and proteinuria. Complement deposits are prominent in MN, but we are still uncertain how the complement system is activated and whether or not it plays a role in podocyte damage. Notwithstanding the advances over the past decade, our treatments have not changed substantially.

Summary: This review identifies opportunities to extend the advances that have been made to better understand the immunopathogenesis and genetic basis of primary MN and apply the knowledge to design more specific therapies.

Keywords

Membranous nephropathy; Phospholipase A2 receptor (PLA2R); Thrombospondin type I domain containing 7A (THSD7A); IgG4 autoantibodies; Epitope spreading; Class II MHC alleles

Introduction

There have been very substantial advances in the field of human membranous nephropathy (MN) over the past decade since the description of transplacental passage of alloantibodies to neutral endopeptidase (NEP) in a patient with neonatal MN [1] and the discovery of the

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Conflicts of interest

Dr. Salant is co-inventor on a patent entitled "Diagnostics for Membranous Nephropathy" and he receives royalties from EUROIMMUN for an immunoassay for anti-PLA2R. Dr. Salant is also co-author of chapters in Up-To-Date on Membranous Nephropathy for which he receives royalty payments.

M-type phospholipase A2 receptor (PLA2R) as a major target autoantigen on podocytes in primary MN [2], however there is still much to be discovered (Table 1). We have learned about genetic susceptibility and especially the role of class II MHC alleles [3]; anti-PLA2R assays are being used almost universally for diagnosis, treatment monitoring, and prognostication, and PLA2R in relation to MN has been cited in more than 680 papers since 2009. We are beginning to appreciate the clinical significance of epitope spreading of the anti-PLA2R response [4], and thrombospondin type I domain containing 7A (THSD7A) has been identified as a second, albeit minor antigen, in primary MN [5], as well as its possible role in cancer-associated MN [6*]. Importantly, the expression of THSD7A on rodent podocytes has enabled fulfillment of the 3rd of Koch's postulates and established the pathogenicity of the autoantibodies, namely infusion of anti-THSD7A sera into mice produced MN-like immune deposits and proteinuria [7**]. So much for the achievements, what are the remaining challenges?

What is the physiological function of PLA2R and THSD7A in the kidney and how do anti-PLA2R and anti-THSD7A autoantibodies cause podocyte injury and proteinuria?

We still don't know how antibodies to PLA2R and THSD7A cause podocyte injury in vivo and whether this is a direct effect or whether other agents, such as complement are involved as in the Passive Heymann Nephritis (PHN) model in rats (reviewed in [8]). We also don't know what PLA2R and THSD7A are doing on human podocytes, neither their physiological functions nor after ligation by the autoantibodies. We have some clues about cell-matrix adhesion and cytoskeletal alterations from vitro studies with THSD7A [7] but the relevance to what is happening in vivo has yet to be established.

Although PLA2R was originally identified as a receptor for small, secreted phospholipase A2s, perhaps for their detoxification, its physiological function in tissues in general, and in glomeruli in particular, remains an enigma. Though variably expressed in other organs in different species, PLA2R is expressed most highly on human podocytes but not on the podocytes of most other species. In cell culture systems it has been shown to undergo constitutive recycling, pH-dependent conformational changes, and to promote replicative senescence, mammary epithelial tumor suppression, matrix adhesion and binding and internalization of collagen and fibronectin (reviewed in [9**]). Whether or not any of these functions contribute to human podocyte structure and function is unknown. The fact that rodent podocytes that normally lack PLA2R form a perfectly healthy filtration barrier argues for a redundant function in humans but one that renders susceptible individuals prone to autoantibody-mediated injury.

Likewise, the physiological role of THSD7A in podocytes is unclear. It was originally identified in placental vessels and in human umbilical vein endothelial cells (HUVECs) where it was shown to co-localize with $\alpha(V)\beta(3)$ integrin and paxillin, link to the actin cytoskeleton and regulate endothelial migration [10]. Interestingly, although THSD7A was also demonstrated in the neural tube of developing zebrafish embryos along the growth path of angiogenic intersegmental vessels and in intestinal vessels [11, 12], THSD7A is not

expressed on glomerular endothelial cells [5]. Nonetheless, the ability of thrombospondin type 1 repeats to engage in cell-cell and cell-matrix interactions, as well as the studies with THSD7A in HUVECs and zebrafish, suggest that THSD7A may contribute to podocyte adhesion to the GBM. Moreover, it is possible that proteolytic cleavage and release of soluble THSD7A during antibody-mediated injury may influence such adhesion as has been shown in HUVECs [12] and thereby cause podocyte foot process effacement.

There are two possible mechanisms by which autoantibodies to PLA2R or THSD7A might cause podocyte injury. The first would implicate the autoantigens as passive targets for the assembly of immune complexes that activate complement as in the case of megalin expressed on rat podocytes in the PHN model of experimental MN (discussed below). The other would involve a direct effect of the autoantibodies on the target antigens. Support for this latter mechanism in the case of PLA2R comes from a study showing that patient sera containing anti-PLA2R inhibited the binding of PLA2R-transfected HEK293 cells and podocytes to collagen type 4 [13]. However this finding is at odds with other studies that failed to show PLA2R binding to collagen types 1 or 4 [14]. The case is somewhat stronger for a direct mechanism with THSD7A as mice injected with human serum containing anti-THSD7A autoantibodies developed proteinuria and subepithelial immune deposits containing human IgG despite the absence of complement deposits until much later in the course [7]. Moreover, primary cultured mouse podocytes exposed to affinity-purified anti-THSD7A IgG from a patient with MN exhibited alterations in the actin cytoskeleton and activation of focal adhesion complexes [7].

What have we learned from genetics in MN?

Excitement followed the report of a genome-wide association (GWA) study by a UK and European consortium identifying a high risk of idiopathic MN in patients carrying *HLA-DQA1* and an intronic single-nucleotide polymorphism (SNP) in *PLA2R1* [3]. Remarkably, genetic polymorphisms in *PLA2R1* and HLA-D alleles confer a very high risk of primary MN in Caucasians and South Asians, however we still don't know what it is about genetic variation in *PLA2R1* that confers a 4 fold increased risk of primary MN in Caucasians and, when combined with homozygosity for HLA-DQA1, increases the risk to 80 fold. Whole exome sequencing, additional GWA studies and genotyping have identified additional intronic SNPs, but they have not revealed any significant MN-specific mutations [15–17] and the variants in the coding region that have been identified are common in the general population [18, 19]. Rather than altering the amino acid composition or conformation of PLA2R by rare and disease-specific coding mutations, genetic susceptibility might instead reside within non-coding mutations in the promoter region to influence the level or site of expression or conformation of PLA2R to enable engagement by HLA-D molecules on antigen presenting cells (APCs) or the exposure of nephritogenic epitopes for autoantibody access.

The association of MHC class II with MN has been recognized since 1979 [20] and the HLA-DQA1 connection was first reported in 1989 [21]. This is not all that surprising given the known risk of autoimmunity linked to HLA-DQ and HLA-DR. While the HLA-D risk alleles for MN differ among ethnic groups, the interaction between *PLA2R1* and HLA-D is

strong across different ethnicities. Rather than HLA-DQA, studies of Han Chinese patients with MN from Beijing identified DRB1*1501 and DRB1*0301 as risk alleles for MN [22*]. Another study of Han Chinese patients with PLA2R-associated MN reported a strong association with DRB3*0202 [23*], which probably accounts for the DRB1*0301 signal as they are on the same haplotype. Based on *in silico* modeling studies, the Beijing group reported on three amino acids in the antigen-binding pocket of the HLA-DR beta1 chain that might help to present PLA2R T cell epitopes to activate the immune response, and the authors have suggested that this confers a genetic susceptibility to environmental factors such as industrial pollution [24]. While these are encouraging observations, the functional studies to document immune cell activation and identification of T cell epitopes have yet to be done.

Thus, while the risk polymorphisms in PLA2R1 are highly specific for primary MN, the explanation for the increased risk is still unknown. On the other hand, although the association with HLA-D risk alleles is less specific for MN and confers a more general propensity to autoimmunity, the inheritance of *PLA2R1* risk polymorphisms on such a background confers a greatly increased risk of developing primary MN. Future studies will need to examine this interaction in detail by defining the T cell epitopes on PLA2R, the interaction with antigen-specific T helper cells, and the possibility that non-coding SNPs alter PLA2R expression.

What triggers the loss of self-tolerance to PLA2R?

The site of initial exposure of PLA2R to the immune system has not been identified, and it is unlikely that it occurs on the podocyte. A recent increase in the incidence of MN in parts of China has been attributed to environmental air pollution, especially in regions with the highest level of pollution with PM_{2.5} particles [25*]. The mechanisms for this are still undefined but gene/environmental interactions and release of pro-inflammatory cytokines have been proposed [24]. Although human PLA2R is most strongly expressed in kidney (and in glomerular podocytes in particular), it is also expressed in lung, placenta, liver and skeletal muscle [26]. Thus, there is the potential that up-regulation of PLA2R in the lung in response to airway damage from environmental pollutants could be the site of its interaction with antigen presenting cells that abound in the inflamed lung. Molecular mimicry resulting from exposure to an antigen from a microorganism that shares morphological, but not necessarily sequence, homology to PLA2R has also been proposed as a trigger for anti-PLA2R reactivity. With regard to linear sequences of PLA2R that could potentially serve as a T cell epitope, an amino acid sequence (LTLENCK) contained within a peptide that forms part of the dominant epitope in the N-terminal cysteine-rich (ricin) domain of PLA2R also exists in D-alanyl-D alanine carboxypeptidase, a cell wall enzyme in several bacterial strains, including *Clostridia* species [27]. It has not been reported yet if anti-PLA2R autoantibodies actually react with carboxypeptidase but the concept has merit nonetheless.

Are IgG4 autoantibodies to PLA2R and THSD7A truly pathogenic?

We know that IgG4 is the predominant circulating anti-PLA2R and anti-THSD7A IgG isoform in most cases of primary MN, and the glomerular immunize deposits and

glomerular eluates also contain mostly IgG4 [2, 5], but we still don't know if IgG4 alone is truly pathogenic or requires cooperation with lesser amounts of IgG1 or IgG3. Reasons to question the pathogenic role of IgG4 are that it may undergo Fab arm exchange, meaning that each antigen-binding arm may recognize a different antigen, which renders the IgG essentially monovalent for reactivity to PLA2R or THSD7A. This would limit the ability of the antibody to cross-link the antigen and form large lattice immune complexes [28]. Another characteristic of IgG4 is its inability to bind C1q and activate the classical complement pathway, yet there is typically abundant C3 present in the glomerular immune deposits (discussed below). Experimental studies have not yet resolved this issue in primary MN. There is no suitable experimental model to test if purified anti-PLA2R IgG4 is able to form immune deposits in vivo with or without cooperation with IgG1 or IgG3, and the reported in vivo mouse studies with anti-THSD7A used unfractionated serum or IgG. Although IgG4 was present within the immune deposits, it is not known if there was also human IgG1 or IgG3 [7].

Primary MN is not unique in being an IgG4-associated autoimmune disease. Indeed IgG4 autoantibodies have been shown to be pathogenic in passive transfer studies in a form of myasthenia gravis (MuSK-MG) caused by antibodies to a muscle-specific kinase, as well as in pemphigus due to anti-desmoglein antibodies and in thrombotic thrombocytopenic purpura due to antibodies to ADAMTS13 [28]. In these conditions the effect of the monovalent antibodies is believed to be due to disruption of protein-protein, receptor-ligand or enzyme-substrate interactions [28]. It is also conceivable that IgG4 anti-PLA2R and anti-THSD7A could have a partially protective effect by interfering with IgG1 or IgG3 lattice formation. These issues need resolution, especially if one were to consider immunoabsorption therapy to deplete IgG4, a not implausible strategy considering the low levels of IgG4 in circulation.

Is complement a therapeutic target in primary MN?

There is compelling evidence to suggest that complement plays an important role in human MN (Table 2). Functional evidence derives from studies of the heterologous phase in PHN induced by complement fixing sheep anti-Fx1A, which causes podocyte injury and proteinuria by assembly of C5b-9 [29]. There is also experimental evidence in MN that complement regulatory proteins (CRPs) may be compromised. Thus, Schiller et al showed that neutralization of cell surface CRPs was essential for the development of proteinuria in Heymann nephritis [30, 31], and Couser et al found that administration of a soluble form of the complement inhibitor CRI reduced proteinuria in PHN [31]. Evidence that complement is responsible for podocyte injury in human primary MN is more circumstantial and includes glomerular deposition of C3, C4 and C5b-9 (reviewed in [32]) and urinary excretion of C5b-9 in the active phase of MN [33, 34]. Interestingly, C1q is typically absent from the deposits, which is consistent with the inability of IgG4 to bind C1q and suggests that the alternative pathway (AP) or lectin pathway (LP) may initiate complement activation. The presence of C4 points to LP activation, however a case of MN with deficiency in mannose binding lectin (MBL) [35*] and another with ficolin-3 deficiency [36], two major activators of the LP, have been reported. While the LP may activate complement in most cases, the deposition of components of the AP in the MBL-deficient case suggests that the AP might

also be activated directly by IgG4 anti-PLA2R [35]. MBL and the ficolins bind to acetylated glycans such as N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) and form complexes with MBL-associated serine proteases (MASPs) to cleave and activate C4. It is noteworthy that immunoglobulins in which both arms of the biantennary N-glycan on the Fc domain lack a terminal galactose residue exposes GlcNAc residues, which allows MBL to directly bind IgG and activate the LP [37]. We have presented in vitro evidence that that IgG4 PLA2R is able to bind MBL and activate C4 and, at least in some cases is hypogalactosylated [32]. Similar findings have been reported in other preliminary studies [38], however confirmation that anti-PLA2R or anti-THSD7A are able to activate complement in vivo is still lacking.

One might asked why this is important. Although we are able to induce clinical and immunological remission in a fairly high proportion of patients with primary MN using immunosuppressive drugs, most remissions are partial. I believe this is because immunosuppression takes time to have its effect as shown by Beck et al [39]. Immunological remission may take several months; in the meantime there is ongoing podocyte injury and glomerular basement membrane remodeling which reduces the potential for complete restoration of a normal filtration barrier. This creates a window of opportunity to prevent ongoing injury by inhibiting complement until immunosuppressive therapy takes effect and eliminates the autoantibodies. It is conceivable however, that complement deposition is an epiphenomenon (Table 2). There is experimental data in C6-deficient rats with PHN that podocyte injury and proteinuria can occur in the absence of C5b-9 [40]. Moreover, injection of human anti-THSD7A autoantibodies in mice caused proteinuria and immune deposits without C3 deposition [7]. Therefore, before undertaking clinical trials with one or more of the many complement inhibitors and regulators that have been developed, we need more concrete evidence that the autoantibodies in primary MN are indeed responsible for complement-mediated injury in vivo.

Are there prospects for targeted immunotherapy in primary MN?

As noted earlier, our therapeutic strategy in MN has hardly changed over the past three decades. Diuretics, inhibitors of the renin-angiotensin system, statins, anticoagulants and vitamin D replacement are helpful to control the symptoms and complications of the nephrotic syndrome, however we still rely on global immunosuppression to treat the disease. None of the discoveries and advances in the field has changed the way we treat patients. We have learned to select patients for treatment and monitor the response more intelligently but our treatment is still non-specific. Even rituximab targets all CD20-positive B cells rather than only those responsible for anti-PLA2R or anti-THSD7A.

The emergence of engineered T cell-based therapy for the treatment of B cell lymphomas and leukemias, so-called CAR-T cell therapy, has real potential in autoimmune diseases with the use of chimeric autoantigen receptor expressing T (CAAR-T) cells to eliminate the B cells that specifically express the B cell receptor for PLA2R2 or THSD7A and thereby prevent the generation plasmablasts responsible for production of the autoantibodies. The feasibility of this approach has been pioneered in an experimental model of pemphigus vulgaris to eliminate B cells expressing the receptor for the target antigen, desmoglein and

reverse skin blistering [41**]. Knowledge of the nephritogenic epitopes on PLA2R, including those that portend a more aggressive course, would enable production of a panel of CAAR-T cells to target the oligoclonal B cell repertoire. As appealing as this concept and its technical feasibility are with current technology, such therapy for non-lethal autoimmune diseases like MN is not without potential drawbacks. While the cytokine release and tumor lysis syndromes reported with CAR-T cell therapy for lymphoma and leukemia [42] are unlikely to occur in MN and similar autoimmune diseases because of the much smaller target cell burden, it remains to be determined if the pre-conditioning myeloablative protocols used for CAR-T cell treatment of lymphoma are necessary to create a niche for the infused CAAR-T cells to engraft and expand. Such preconditioning may be as or even more toxic than current immunosuppressive protocols that are effective in inducing remission and prolonging kidney survival. In addition, the lack of a suitable preclinical model to test the in vivo efficacy of CAAR-T cell therapy of PLA2R-associated MN is a significant limitation before proceeding to human trials.

Restoration of self-tolerance in autoimmune diseases has been a long-standing aspirational goal that appears to be a realistic possibility [43*]. Studies in type 1 diabetes [44**], multiple sclerosis [45] and Graves disease [46] have demonstrated the potential to induce antigen-specific protective immunity while avoiding non-specific immunosuppression using a desensitization technique in which antigen-derived peptides are coupled to the cognate class II MHC and presented to CD4-positive T cells in the absence of co-stimulatory signals derived from APCs. This converts the T cells into T regulatory cells that suppress APCs and induce B regulatory cells, which quell the immune response in an antigen-specific way [43]. Making the reasonable assumption that the initiation of the immune response in MN is driven by CD4-positive T cells engaging class II MHC molecules and our expanding knowledge of the nephritogenic PLA2R epitopes and MHC class II alleles, there is potential to induce antigen-specific protective immunity in MN by employing PLA2R peptides coupled with the relevant class II MHC molecules. Such therapy has advanced to clinical trials in relapsing multiple sclerosis [45].

In conclusion

I believe there are real opportunities to extend the advances that have been made over the past decade to better understand the immunopathogenesis and genetic basis of primary MN and apply the knowledge to design new and more specific therapies while avoiding their potential harmful effects.

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Key points:

- Autoantibodies to PLA2R and THSD7A account for about 80% of cases of primary membranous nephropathy (MN) but the mechanisms of injury remain undefined.
- Genetic association with PLA2R1 and HLA-D class II MHC alleles confers a high degree of susceptibility to primary MN but the basis for the susceptibility and mechanisms of loss of self-tolerance to the autoantigens is as yet unknown.
- There are abundant deposits of complement in the glomeruli together with IgG4 autoantibodies to PLA2R or THSD7A but it is uncertain how the complement system is activated and whether or not the IgG4 antibodies are pathogenic.
- The discoveries open the way to develop more specific immunotherapies but some obstacles still remain.

Table 1:

Recent advances and remaining challenges in primary membranous nephropathy

Advances since 2009:	Remaining challenges:
<ul style="list-style-type: none">• Anti-PLA2R assays are used widely• Genetic susceptibility associated with <i>PLA2R1</i> and class II MHC• Clinical significance of PLA2R epitope spreading• THSD7A identified as a second (minor) antigen• Possible role of THSD7A in cancer-associated MN• 3rd of Koch's postulates fulfilled for THSD7A in rodents	<ul style="list-style-type: none">• Physiological role of PLA2R and THSD7A on podocytes• Podocyte response to injury by anti-PLA2R and anti-THSD7A• Genetic influence on disease susceptibility• Influence of environmental factors• Mechanisms of antibody-induced injury• Defining the pathogenicity of IgG4 anti-PLA2R and anti-THSD7A• Mechanisms of GBM disorganization• Role of complement• No new therapies

Table 2:

Factors in favor and against a role for complement in primary membranous nephropathy

In favor:	Against:
<ul style="list-style-type: none"> • C4 – C5b-9 are present in human primary MN • Urine C5b-9 excretion in active MN • In vitro – anti-PLA2R-dependent complement activation • PHN model – C5b-9-dependent podocyte injury • AP dysregulation favors Heymann nephritis 	<ul style="list-style-type: none"> • IgG4 doesn't activate C1q • Human anti-THSD7A did not cause C3 deposits in mice • C6-deficient PHN rats get proteinuria

AP: alternative complement pathway; PHN: passive Heymann nephritis