Transglutaminase is essential for IgA nephropathy development acting through IgA receptors

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IgA nephropathy (IgAN) is a common cause of renal failure worldwide. Treatment is limited because of a complex pathogenesis, including unknown factors favoring IgA1 deposition in the glomerular mesangium. IgA receptor abnormalities are implicated, including circulating IgA–soluble CD89 (sCD89) complexes and overexpression of the mesangial IgA1 receptor, Tfr1 (transferrin receptor 1). Herein, we show that although mice expressing both human IgA1 and CD89 displayed circulating and mesangial deposits of IgA1–sCD89 complexes resulting in kidney inflammation, hematuria, and proteinuria, mice expressing IgA1 only displayed endocapillary IgA1 deposition but neither mesangial injury nor kidney dysfunction. sCD89 injection into IgA1-expressing mouse recipients induced mesangial IgA1 deposits. sCD89 was also detected in patient and mouse mesangium. IgA1 deposition involved a direct binding of sCD89 to mesangial Tfr1 resulting in Tfr1 up-regulation. sCD89–Tfr1 interaction induced mesangial surface expression of Tgase2 (transglutaminase 2), which in turn up-regulated Tfr1 expression. In the absence of Tgase2, IgA1–sCD89 deposits were dramatically impaired. These data reveal a cooperation between IgA1, sCD89, Tfr1, and Tgase2 on mesangial cells needed for disease development. They demonstrate that Tgase2 is responsible for a pathogenic amplification loop facilitating IgA1–sCD89 deposition and mesangial cell activation, thus identifying Tgase2 as a target for therapeutic intervention in this disease.
the joining (J) chain. In healthy individuals (contrary to other species like the mouse), circulating IgAs are essentially monomeric. IgA receptors (IgARs) have been proposed to play a role in IgAN pathogenesis (Monteiro et al., 2002). Within the family of multiple IgARs, the myeloid FcαRI (CD89) and TIR1 (transferrin receptor 1; CD71) were identified as putative pathogenic factors in IgAN patients with altered expression on monocytes (Grossetête et al., 1998) and mesangial cells (Moura et al., 2001), respectively. Although CD89 shedding from myeloid cells results in pathogenic soluble forms complexed to IgA (Launay et al., 2000), TIR1 is overexpressed on mesangial cells after IgA1 complex deposition (Haddad et al., 2003). Deposits of IgA1 immune complexes in the mesangium could thus be formed through interaction of these complexes with the mesangial TIR1, but this could not be experimentally demonstrated in vivo because of the lack of a valid animal model reproducing the human IgA1 system. Previously, we have shown that transgenic (Tg) mice expressing the human CD89 on monocytes/macrophages display mouse IgA–human CD89 interaction on these cells and spontaneously develop mouse IgA deposits in their mesangium at 24 wk (Launay et al., 2000). However, it has been claimed that mouse IgAs fail to bind to human CD89 in vitro (Pless et al., 1999) and that injection of soluble CD89 (sCD89) does not induce mouse IgA deposition in the mesangium (van der Boog et al., 2004). The role of mouse IgA–human CD89 complexes in IgAN development in CD89Tg mice was indirectly demonstrated by serum transfer experiments from CD89Tg into BALB/c mice, leading to disease development, which was lost by anti-CD89 immunoabsorption (Launay et al., 2000). More recently, patients with severe IgAN were shown to present decreased levels of IgA–sCD89 complexes in the circulation (Vuong et al., 2010). Whether sCD89 plays a deleterious or protective role in IgAN pathogenesis is a question that has been raised (Monteiro et al., 2002). Within the family of multiple IgARs, the myeloid FcαRI (CD89) and TIR1, involves TGase2 as a key player in the complex pathogenic mechanism of this disease.

RESULTS

Mice expressing human IgA1 and CD89 developed early mesangial IgA1 deposits with hematuria and overt proteinuria

The IgA system differs between humans and mice (Kerr, 1990). Although humans display two IgA subclasses and a myeloid IgAR belonging to the Fc receptor family, the CD89 (or FcαRI), mice have only one IgA subclass differing from the human counterpart by its shorter hinge region (where O-glycosylation occurs) and lack a CD89 homologue (Monteiro and Van De Winkel, 2003). Therefore, to establish a model that mimics human IgAN, we backcrossed CD89Tg mice with α1KI mice, which produce significant amounts of human IgA1 in the circulation (0.5–1 mg/ml; Duchez et al., 2010). Although both α1KI and α1KI-CD89Tg mice presented glomerular deposits of IgA1 at 12 wk of age, α1KI-CD89Tg mice displayed typical mesangial IgA1 deposits, whereas α1KI mice exhibited diffuse endocapillary deposits of IgA1 (Fig. 1 A). The latter was demonstrated by confocal immunofluorescence analysis showing extensive colocalization of the vascular marker CD31 with IgA1 deposits within the capillary walls of α1KI but not α1KI-CD89Tg mice (Fig. 1 A). Moreover, dense materials were observed in the mesangial area for α1KI-CD89Tg mice and in the endocapillary area for α1KI mice using transmission electron microscopy (Fig. 1 B). Mesangial IgA1 deposits started at 6 wk of age in α1KI-CD89Tg mice with an intense pattern detected starting at 12 wk (Fig. 1 C). This contrasted with the late detection of mouse IgA1 staining observed in 24-wk-old CD89Tg mice in similar 129- C57BL/6 background, confirming our previous observations in NOD.C57BL/6 CD89Tg mice (not depicted; Launay et al., 2000). To address whether these differences in IgA1 deposition kinetics resulted from affinity differences between mouse and human IgA to CD89, we performed surface plasmon resonance experiments using recombinant sCD89. Mouse IgA interacted with sCD89 with a very low affinity (Kd ≈ 7.6 × 10^-5 M; Fig. 1 D), which was 100 times lower than that of human IgA1 and of monoclonal human IgA1 obtained from α1KI mice (Fig. 1 E). Moreover, morphological analyses of α1KI-CD89Tg kidneys showed expansion of mesangial extra cellular matrix as indicated by periodic acid Schiff (PAS) staining (Fig. 1 A), which was also accompanied by fibronectin overexpression (Fig. 1 A).

α1KI-CD89Tg mice exhibited marked MBL and C3 glomerular deposits, hallmarks of IgAN, whereas in α1KI and WT mice, these deposits were notably less intense (Fig. 2 A). As anti-IgA1 IgG have been reported in patients (Suzuki et al., 2009), IgA1 binding on mesangial cells, whereas no disease was induced in TGase2-deficient animals. Therefore, our observations in a humanized mouse model for the IgA1 system as well as in humans document the importance of sCD89 in the induction of the mesangial IgA1 receptor TIR1 expression and in the development of IgAN and support a model that, besides IgA1, sCD89, and TIR1, involves TGase2 as a key player in the complex pathogenic mechanism of this disease.
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was significantly observed in glomeruli and periglomerular interstitial areas of α1KI-CD89Tg mice, indicating that a local inflammation occurs in kidneys of these mice that express human CD89. α1KI-CD89Tg mice also presented features of altered kidney function such as proteinuria, albuminuria, hematuria, and increase in serum creatinine levels (Fig. 2, C–G). Proteinuria started at 12 wk and was more intense at 24 wk (Fig. 2 D). Although no significant proteinuria was found in α1KI mice, CD89Tg mice in the 129-C57BL/6 background displayed very mild proteinuria (not depicted) as previously described (Launay et al., 2000). However, proteinuria was completely abolished in NOD.SCID-CD89Tg mice that lack IgA (not depicted). Collectively, these results show that the presence of both human IgA1 and CD89 promotes increased mesangial IgA1 deposition, hematuria and proteinuria, and altered renal function in mice.

sCD89 induces high molecular mass circulating IgA1 complexes and mediates mesangial IgA1 deposition

As α1KI-CD89Tg mice developed pathogenic mesangial IgA1 deposits, we next investigated whether circulating sCD89 was complexed with IgA1. Cell surface expression of CD89 on blood monocytes, absent in α1KI and WT mice, was lower in α1KI-CD89Tg than in CD89Tg mice (Fig. 3 A), suggesting that expression of human IgA1 in mice may enhance shedding of CD89 as previously shown in vitro (Launay et al., 2000). Similar results were significantly observed in glomeruli and periglomerular interstitial areas of α1KI-CD89Tg mice, indicating that a local inflammation occurs in kidneys of these mice that express human CD89. α1KI-CD89Tg mice also presented features of altered kidney function such as proteinuria, albuminuria, hematuria, and increase in serum creatinine levels (Fig. 2, C–G). Proteinuria started at 12 wk and was more intense at 24 wk (Fig. 2 D). Although no significant proteinuria was found in α1KI mice, CD89Tg mice in the 129-C57BL/6 background displayed very mild proteinuria (not depicted) as previously described (Launay et al., 2000). However, proteinuria was completely abolished in NOD.SCID-CD89Tg mice that lack IgA (not depicted). Collectively, these results show that the presence of both human IgA1 and CD89 promotes increased mesangial IgA1 deposition, hematuria and proteinuria, and altered renal function in mice.

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were obtained with splenic macrophages (not depicted). sCD89 was found complexed with IgA1 (Fig. 3 B), and no free sCD89 (not depicted) was detected in the serum of α1KI-CD89Tg mice using two mAbs, anti-CD89 A3 and MIP8a, an mAb that recognizes CD89 IgA binding site (Zhang et al., 2000). Similar data were obtained in IgAN patient sera (Fig. 3 C), confirming our previous observations (Launay et al., 2000). Despite the fact that α1KI and α1KI-CD89Tg mice presented similar levels of IgA1 in their sera (Fig. 3 D), only α1KI-CD89Tg mice exhibited large molecular mass IgA1 complexes (>670 kD) in the serum as detected by Western blotting and HPLC analysis (Fig. 3, E and G), whereas α1KI displayed essentially monomers and dimers (Fig. 3, E and F). IgA1–sCD89 complexes were detected by ELISA in size-fractionated serum (fractions 6–9), corresponding to high mass molecular forms (Fig. 3 G).

The presence of sCD89 was further demonstrated in fractions containing high molecular mass IgA1 complexes by immunoprecipitation assays using anti-CD89 mAb A3 coupled to beads followed by SDS-PAGE at nonreducing conditions and Western blotting using a cocktail of anti-CD89 mAbs (Fig. 3 H). A 50–70-kD sCD89 protein was detected under reducing conditions overlapping the α chain (not depicted) as previously described (Launay et al., 2000).

We next assessed the presence of CD89 in the mesangium by using an anti-sCD89 polyclonal antibody. α1KI-CD89Tg kidney sections displayed positive stainings for CD89 with a typical mesangial pattern (Fig. 4 A). We then addressed whether anti-sCD89 polyclonal antibody stained CD89 on biopsies from IgAN patients. As shown in Fig. 4 B, a mesangial pattern of CD89 staining was observed that was abolished in the presence of an excess of sCD89. This staining was confirmed in biopsies from two additional IgAN patients but not in biopsies from patients with minimal change disease (not depicted). To formally demonstrate that sCD89 is responsible for mesangial IgA1 deposition, we injected recombinant sCD89 into 6-wk-old α1KI mice because they display very few deposits of IgA1 in their glomerular capillary walls. sCD89 induced massive mesangial IgA1 deposits when compared with mice that received an irrelevant control protein BSA (Fig. 4 C).
IgA1 mesangial deposits and hematuria 24 h after injection of serum from α1KI-CD89Tg mice (not depicted). Together, α1KI-CD89Tg mice data indicated that circulating high molecular mass IgA1–sCD89 complexes are responsible for mesangial IgA1 deposition in IgAN.

sCD89 binds to TfR1 and induces its overexpression on mesangial cells

TfR1 is an IgA1 receptor that is overexpressed in the mesangium of patients with IgAN (Moura et al., 2001; Haddad et al., 2003) and enterocytes from patients with celiac disease (Matysiak-Budnik et al., 2008). As human IgA1 interacts with mouse TfR1 (Coulon et al., 2011), we next examined whether knockin expression of human IgA1 in the mouse could induce mesangial TfR1 expression and whether this overexpression was dependent on CD89. Although α1KI mice displayed a slight increase in TfR1+ cells in their mesangium, the presence of CD89 markedly enhanced (more than threefold) the TfR1 expression in the mesangium of α1KI-CD89Tg mice (Fig. 5 A). CD89-mediated overexpression of TfR1 increased with age.
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TfR1 microRNA (miRNA) treatment, which down-regulated TfR1 cell membrane expression (50% decrease; Fig. 5 E, inset), resulted also in a decreased binding of sCD89 to HMCs (49% decrease; Fig. 5 E). A direct molecular binding was detected between sCD89 and TfR1 by ELISA (Fig. 5 F) and by pull-down experiments (Fig. 5 G). To formally demonstrate the role of sCD89 in TfR1 expression, we next stimulated quiescent HMCs in culture with recombinant sCD89 in the absence of IgA. This treatment resulted in TfR1 overexpression on these cells (96% increase) over a 48-h period (Fig. 5 H) with an increase of its messenger RNA (mRNA) levels (Fig. 5 I). All of the aforementioned data demonstrate that sCD89 can directly interact with TfR1 and induce its overexpression on mesangial cells even in the absence of IgA.

To examine whether sCD89 is directly involved in IgA1 binding to HMCs, we incubated these cells on ice with preformed IgA1–sCD89 complexes for 1 h and assessed for IgA binding by flow cytometry using an anti-IgA antibody. sCD89 complexed to IgA1 led to increased IgA1 binding to mesangial cells (Fig. 6 A). To precisely localize the interactions of IgA1–sCD89 complexes on HMCs, we next visualized these interactions in vitro using confocal microscopy. IgA1 and sCD89 were colocalized on the cell surface of HMCs (Fig. 6 B). Therefore, sCD89 plays an essential role in tissue IgA1 deposit formation, both by increasing TfR1 expression and by facilitating IgA1 binding on mesangial cells. To evaluate the functional role of these proteins, we then analyzed the effect of IgA1 or IgA1–sCD89 complexes on the secretion of IL-8, IL-6, and TNF on HMCs in culture. In line with the increased binding of IgA1–sCD89 complexes (Fig. 6 A), these complexes induced strong secretion of IL-6 and IL-8 cytokines as compared with stimulation with IgA1 alone (Fig. 6 C). However, IgA1 alone induced TNF secretion and only marginal IL-8 and IL-6 secretion as compared with sCD89 (Fig. 6 C). TfR1 miRNA treatment of HMCs markedly decreased the ability of IgA1–sCD89 complexes to stimulate cytokine secretion (Fig. 6 D). Because sCD89 was required for mesangial IgA1 deposits, we next explored whether IgA1 and sCD89 expression in the mouse had renal functional consequences by analyzing tissue mRNA levels of proinflammatory cytokines using saline-perfused kidneys. α1KI-CD89Tg mice presented a marked increase in mRNA levels for MIP-2, IL-6, and TNF cytokines (Fig. 6 E). Therefore, both IgA1 and sCD89 within the IgA1–sCD89 complexes cooperate to induce mesangial TfR1 expression and kidney inflammation in IgAN.

sCD89 induces TGase2 mesangial surface expression, which is associated with TfR1 and is crucial for IgA1 deposit formation

TfR1 is ubiquitously expressed to perform its major function of iron uptake through transferrin (Ganz, 2008). The observation of TfR1 as an IgA1 receptor has been established in pathological settings where TfR1 is overexpressed by tissues, notably by mesangial and epithelial cells (Moura et al., 2001; Matysiak-Budnik et al., 2008). Because IgA1 binding depends on the density of TfR1 expressed on the cell surface (Moura et al., 2004b),
we investigated whether tissue mesangial cells express a protein associated to TIR1 that may control the expression of TIR1 and therefore favor IgA1 binding. Because TGase2, a cross-linking enzyme with pleiotropic functions (Lorand and Graham, 2003), is overexpressed in the mesangium of IgAN patients and correlated with renal function decline (Ikee et al., 2007), we examined whether it could modulate IgA complex deposition and TIR1 expression. TGase2 expression was markedly up-regulated in the mesangium of α1KI-CD89Tg mice, whereas it was only weakly detected in α1KI and absent in WT mice (Fig. 7A), indicating a role of sCD89 in its regulation. Mesangial TGase2 overexpression was confirmed in biopsies of IgAN patients (Fig. 7B), as reported by others (Ikee et al., 2007). This was also supported by experiments in which injection of recombinant sCD89 in α1KI mice resulted in increased TGase2 staining (Fig. 7 C). Arrows indicate TIR1+ cells. The corresponding graphs represent the numbers of positive cells per glomerulus counted in 20 randomly chosen fields for each mouse at 200 magnification. n = 3 mice per group. Bars: (A) 10 µm; (B and C) 5 µm. (D) Flow cytometry histogram (one representative experiment of six) of HMCs binding sCD89 (white layer) and stained with anti-C89. The dashed layer represents incubation with sTfR1. The grey layer corresponds to the isotypic-matched control. The inset graph shows the mean binding values of indicated doses of sCD89 (±SEM) to HMCs as a ratio to background mean fluorescence intensity of seven independent experiments. (E) Flow cytometry analysis of sCD89 binding on HMCs untreated (white layer) or treated with TIR1 miRNA (dotted layer). The inset shows flow cytometry analysis of TIR1 expression on HMCs untreated (white layer) or treated with TIR1 miRNA (dotted layer). The histogram shows one representative experiment of six. The grey layers correspond to the isotypic-matched controls. (F) Binding of sCD89 to TIR1-His by ELISA using biotinylated sCD89. 10 µg/ml sTfR1 was coated in ELISA plates and incubated with the indicated doses of biotinylated sCD89. β-Synuclein is a His-tagged protein used as a negative control and A3 an anti-CD89 mAb as a positive control. n = 4 experiments. (G) sCD89 interaction with recombinant sTfR1. sCD89 was loaded on a column of nickel beads preincubated or not with sTfR1-His. Eluates were analyzed by SDS–10% PAGE followed by Western blot using a cocktail of anti-CD89 antibodies. (H) Flow cytometry analysis of TIR1 expression on HMCs unstimulated (gray layer) or stimulated with sCD89 (white layer). The histogram shows one representative experiment of six. (I) miRNA levels of TIR1 normalized to GAPDH mRNA levels in HMCs stimulated with sCD89 or BSA. (A–C, F, and I) Error bars indicate SEM. *, P < 0.05 (unpaired Student’s t test); **, P < 0.01; and ***, P < 0.001 (Mann–Whitney U test).
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Since our first description of the putative role of sCD89 in generating mouse IgA deposits in the mesangium (Launay et al., 2000), there was a lack of formal demonstration that sCD89 actively participates in IgA1 mesangial deposition in mice and humans. The production of mice expressing both human IgA1 and human CD89 on monocytes/macrophages allowed us to readdress this question and to demonstrate that sCD89 is required for pathogenic mesangial IgA1 deposits leading to disease development. Interestingly, in the absence of CD89, IgA1 deposition occurring in α1KI mouse glomeruli did not lead to any detectable renal dysfunction, which is in line with observations in humans that not all subjects with IgA glomerulus deposition express overt signs of glomerulonephritis (Varis et al., 1993; Glassock, 2011). The spontaneous sCD89 production observed in mice that express human IgA1 and CD89 may be related to the high polymeric/monomeric ratio of IgA in the mouse. Indeed, although ~30% of circulating chimeric IgA1 (composed by a human α1 heavy chain and mouse λ and κ chains) were polymeric in both α1KI and α1KI-CD89Tg mice, in human normal serum, this form represents ~10% of total IgA (Mestecky, 1988; Kerr, 1990). Thus, small IgA1 complexes, mimicking complexes formed by underglycosylated IgA1 found in patients, may induce CD89 aggregation and shedding from myeloid cells in α1KI-CD89Tg mice as reported for human myeloid cells (Launay et al., 2000). This would result in the formation of circulating complexes containing IgA1 and sCD89 that would deposit in kidney mesangium. However, so far, there was no published evidence for mesangial sCD89 deposition in patients. Using a new polyclonal antibody raised against sCD89, we clearly detected CD89 in the mesangium of α1KI-CD89Tg mice and of IgAN patients. Acid elution procedure, a standard method to isolate antibodies and complexes from glomeruli (Woodroffe and Wilson, 1977), confirmed the presence of sCD89 in mesangial IgA1 deposits, further suggesting that the molecular mass of IgA1 complexes is a major factor promoting IgAN. In line with this observation, acidic elution of

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IgA1–sCD89 complexes from kidney eluates was predominantly of high molecular mass, confirming previous data obtained with IgAN patients (Monteiro et al., 1985). Interestingly, sCD89 was also detected in serum as high molecular mass forms, suggesting that sCD89 could be covalently linked to IgA1. This is in agreement with previous data showing that sCD89 can be covalently linked to IgA in a polymeric form (van der Boog et al., 2002). However, although in a previous study authors found a 30-kD sCD89 protein complexed with IgA in normal human sera (van der Boog et al., 2002), we observed a 50–70-kD sCD89 form that is exclusively found in the serum of IgAN patients (Launay et al., 2000). It is noteworthy that all sCD89 found in serum in α1KI-CD89Tg mice was associated with IgA1, demonstrating that no IgA-free sCD89 may exist in the circulation. It is also remarkable that in the absence of CD89, no high molecular mass IgA1 complexes were found in the circulation of α1KI mice. These IgA1–CD89 complexes appeared nephrotoxic because only α1KI-CD89Tg mice developed renal failure as indicated by proteinuria, hematuria, and increased serum creatinine levels. Our data may support recent observations by others in which severity of renal dysfunction in IgAN patients correlates with the disappearance of IgA1–sCD89 complexes in the circulation, suggesting that this could be caused by their increased deposition in kidneys (Boyd and Barratt, 2010; Vuong et al., 2010). The large size of the deposited pathogenic aggregates observed in these mice that include IgA1, TfR1, TfR1-associated TGase2, and sCD89 and that may also include IgA1-associated MBL with their associated serine proteases (MASP; Roos et al., 2006) may explain why deposited mesangial sCD89 has escaped detection so far. Yet, the role of sCD89 in pathogenic mesangial deposits in our IgAN murine model stresses the importance of a systematic evaluation of CD89 presence in IgAN patient biopsies. Generation of adapted molecular tools, such as our new polyclonal anti-sCD89 antibody, will be important in this regard.

TfR1, a multiligand receptor, participates in several cellular functions (Lebrón et al., 1998; Radoshitzky et al., 2007; Schmidt et al., 2008). TfR1 is a receptor for IgA1 whose binding depends on the size and the glycosylation of IgA1 (Moura et al., 2004a). TfR1–IgA1 interaction plays a crucial role in physiology where polymeric IgA1 controls erythroblast proliferation and accelerates erythropoiesis recovery in anemia (Coulon et al., 2011). In pathology, cell surface overexpression of TfR1 is a major characteristic of IgAN and celiac disease (Moura et al., 2001; Haddad et al., 2003; Matysiak-Budnik et al., 2008).
It acts predominantly as a cytosolic enzyme but can be externalized from cells by an unknown secretory pathway, after which it cross-links proteins of the extracellular matrix and induces renal fibrosis development (Fesus and Piacentini, 2002; Shweke et al., 2008). Our data reveal that TGase2 surface expression is induced by sCD89 and is up-regulated in the mesangium of α1KI-CD89Tg mice and in IgAN patients. The latter observation is in agreement with data reported by others showing increased mesangial TGase2 expression in IgAN patients (Ikee et al., 2007). Moreover, the correlation of TGase2 mesangial expression with deterioration of renal function (Ikee et al., 2007) supports our observations that only α1KI-CD89Tg mice that overexpress TGase2 develop proteinuria, whereas α1KI mice alone show no proteinuria and mild TGase2 overexpression, further suggesting that TGase2 controls the triggering of IgAN pathology. The mechanism by which TGase2 surface expression increases in turn surface expression of TIR1 remains unresolved whether it involves increased TIR1 gene transcription or interference in TIR1 cycle by favoring its recycling to and stabilization at, the plasma membrane. Mesangial fibronectin expression is also up-regulated in α1KI-CD89Tg mice.

However, molecular mechanisms involved in TIR1 overexpression in primary cells are not yet identified. Several factors have been shown to up-regulate TIR1 expression, including iron deprivation and human hemochromatosis protein, as well as polymeric IgA1 from patients with IgAN (Moura et al., 2005). In this study, we show that sCD89 is a new ligand for TIR1 able to up-regulate its expression on mesangial cells and induce secretion of proinflammatory cytokines such as IL-8, IL-6, and TNF. sCD89–TIR1 interaction has a marked effect on cell stimulation, suggesting that IgA1–sCD89 complexes might be responsible for a local mesangial cell activation and the development of IgAN.

Our study demonstrates that sCD89 plays a pivotal role in IgAN in mice by (a) the formation of IgA1 circulating complexes allowing their deposition in mesangium, (b) the induction of increased mesangial expression of TIR1, and (c) the induction of surface expression of TGase2. TGase2, a calcium-dependent multifunctional protein, is ubiquitously expressed in almost all cells and tissues (Lorand and Graham, 2003).
and colocalizes with TGase2 and IgA1. Interestingly, it has been shown that TGase2 binds to fibronectin and acts as an integrin coreceptor (Lorand and Graham, 2003), emphasizing that TGase2 could intervene at multiple levels in mesangial cell activation for inflammation and fibrosis in IgAN.

In conclusion, our study demonstrates that IgA1–CD89 complex models could initiate a process of auto-amplification involving hyperexpression of TR1 and TGase2, allowing increased mesangial deposition of pathogenic IgA1 complexes and chronic mesangial cell activation. The critical role played by TGase2 revealed by our humanized mouse model opens new perspectives for pharmacological modulation of excessive TGase2 expression as a promising strategy for therapeutic intervention in IgAN.

MATERIALS AND METHODS

Subjects. Sera were obtained from 15 patients with biopsy-proven IgAN and no steroid treatment and from 25 healthy controls with their informed consent as approved by the hospital ethical committee. Renal biopsy specimens from three patients with IgAN and two with minimal change disease were studied.

Mouse procedures. α1KI mice (Duchez et al., 2010), expressing the human IgA1, were backcrossed for 15 generations with C57BL/6 CD89Tg mice expressing the WT human CD89 on monocytes/macrophages (Launay et al., 2000). Mice on 129S6/SvPasCrl-C57BL/6 mixed background were used as WT controls to α1KI, CD89Tg, and α1KI–CD89Tg mice. TGase2+/− C57BL/6 mice generated as described previously (De Laurenzi and Melino, 2001) were provided by G. Melino (University of Rome Tor Vergata, Rome, Italy) and P.-L. Tharaux (Institut National de la Santé et de la Recherche Médicale Unité 970, Paris, France) and were backcrossed with α1KI–CD89Tg mice. All strains were raised and maintained at the mouse facilities of the Claude Bernard Institute. All experiments were performed in accordance with the national ethical guidelines and with the approval of local authorities of the Comité d’Éthique d’Expérimentation Animale Bichat-Debré. Serum and kidneys were collected from 3-, 6-, 12-, 24-, and 40-wk-old male mice. 200 µg sCD89 was injected i.v. on days 0, 3, and 6 to α1KI or NOD.C.B.17-Prkd−/− mice (6 wk old), and the mice were sacrificed on day 7.

Production of soluble proteins and antibodies. sCD89 and human sTfR1 were expressed and produced in lytic baculovirus/insect cell expression systems (Lebrón et al., 1998). The murine IgG1 anti-CD89 mAb A3 was produced and purified in our laboratory. The polyclonal anti-CD89 antibody was produced after injection of sCD89 in a rabbit and purified on a DEAE-Trisacryl column (BioSepra; Pall).

Histology, immunohistochemistry, and immunofluorescence. Paraffin-embedded kidney sections 4 µm in thickness were stained with PAS for morphological analysis. For immunohistochemistry, frozen kidney sections were incubated with biotinylated antibodies against human IgA, mouse IgA, mouse CD11b, mouse F4/80, human sCD89, and mouse TR1 (BD) or with primary antibodies against mouse C3 (Abcam), mouse MBL (R&D Systems), and TGase2 (Thermo Fischer Scientific) for 1 h at room temperature. When necessary, the primary antibody incubation was followed by incubation with anti-rabbit IgG or anti-goat IgG (SouthernBiotech). Slides were mounted with the Eukitt mounting medium (Electron Microscope Sciences) and read with an upright microscope (DM2000; Leica) at 200 magnification using the IMSO software (Leica). Human TGase2 expression in paraffin-embedded tissue sections from human normal and IgAN kidney patients was performed using an anti-TGase2 polyclonal antibody from rabbit (pab0063; Covablab). For colocalization experiments, frozen kidney sections were incubated successively with each antibody for 2 h (anti-IgA FITC, anti-CD31-biotin, rabbit anti-TGase2, goat antifibronecin, and anti–IgA-biotin) followed by incubation with streptavidin–Alexa Fluor 568 or anti–rabbit–Alexa Fluor 568 or anti–goat FITC at room temperature. Tissue sections were mounted with Vectashield (Vector Laboratories). Slides were read with a laser-scanning confocal microscope (LSM 510; Carl Zeiss) at ×630 magnification (except for IgA1–CD31 and IgA1–TGase2 staining at 400 magnification) using the LSM Image Browser (Carl Zeiss). For transmission electron microscopy, kidneys were fixed with 2.5% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide in PBS, dehydrated in a graduated series of ethanol dilutions, and embedded in Epoxy Resin 812. Blocks were cut using an ultramicrotome (Ultracut; Leica). Ultrathin sections placed on 200-mesh copper were stained with 1% uranyl acetate in 50% ethanol and Reynolds lead citrate and viewed on an electron microscope (model 1010; Jeol) coupled with a camera (MegaView III; Olympus) and SiS Analysis System version 3.2 software (Olympus) at 1,800 magnification.

Elution from mouse kidney tissues. Seven frozen kidneys per group, obtained from α1KI and α1KI–CD89Tg mice perfused with 0.9% NaCl, were thawed, pooled, and cut into small pieces, suspended in PBS, and homogenized at 4°C. After centrifugation at 4,000 g, the pellets were washed three times in PBS and incubated in 20 ml of 0.02 M citrate buffer, pH 3.2, for 2 h at 37°C as described previously (Jacob et al., 1987). Then, the suspension was centrifuged for 30 min at 4,000 g. The supernatants were neutralized at pH 7, concentrated, and adjusted to 1 ml.

Kidney functional parameters. Protein, albumin, and creatinine levels were measured in urines and creatinine levels in sera of mice using the AU400 chemistry analyzer (Olympus). For hematuria, 10 µl of fresh urines were mounted on a Malassez hemocytometer, and red cells were counted.

HPLC. 300 µl of serum or 500 µg of kidney-related proteins diluted in PBS was resolved by gel filtration through a Superdex 200 10/30 column (GE Healthcare) connected to an HPLC AKTA-basic automated liquid chromatography system (GE Healthcare).

Immunoblot analysis. Sera or HPLC fractions were solubilized in SDS sample buffer under nonreducing conditions and subjected to electrophoresis in 6% polyacrylamide gels. Proteins were electrobotted on polyvinylidene difluoride membranes (Millipore) and subjected to Western blot analysis using a biotinylated goat anti-human IgA (SouthernBiotech) and streptavidin coupled to horseradish peroxidase (HRP). Membranes were developed by enhanced chemiluminescence treatment (GE Healthcare). For serum immunoprecipitation assays, CD89-containing complexes were immunoprecipitated for 1 h at room temperature with mAb A3 coupled to CNBr-activated Sepharose 4B beads (GE Healthcare) followed by SDS–10% PAGE at nonreducing conditions and analyzed by Western blotting using a cocktail of anti-CD89 mAbs. For TGase2 or sCD89 with sTfR1 interaction experiments, TGase2 or sCD89 was pulled down in a column prepared with nickel beads preincubated or not with histidine-tagged TR1 for 4 h at 4°C. Eluates were analyzed by SDS–10% PAGE followed by Western blot using an anti-TGase2 or anti-CD89 antibody plus anti–rabbit coupled with HRP.

Cells, flow cytometry, and immunofluorescence. Spleenocytes and cells from blood samples from WT, α1KI, CD89Tg, and α1KI–CD89Tg mice were stained with anti–mouse CD11b–PE-Cy7 and anti–CD89–PE antibodies for 1 h at room temperature. After centrifugation, the cells were resuspended in 300 µl of serum or 500 µg of kidney-eluted proteins diluted in PBS washed by gel filtration through a Superdex 200 10/30 column (GE Healthcare) followed by SDS–10% PAGE at nonreducing conditions and analyzed by Western blotting using a cocktail of anti-CD89 mAbs. For TGase2 or sCD89 with sTfR1 interaction experiments, TGase2 or sCD89 was pulled down in a column prepared with nickel beads preincubated or not with histidine-tagged TR1 for 4 h at 4°C. Eluates were analyzed by SDS–10% PAGE followed by Western blot using an anti-TGase2 or anti-CD89 antibody plus anti–rabbit coupled with HRP.
and TGase2 expression was analyzed by flow cytometry using an anti–TfR1-PE mAb (BD) or a biotinylated anti-TGase2 polyclonal antibody (Thermo Fisher Scientific), respectively. Cytokine (IL-8, IL-6, and TNF-α) production in the HMC culture supernatants was measured using ELISA DuoSet kits (R&D Systems) at 8 or 48 h. For immunofluorescence, HMCs were cultured on 4-well plates (Nunc) for 1 h at 4°C, and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Non-specific labeling was avoided by two 10-min incubations with sodium borohydride (NaNBH4) at 1 mg/ml followed by incubation in 4% BSA for 30 min. Cells were then labeled with a rabbit polyclonal anti–CD89 in PBS for 1.67 h, anti–human IgA–FITC (Southern Biotech) for 1.67 h, and anti–rabbit–Alexa Fluor 568 (Invitrogen) for 1 h. After washes, HMCs were then incubated with Alexa Fluor 647–conjugated wheat germ agglutinin (Invitrogen), a cell membrane marker, in HBSS for 4 min at 4°C, followed by two washes with HBSS. Slides were mounted with Vectashield (Dako) and read with a confocal microscope (TCS SP5 II, Leica).

Human transferrin receptor gene silencing. Lentiviral vectors for human TfR1 and irrelevant control miRNA were built with the BLOCK-it LentiViral Pol II miRNA RNA Expression System (Invitrogen). DNA sequences were designed using the BLOCK-it RNAi Designer according to the manufacturer's recommendations and synthesized (Operon MWG). DNA oligonucleotide sequences were inserted into pcDNA6.2 vectors and transferred to pLent6.2 BLOCK-it vectors by DNA recombination. To generate lentiviral stocks, 6 x 10^6 293T cells were transfected with 3 µg pLent6.2/BLOCK-it–miR-human TfR1, along with 9 µg ViraPower (pLP1, pLP2, and pLP/VSVG; Invitrogen) in 6 × 106 293T cells were transfected with 3 µg pLent6.2/BLOCK-it–miR-human TfR1, along with 9 µg ViraPower (pLP1, pLP2, and pLP/VSVG; Invitrogen) in Opti-MEM I (Invitrogen) without antibiotics. Cells were cultured for 72 h, and culture medium was collected, centrifuged at 4°C, 3,000 rpm for 15 min, and stored at −80°C. HMCs were infected with lentivirus in RPMI (Invitrogen) and in the presence of 6 µg/ml Polybrene (Sigma-Aldrich). Cell phenotype was analyzed 48–72 h after infection.

Quantitative real-time PCR analysis. Kidneys were homogenized, and RNA was prepared using the RNeasy Plus Mini kit (Qiagen). 700 ng RNA was reverse transcribed using a QuantiTect RT kit (Qiagen). Resulting cDNA was used as template for quantitative PCR analysis. Primers were purchased from Eurofins and were as follows: GAPDH, 5′-AAGCCTTCCCTACTTCAAGG-3′ (sense) and 5′-GCTTGAGTGTGACGCCCCTC-3′ (antisense); IL-6, 5′-GCTTGAGTGTGACGCCCCTC-3′ (sense) and 5′-GCTTGAGTGTGACGCCCCTC-3′ (antisense); IL-8, 5′-GCTTGAGTGTGACGCCCCTC-3′ (sense) and 5′-GCTTGAGTGTGACGCCCCTC-3′ (antisense); IL-6, 5′-GCTTGAGTGTGACGCCCCTC-3′ (sense) and 5′-GCTTGAGTGTGACGCCCCTC-3′ (antisense); TNF-α, 5′-AGGCATGCTCGCCAAAGATG-3′ (sense) and 5′-TCCACCCGAGTCTGACAGACA-3′ (antisense). Gene quantification was performed in duplicate using a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories). Data were normalized to GAPDH values.

ELISA. Plates were coated overnight with F(ab′)2 goat anti–human IgA (10 µg/ml; SouthernBiotech) or A3 mAb (10 µg/ml) or were directly coated with histidine-tagged TfR1 or β-synuclein or IgA1 and albumin (10 µg/ml). Sera or HPLC fractions were incubated in the wells in PBS, containing 0.05% Tween, 0.1% sodium azide, and 1% BSA overnight. After washing, the anti–human IgA mAb coupled with the alkaline phosphatase (AP; BD) was added at 1:2,000 dilution for 1 h. For binding tests, biotinylated cDNA9 or T-Gase was incubated for 2 h, and after washing, streptavidin–AP (Jackson ImmunoResearch Laboratories, Inc.) was added at 1:10,000 dilution for 30 min. The reaction was developed by adding the AP substrate (SIGMAFAST p-nitrophenyl phosphate tablets; Sigma–Aldrich).

Surface plasmon resonance assays. All the assays were performed on a Biacore X100 (GE Healthcare). The antibody A3 against CD89 was immobilized on two flow cells of a CMS carboxymethylated dextran biosensor chip (GE Healthcare) using carbodiimide chemistry. After capture of CD89 on one flow cell, the different purified IgAs, mouse IgA (methylene IgA; MP Biomedicals), human IgA1 (purified from healthy control serum using jactalin, and α1IK mouse IgA (methylene IgA1, purified using DEAE column), were injected for 2 min on the two flow cells. The association and dissociation profiles were double-referenced (i.e., both the signal from the reference surface with A3 alone and from blank buffer injections were subtracted) and analyzed using the BIAevaluation software.

Statistical analysis. Nonparametric Mann-Whitney tests were performed for the different comparisons between different mice and between healthy controls and IgAN patients. A p-value of <0.05 was considered significant.

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