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The Tetraspanin CD37 Protects Against Glomerular IgA Deposition and Renal Pathology

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The tetraspanin protein CD37 is a leukocyte-specific transmembrane protein that is highly expressed on B cells. CD37-deficient (CD37^{-/-}) mice exhibit a 15-fold increased level of immunoglobulin A (IgA) in serum and elevated numbers of IgA⁺ plasma cells in lymphoid organs. Here, we report that CD37^{-/-} mice spontaneously develop renal pathology with characteristics of human IgA nephropathy. In young naïve CD37^{-/-} mice, mild IgA deposition in glomeruli was observed. However, CD37^{-/-} mice developed high titers of IgA immune complexes in serum during aging, which was associated with increased glomerular IgA deposition. Severe mesangial proliferation, fibrosis, and hyalinosis were apparent in aged CD37^{-/-} mice, whereas albuminuria was mild. To further evaluate the role of CD37 in glomerular disease, we induced anti-glomerular basement membrane (GBM) nephritis in mice. CD37^{-/-} mice developed higher IgA serum levels and glomerular deposits of anti-GBM IgA compared with wild-type mice. Importantly, glomerular macrophage and neutrophil influx was significantly higher in CD37^{-/-} mice during both the heterologous and autologous phase of anti-GBM nephritis. Taken together, tetraspanin CD37 controls the formation of IgA-containing immune complexes and glomerular IgA deposition, which induces influx of inflammatory myeloid cells. Therefore, CD37 may protect against the development of IgA nephropathy. (*Am J Pathol* 2010, 176:2188–2197; DOI: 10.2353/ajpath.2010.090770)

Tetraspanins are small four-transmembrane spanning proteins that are expressed on all nucleated cells. Tetraspanins associate noncovalently with (immuno-)receptors, signaling molecules, and each other, whereby they create 'tetraspanin microdomains,' also known as the tetraspanin web.^{1–3} These domains bring together receptors and signaling molecules into functional complexes. Consequently, tetraspanins are important in several fundamental cellular processes including migration, proliferation, differentiation, and cancer.^{4–6}

In the immune system, tetraspanins have been reported to interact with antigen-presenting major histocompatibility complex molecules, integrins, and C-type lectins.^{7,8} The importance of tetraspanins in immunology has been validated by recent studies with tetraspanin-deficient mice.⁸ Tetraspanin CD37 has a rather confined expression; whereas the majority of tetraspanins (CD9, CD63, CD81, CD151, among others) have a broad tissue expression, CD37 is only expressed on cells of the immune system. CD37 is present on lymphocytes, monocytes, macrophages, neutrophils, and immature dendritic cells, with highest expression on B cells.⁹

CD37 associates with other tetraspanins (CD53, CD81, and CD82), major histocompatibility complex class II molecules, and the β -glucan receptor dectin-1.^{9,10} CD37-deficient mice (CD37^{-/-}) display defects in various arms of the immune system, including impaired antibody responses, T cell hyperproliferation, and increased antigen-presenting capacity by dendritic cells.^{11–13} Recently, we observed that tetraspanin protein CD37 inhibits immunoglobulin (IgA) responses both in steady-state conditions and during infection.¹⁴ CD37^{-/-} mice exhibit a 15-fold increased level of IgA in serum and significantly elevated numbers of IgA⁺ plasma cells in lymphoid organs.

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Immunoglobulin A is critical for protecting the host from environmental and microbial infections. However, systemic IgA overproduction has been linked to IgA deposition in the kidney and development of IgA nephropathy (IgAN; Berger's disease).¹⁵ The special characteristics of IgA antibodies in serum of IgAN patients (predominantly polymeric IgA₁, lambda light chains, and aberrant O-glycosylation) promote IgA immune complex formation and mesangial deposition, but the precise molecular mechanisms underlying IgAN remain unclear.^{16,17} The high levels of circulating IgA antibodies in CD37^{-/-} mice stimulated us to investigate the possible involvement of CD37 in the development of IgAN and experimental nephritis. The model of anti-glomerular basement membrane (GBM) nephritis is induced by injection of heterologous anti-GBM antibodies, which bind immediately to the GBM. This initiates complement activation and deposition resulting in leukocyte attraction and inflammation. During the acute heterologous phase, glomerular polymorphonuclear neutrophil (PMN) influx peaks 2 hours after injection, whereas albuminuria is manifested from 1 day on.^{18–21}

We report that CD37^{-/-} mice spontaneously develop high titers of IgA immune complexes in serum, glomerular IgA deposition, and mesangial proliferation, which are the hallmarks of IgAN.¹⁶ Moreover, tetraspanin CD37 controls glomerular IgA deposition and influx of inflammatory myeloid cells during anti-GBM nephritis. Taken together, the B cell protein CD37 controls glomerular IgA deposition and may be protective against the development of IgA nephropathy.

Materials and Methods

Mice

CD37-deficient (CD37^{-/-}) mice on a C57BL/6J background and age- and sex-matched C57BL/6J wild-type mice (Charles River, France) were bred at the Central Animal Laboratory (Radboud University Nijmegen, The Netherlands) and handled according to the guidelines of the local animal ethics committee. For aging studies, groups of twelve CD37^{-/-} and wild-type mice were matured in the same room and used at the age of 1 or 1.5 years.

Immunoglobulin A Western Blot Analysis

Serum (1, 0.05, or 0.001 μ l) was loaded on 8% SDS-PAGE gels, separated under nonreducing conditions, and blotted onto PVDF membranes. Membranes were blocked in PBS, 3% bovine serum albumin, 1% skim milk powder at 4°C overnight, and probed with biotinylated rat anti-mouse IgA (BD Pharmingen, San Diego, CA), followed by coupled streptavidin-horseradish peroxidase (Invitrogen Life Technologies, Breda, The Netherlands) and enhanced luminescence detection kit (Pierce, Rockford, IL).

Immunofluorescence Staining

Cryosections (4 μ m) were fixed in ice-cold acetone for 10 minutes and incubated with 5 μ g/ml primary antibodies diluted in PBS containing 1% bovine serum albumin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and 0.05% sodium azide (IF-buffer) for 45 minutes at room temperature. Directly labeled antibodies included biotinylated rat anti-mouse IgA (BD Pharmingen), goat anti-mouse C3c- and fibrinogen-fluorescein isothiocyanate (FITC; Nordic, Tilburg, The Netherlands), goat anti-rabbit IgG-Alexa 488, goat anti-mouse IgM-, IgG- (H + L), IgG1-, and IgG2a-Alexa 488, and goat anti-rat Alexa 488 (Invitrogen Life Technologies, Breda, The Netherlands). Unlabeled antibodies included anti-mouse GR-1 (RB6.8C5), CD4 (L3T4), CD8a (Ly-2; all from BD Biosciences, Alphen aan de Rijn, The Netherlands), CD68 (MCA1957; Serotec, Oxford, United Kingdom), and goat anti-mouse lambda light chain (Serotec). Sections were washed in PBS and incubated with the appropriate secondary Alexa 488 antibodies in IF-buffer for 45 minutes at room temperature. Glomeruli were visualized using the hamster anti-agrin antibody (MI91)²² followed by a Cy3-labeled antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Finally, sections were postfixed with 1% paraformaldehyde-PBS and embedded in VectaShield mounting medium H-1000 (Brunschwig Chemie, Amsterdam, The Netherlands). The amount of rabbit IgG or mouse IgA, IgM, IgG, IgG1, and IgG2a deposited in glomeruli was quantified by titrating the appropriate secondary antibodies. The titer was defined as the reciprocal of the dilution that was still positive and expressed as arbitrary units (AU). The staining intensities of all antibodies were scored in 50 glomeruli on a scale between 0 and 10 (0 = no staining, 1 = 10% staining intensity, with a maximum score of 10 for 100% staining intensity).

Renal Histology

Renal sections (5 μ m) were deparaffinized, rehydrated, and stained with Periodic acid-Schiff, Chromotop Anilinblue, and Jones' methenamine. Sections were counterstained with hematoxylin. Sections were evaluated by an experienced pathologist. At least 30 glomeruli were analyzed for presence of mesangial proliferation, crescents, hyalinosis, and sclerosis. Mesangial proliferation was scored as absent (≤ 3 mesangial cells/area), mild (4 to 5 mesangial cells/area), moderate (6 to 7 mesangial cells/area), or severe (≥ 8 mesangial cells/area).²³

Transmission Electron Microscopy

For electron microscopy, kidneys were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C and washed in the same buffer. Tissues were postfixed in palade-buffered 2% OsO₄ for 1 hour, dehydrated, and embedded in Epon812 according to the Luft procedure (Merck, Darmstadt, Germany). Ultrathin sections were contrasted with 4% uranyl acetate for 45 minutes and subsequently with lead citrate

for 5 minutes at room temperature. Sections were examined in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Detection of IgA Immune Complexes in Serum

MaxiSorp 96-wells plates (Nunc, Roskilde, Denmark) were coated with 2 μ g/ml rat anti-mouse IgG1 or IgG2a (BD Pharmingen) overnight at 4°C. Plates were washed with 0.05% Tween/PBS, blocked with 2% milk/PBS, and serial dilutions of mouse sera were incubated for 1 hour at room temperature. Subsequently, wells were washed and incubated with 1 μ g/ml biotin-conjugated rat anti-mouse IgA (BD Pharmingen) in 0.2% milk/PBS for 1 hour at room

temperature followed by streptavidin-horseradish peroxidase (Invitrogen) and tetramethylbenzidine substrate (SFRI Laboratories, Berganton, France). The reaction was stopped with 2M H₂SO₄, and adsorption was measured at 450 nm using a Bio-Rad Multiplate Reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Induction of Anti-GBM Glomerulonephritis and Determination of Albuminuria

Experimental anti-GBM nephritis was induced as described.²¹ Briefly, groups of five wild-type and CD37^{-/-} mice (14 to 18 weeks old) were injected in the tail vein

Figure 1. IgA deposition in glomeruli of young CD37^{-/-} mice. **A:** Characterization of molecular forms of IgA (m = monomeric, d = dimeric, p = polymeric IgA) in serum of naïve 3-month-old wild-type and CD37^{-/-} mice. 1, 0.05, or 0.001 μ l of serum was loaded on 8% SDS-PAGE gels, and Western blots were probed with anti-mouse IgA. Note the predominance of high molecular weight IgA (pIgA) in CD37^{-/-} serum. **B:** Kidneys of 3-month-old wild-type (left) and CD37^{-/-} (right) mice were stained for presence of IgA by immunohistochemistry. Scale bars represent 50 μ m. **C:** Immunohistochemistry stainings of lambda light chains in kidneys of wild-type and CD37^{-/-} mice. Scale bars represent 50 μ m. Quantification of lambda light chain deposits in glomeruli of CD37^{-/-} mice as compared with wild-type mice (**P* < 0.01) as described in *Materials and Methods* (right). Isotype stainings were negative. AU indicates arbitrary units. **D:** Histological analysis of kidneys (Periodic acid-Schiff staining) of young wild-type (left) and CD37^{-/-} (right) mice did not reveal any abnormalities. Scale bars represent 60 μ m. **E:** Albuminuria was not detected in young wild-type and CD37^{-/-} mice by Mancini test. Urine of mice with anti-GBM nephritis was used as positive (+) control.

with 1, 3, or 8 mg purified rabbit IgG anti-mouse GBM in sterile PBS. Urine was collected and mice were killed after 2 hours and 4 days. Blood was taken for antibody analysis (described below), and kidneys were fixed in 10% buffered formalin and paraffin embedded or snap-frozen in liquid nitrogen for histology and immunohistochemistry, respectively. Albumin in urine was measured by radial immunodiffusion (Mancini).

Determination of Serum Levels of Mouse IgA, IgG, IgG1, and IgG2a Anti-Rabbit IgG Antibodies during Anti-GBM Nephritis

Serum levels of mouse antibodies directed against rabbit IgG were determined by ELISA as described.²¹ Briefly, 96-wells plates were coated with 10 μ g/well rabbit IgG. Serial dilutions of mouse plasma from wild-type and CD37^{-/-} mice with anti-GBM nephritis were added, and anti-rabbit antibodies were detected by peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a (Sanbio BV, Uden, The Netherlands), or biotin-conjugated goat anti-mouse IgA, followed by streptavidin-horseradish peroxidase and tetramethylbenzidine substrate.

Purification of Mouse IgA and Adoptive Transfer Experiments

IgA was purified from pooled sera of twelve CD37^{-/-} mice by affinity chromatography. Rabbit anti-mouse IgA (α chain; Abnova GmbH, Heidelberg, Germany; 9 mg) was coupled to cyanogen bromide-activated (CNBr) Sepharose 4B. The column was first absorbed with pooled wild-type serum to block nonspecific binding. Subsequently, pooled CD37^{-/-} serum was applied to the column, bound IgA was eluted in a 0.05 M citrate (pH 2.6)/1 M NaCl/0.5 M acetate (pH 3.0) buffer, neutralized, and concentrated. IgA was sterilized through 0.2 μ m filter before *in vivo* use. Wild-type mice (14 to 18 weeks old; $n = 3$) were injected in the tail vein with 0.9 mg purified mouse IgA and 3 mg rabbit anti-mouse GBM IgG. Control wild-type and CD37^{-/-} mice (14 to 18 weeks old) were injected with 3 mg rabbit anti-mouse GBM IgG only. Mice were killed after 4 days, and kidneys were processed for immunohistochemistry as described above.

Statistical Analysis

Values are expressed as means $\pm$ SEM and significance was evaluated by Student *t* test using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Results

CD37-Deficiency Leads to Glomerular IgA Deposition

The high levels of circulating IgA antibodies in CD37^{-/-} mice¹⁴ stimulated us to investigate the possible involvement

of CD37 in the development of IgAN. Because the circulating IgA antibodies in IgAN patients that promote mesangial deposition have special characteristics (ie, predominantly polymeric IgA, lambda light chains, and aberrant O-glycosylation),¹⁶ we first analyzed the properties of IgA in CD37^{-/-} serum at the protein level. CD37^{-/-} serum contained mainly polymeric IgA, whereas IgA in wild-type serum was predominantly present as dimeric and monomeric forms (Figure 1A). Next, we evaluated glomerular IgA deposition—the hallmark of IgAN—and renal histology in wild-type and CD37^{-/-} mice of different ages. All evaluated young CD37^{-/-} mice (3 months old) showed clear mesangial deposition of mouse IgA in glomeruli, in contrast to none of wild-type mice (Figure 1B) as detected by immunohistochemistry. Moreover, lambda light chains were readily detected in glomeruli of CD37^{-/-} mice (Figure 1C). Quantification revealed that glomeruli of CD37^{-/-} mice contained significantly more lambda light chain deposition than glomeruli of wild-type mice (21.5 versus 8.8 AU; $P < 0.01$). However, renal histology did not reveal abnormalities in these young CD37^{-/-} mice (Figure 1D). Furthermore, these mice presented no albumin in the urine (Figure 1E).

CD37-Deficiency Leads to Severe Renal Pathology During Aging

In contrast to young CD37^{-/-} mice, 1.5-year-old mice developed mild albuminuria (Figure 2A) without hematuria (data not shown). Although statistically not significant, the urinary albumin excretion in aged CD37^{-/-} mice tended to be higher compared with aged-matched wild-type mice. Kidneys of aged CD37^{-/-} mice showed intense glomerular IgA deposition in contrast to kidneys of aged wild-type mice, as revealed by immunohistochemistry (Figure 2B). These results indicate that, normally, tetraspanin CD37 controls glomerular IgA deposition. Glomerular IgM and IgG depositions were similar in the young and aged CD37^{-/-} and wild-type mice (data not shown). It has been shown that serum IgA-IgG2a immune complex levels correlate with the severity of glomerular lesions in a mouse model of IgAN.²⁴ IgA-containing immune complexes are also involved in the pathogenesis of IgAN in humans.¹⁶ Therefore, we evaluated the IgA-containing immune complex levels in serum of CD37^{-/-} mice. Interestingly, aged CD37^{-/-} mice had significantly higher serum IgA-IgG1 and IgA-IgG2a immune complex levels compared with young CD37^{-/-} and wild-type (both young and aged) mice (Figure 2C), supporting a role for IgA immune complexes in driving mesangial deposition in CD37^{-/-} mice. Next, kidneys of aged CD37^{-/-} and wild-type mice were analyzed for the presence of histological lesions. All kidneys of aged CD37^{-/-} mice showed histological abnormalities with prominent mesangial expansion attributable to mesangial hypercellularity, hyperlobulation, frequent mesangiolysis, and prominent endocapillary hyalinosis (Figure 2, D–F). As a consequence, glomeruli were enlarged and clear matrix deposition was observed (Figure 2F). Crescents were not detected in glomeruli of aged CD37^{-/-} mice. Interstitial fibrosis and occasional segmental adhesions with mild hyperplasia of the parietal epithelium were also observed (Figure 2, D–F). Quantifica-

tion revealed that all CD37^{-/-} kidneys demonstrated moderate/severe mesangial proliferation that was present in 62.5% ($\pm$ 19.6%) of the evaluated glomeruli. Hyalinosis was observed in 50% of the CD37^{-/-} kidneys and in 33.9% ($\pm$ 18.4%) of the glomeruli. None of these lesions were found in kidneys of aged-matched wild-type mice. Electron microscopy of kidneys of aged CD37^{-/-} mice showed severe mesangial expansion and hypercellularity with scattered small electron-dense deposits (Figure 2G). There was frequent mesangiolysis with microaneurysm formation without any endothelial abnormalities, excluding thrombotic microangiopathy as the cause of mesangiolysis. There was occasional subendothelial hyalinosis. The glomerular basement membrane was unremarkable, and podocytes showed normal foot processes (Figure 2G). Kidneys of aged-matched wild-type mice did not demonstrate abnormalities except for some electron-dense deposits in mesangial cells that was attributable to IgG/IgM deposition (data not shown), but not attributable to IgA deposition (Figure 2B). Taken together, these results demonstrate that CD37^{-/-} mice spontaneously develop renal pathology with evident glomerular IgA deposition and mesangial proliferation.

CD37^{-/-} Mice Develop High Levels of Mouse IgA Anti-Rabbit IgG and More Glomerular IgA Deposits during Anti-GBM Nephritis

Because IgAN is triggered under inflammatory conditions,²⁵ we determined whether young CD37^{-/-} were more prone to renal damage by induction of anti-GBM nephritis in CD37^{-/-} and wild-type mice. Inflammation was analyzed during the acute heterologous phase (ie, after 2 hours) and during the autologous phase (ie, after 4 days).²¹ Administration of 8 mg rabbit anti-mouse GBM antibodies to wild-type and CD37^{-/-} mice resulted in similar binding of rabbit IgG along the GBM after 2 hours (Figure 3A), which remained unaffected until 4 days. Evaluation of serum levels of mouse antibodies directed against rabbit IgG revealed significantly higher levels of serum mouse IgA anti-rabbit IgG in CD37^{-/-} mice compared with wild-type mice at day 4 after induction of anti-GBM nephritis (Figure 3B). CD37^{-/-} mice also showed higher serum levels of mouse IgG anti-rabbit IgG compared with wild-type mice after 4 days (data not shown). Furthermore, CD37^{-/-} mice demonstrated significantly higher IgA-IgG1 and IgA-IgG2a immune complex levels in serum compared with wild-type mice at day

4 after induction of nephritis (Figure 3C). The glomerular rabbit IgG binding was accompanied by a linear complement (C3c) deposition in both mice strains after 2 hours (Figure 4A). During the autologous phase of the disease, CD37^{-/-} mice developed more glomerular deposits of mouse IgA anti-GBM compared with wild-type mice as revealed by immunohistochemistry (Figure 4B). Quantification of the glomerular deposition confirmed that CD37^{-/-} mice showed significantly more glomerular mouse IgA deposition compared with wild-type mice for all injected doses evaluated (1, 3, or 8 mg) of rabbit anti-GBM IgG (Figure 4C). IgG deposition along the capillary wall was comparable between CD37^{-/-} and wild-type mice. The administration of rabbit anti-GBM IgG showed a dose-dependent effect on the glomerular IgA deposition. Thus, CD37-deficiency increases specific IgA deposition in glomeruli during experimental nephritis.

CD37-Deficiency Leads to Increased Glomerular PMN and Macrophage Influx During Anti-GBM Nephritis

Next, we evaluated the glomerular leukocyte influx, being one of the key determinants of glomerular damage in glomerulonephritis.²⁶ During anti-GBM nephritis, PMNs and macrophages emerge within hours, whereas CD4⁺ and CD8⁺ T cells influx is observed after a few days.²¹ We observed glomerular PMN influx to be significantly higher in CD37^{-/-} mice compared with wild-type mice 2 hours after injection of anti-GBM IgG (Figure 5A). The glomerular influx of macrophages, CD4⁺, and CD8⁺ T cells was similar between CD37^{-/-} and wild-type mice after 2 hours. However, after 4 days both residual glomerular PMN and macrophage influx were significantly increased in CD37^{-/-} mice compared with wild-type mice (Figure 5, B and C). As control, kidneys of naïve wild-type and CD37^{-/-} mice were analyzed and did not stain positive for leukocytes, indicative for the absence of inflammation (data not shown). These results indicate that tetraspanin CD37 controls glomerular PMN and macrophage influx during anti-GBM nephritis. In contrast, the glomerular influx of CD4⁺ and CD8⁺ T cells was not different between both mouse strains after 2 hours or 4 days. Surprisingly, CD37^{-/-} mice did not show more albuminuria compared with wild-type mice after 4 days (Figure 5D). However, analysis of renal histology re-

Figure 2. Pronounced renal pathology in aged CD37^{-/-} mice. **A:** Development of mild albuminuria in wild-type and CD37^{-/-} mice during aging. **B:** Kidneys of 1.5-year-old naïve wild-type (**left**) and CD37^{-/-} (**middle**) mice were stained for presence of IgA by immunohistochemistry. Scale bars represent 40 μ m. Quantification of IgA deposition in glomeruli of CD37^{-/-} mice as compared with 1.5-year-old wild-type mice ($*P < 0.01$) as described in *Materials and Methods* (**right**). Isotype staining was negative. AU indicates arbitrary units. **C:** Development of high titers of IgA-IgG1 (**upper**) and IgA-IgG2a (**lower**) immune complexes in serum of CD37^{-/-} mice detected by ELISA. Aged CD37^{-/-} mice (black triangles) contain significantly higher levels of IgA immune complexes in serum than young CD37^{-/-} mice (black squares) and wild-type (young and old) mice (white symbols; $*P < 0.05$). Evident renal pathology in kidneys of 1.5-year-old CD37^{-/-} mice visualized by Periodic acid-Schiff (**D**), Jones' methenamine silver (**E**), and Chromotop Anilinblue (**F**) staining. Note the presence of severe mesangial proliferation (**1**), hyalinosis (**2**), and prominent parietal epithelium (**3**) in CD37^{-/-} glomeruli (**D**). Matrix deposition (**4**) and interstitial fibrosis (**5**) in CD37^{-/-} kidneys is visualized by Chromotop Anilinblue staining (**F**). Kidneys of 1.5-year-old wild-type mice did not demonstrate abnormalities. Note that histology images of wild-type and CD37^{-/-} kidneys of the same magnification are shown, demonstrating that CD37^{-/-} glomeruli are enlarged (Scale bars represent 60 μ m). **G:** Electron microscopy of kidneys of 1.5-year-old wild-type mice (**left**) and CD37^{-/-} mice (**right**; $\times 4000$). Severe mesangial expansion with scattered electron-dense deposits (**1**) were observed in CD37^{-/-} glomeruli. Hyalinosis (**2**) was apparent whereas the glomerular basement membrane (**3**) was unaltered, and podocytes showed normal foot processes. Kidneys of aged wild-type mice did not demonstrate abnormalities except for some electron-dense deposits in the mesangial cells that was attributable to IgG/IgM deposition (data not shown) but not to IgA deposition (**B**).

Figure 3. IgA response during anti-GBM nephritis in CD37^{-/-} versus wild-type mice. Anti-GBM nephritis was induced by injecting 8 mg rabbit anti-GBM IgG i.v. Wild-type and CD37^{-/-} kidneys were analyzed after 2 hours and 4 days. **A:** Deposition of rabbit IgG in glomeruli was similar between wild-type (left) and CD37^{-/-} (right) mice analyzed by immunohistochemistry 2 hours after injection. Scale bars represent 50 μ m. **B:** Serum of CD37^{-/-} and wild-type mice was analyzed for the presence of IgA directed against rabbit IgG 4 days after injection by ELISA. CD37^{-/-} serum (black symbols) contains rabbit-specific IgA in contrast to wild-type serum (white symbols; * P < 0.001, ** P < 0.02). AU indicates arbitrary units. **C:** Presence of IgA-IgG1 (upper) and IgA-IgG2a (lower) immune complexes in serum of CD37^{-/-} mice (black symbols), but not in serum of wild-type mice (white symbols), 4 days after induction of anti-GBM nephritis (* P < 0.02, ** P < 0.05).

Figure 4. Increased IgA deposition into CD37^{-/-} glomeruli during anti-GBM nephritis. Anti-GBM nephritis was induced by injecting 1, 3, and 8 mg rabbit anti-GBM IgG i.v. in wild-type and CD37^{-/-} mice. Kidneys were analyzed after 2 hours and 4 days. **A:** C3c complement deposition in glomeruli was similar between wild-type (left) and CD37^{-/-} (right) mice analyzed by immunohistochemistry 2 hours after injection of 8 mg anti-GBM. **B:** IgA deposition in wild-type (left) and CD37^{-/-} (right) glomeruli 4 days after injection of 8 mg anti-GBM visualized by immunohistochemistry. Scale bars represent 50 μ m. **C:** Quantification of IgA and IgG deposition in CD37^{-/-} and wild-type glomeruli 4 days after administration of 1 mg (left) and 8 mg (right) anti-GBM. AU indicates arbitrary units. CD37^{-/-} glomeruli contain significantly more IgA deposition than wild-type glomeruli (* P < 0.02, ** P < 0.006).

vealed moderate mesangial proliferation in CD37^{-/-} glomeruli in contrast to wild-type glomeruli (Figure 5E).

To get more insight into the mechanism underlying the increased glomerular leukocyte influx in CD37^{-/-} mice, we performed IgA adoptive transfer experiments. Purified IgA isolated from CD37^{-/-} serum was injected intravenously into wild-type mice (0.9 mg IgA/mouse). Anti-GBM nephritis was induced in untreated (wild-type and CD37^{-/-}) mice and in IgA-treated wild-type mice, and glomerular leukocyte influx was analyzed after 4 days. In line with Figure 5A, neutrophil and macrophage glomerular influx was significantly increased in CD37^{-/-} mice compared with untreated wild-type mice. However, wild-type mice treated with IgA demonstrated leukocyte influx that was comparable with influx observed in glomeruli of CD37^{-/-} mice (Figure 5F). These data indicate that elevated and deposited IgA by itself is sufficient to trigger leukocyte recruitment to glomeruli of CD37^{-/-} mice. Thus, the tetraspanin CD37 controls the glomerular deposition of IgA that induces influx of inflammatory myeloid cells *in vivo*.

Discussion

Systemic IgA overproduction has been linked to IgA deposition in the kidney and development of IgAN,¹⁵ how-

ever the immunological mechanisms underlying IgAN are poorly understood. Recently, it has been shown that tetraspanin protein CD37 regulates IgA responses.¹⁴ Here, we evaluated IgA immune complex formation, IgA deposition, renal function, and pathology in CD37^{-/-} mice. Furthermore, we investigated the possible role of CD37 in experimental anti-GBM nephritis as a model for glomerular disease.

Young (3-month-old) CD37^{-/-} mice demonstrated clear mesangial IgA deposition in contrast to wild-type mice, which corresponded to the increased levels of serum IgA in CD37^{-/-} mice and is consistent with previous studies.²⁷⁻³⁰ High serum IgA levels alone, however, are not sufficient to produce mesangial deposits in IgAN patients but are dependent on special characteristics of IgA, being high molecular weight, lambda light chain overexpression, and decreased O-glycosylation. We established that the circulating IgA antibodies in CD37^{-/-} mice are predominantly in the polymeric form with lambda chain over-representation. O-glycosylation of IgA was not investigated in our study because murine IgA lacks the hinge region. Despite mesangial IgA deposition, renal function and histology did not reveal abnormalities in these young CD37^{-/-} mice. However, aged CD37^{-/-} mice demonstrated, in addition to augmented glomerular IgA deposition, evident renal pathology which was not observed in aged-matched wild-type mice. Severe mesangial proliferation was observed in CD37^{-/-} glomeruli that is the common characteristic histopathological finding in IgAN. Our findings correspond to the glomerular IgA deposition seen in the ddY mouse strain, which is a spontaneous murine IgAN model.^{30,31} In ddY mice, the incidence of IgAN is highly variable and high serum IgA levels by itself are not associated with the severity of glomerular injury. However, the presence of serum IgA-IgG2a immune complexes has been correlated with the severity of glomerular lesions.²⁴ Interestingly, CD37^{-/-} mice developed high serum levels of both

Figure 5. Increased influx of inflammatory myeloid cells into CD37^{-/-} glomeruli. Anti-GBM nephritis was induced by injecting 8 mg rabbit anti-GBM IgG i.v. in wild-type and CD37^{-/-} mice. Kidneys were analyzed for inflammation after 2 hours and 4 days. Neutrophil (PMN; GR-1⁺), macrophage (Mφ; CD68⁺), and T cell (CD4⁺, CD8⁺) influxes were examined in wild-type and CD37^{-/-} kidneys by immunohistochemistry. Numbers of leukocytes per 50 glomeruli were quantified 2 hours (**A**) or 4 days (**B**) after nephritis induction. CD37^{-/-} glomeruli contained significantly more influx of neutrophils and macrophages than wild-type glomeruli (* $P < 0.01$, ** $P < 0.001$). **C:** Immunohistochemistry stainings of macrophage (CD68⁺) influx (arrows) into wild-type and CD37^{-/-} glomeruli (visualized by positive staining for agrin) 4 days after nephritis induction. Glomeruli of untreated wild-type and CD37^{-/-} mice did not contain influx of myeloid or lymphoid cells (not shown). Scale bars represent 60 μm. **D:** Albuminuria in wild-type and CD37^{-/-} mice 4 days after nephritis induction. **E:** Renal pathology in wild-type (left) and CD37^{-/-} (right) mice 4 days after anti-GBM injection was analyzed by histology (Periodic acid-Schiff staining). CD37^{-/-} glomeruli showed mild mesangial proliferation (arrow) in contrast to wild-type glomeruli. Scale bars represent 30 μm. **F:** Wild-type mice were injected in the tail vein with purified mouse IgA and rabbit anti-mouse GBM IgG. Control age-matched wild-type and CD37^{-/-} mice were injected i.v. with rabbit anti-mouse GBM IgG only. Kidneys were analyzed for leukocyte influx by immunohistochemistry 4 days after nephritis induction. Numbers of neutrophils (PMN; GR-1⁺) and macrophages (Mφ; CD68⁺) per 50 glomeruli were quantified in kidneys of untreated wild-type and CD37^{-/-} mice and wild-type mice treated with IgA. CD37^{-/-} glomeruli contained significantly more influx of neutrophils and macrophages than wild-type glomeruli (* $P < 0.02$, ** $P < 0.05$), but not more than wild-type glomeruli of mice that received IgA (n.s. indicates nonsignificant).

IgA–IgG1 and IgA–IgG2a immune complexes during aging, which was not observed in wild-type mice. This suggests that IgA immune complexes play an important role in driving mesangial IgA deposition and renal pathology in CD37^{−/−} mice. It has been hypothesized that IgA–IgG2 complexes (Th₁ response) mediate glomerular injury by interaction with activatory Fc receptors on macrophages.²⁷ Taken together, our study demonstrates that CD37^{−/−} mice spontaneously develop renal pathology with characteristics of IgA nephropathy.

We have previously reported that macrophages of CD37^{−/−} mice make increased levels of interleukin-6 (IL-6),⁹ and that the increased IgA response in CD37^{−/−} mice is dependent on IL-6. IL-6 induces proliferation of mesangial cells *in vitro*, and a relation between IL-6 levels in urine of IgAN patients and disease progression has been found.³² Thus, it is tempting to speculate that high IL-6 levels contribute to renal histopathology in CD37^{−/−} mice. Because IgAN is triggered under inflammatory conditions,²⁵ we determined whether young CD37^{−/−} mice were more susceptible to renal damage than wild-type mice during experimental anti-GBM nephritis. The deposition of the injected rabbit anti-mouse GBM IgG, followed by C3c along the capillary wall, was not affected by CD37-deficiency. However, the humoral immune response against the injected rabbit anti-GBM antibodies was different between wild-type and CD37^{−/−} mice. CD37^{−/−} mice had markedly higher serum levels of mouse anti-rabbit IgA and glomerular mouse IgA deposits in contrast to wild-type mice. In line with this, CD37^{−/−} mice showed significantly higher serum IgA–IgG1 and IgA–IgG2a immune complex levels compared with wild-type mice after induction of anti-GBM nephritis.

Analysis of glomerular leukocyte influx demonstrated that recruitment of PMNs and macrophages was increased in CD37^{−/−} mice compared with wild-type mice. The importance of IgA in this process was shown in adoptive transfer experiments that revealed that IgA purified from CD37^{−/−} serum was able to exacerbate glomerular inflammation in wild-type mice during anti-GBM nephritis. The polymeric nature of IgA in CD37^{−/−} mice may play a pathogenic role in stimulating the glomerular influx of inflammatory leukocytes and development of mesangial proliferative nephritis in these mice. Thus, our data demonstrate that elevated and deposited polymeric IgA by itself is sufficient to enhance the acute anti-GBM induced inflammation by attracting myeloid cells to the glomeruli. However, we do not exclude that CD37-deficiency also increases the intrinsic migratory capacity of CD37^{−/−} leukocytes since the role of tetraspanins in integrin-mediated migration is well-established.⁴ Surprisingly, CD37^{−/−} mice did not show more albuminuria compared with wild-type mice after 4 days, which suggests that albuminuria is not strictly leukocyte-dependent in CD37^{−/−} mice with experimental anti-GBM nephritis. Indeed other studies also showed that complement-dependent mechanisms may be operative in anti-GBM nephritis.³³ Notwithstanding, analysis of renal histology revealed increased mesangial proliferation in CD37^{−/−} glomeruli compared with wild-type glomeruli, supporting a role for

CD37 in initiation of renal pathology during anti-GBM nephritis.

Importantly, two other members of the tetraspanin family, CD63 and CD151, have recently been implicated in preventing renal pathology. CD63-deficient mice have a defect in the collecting duct leading to an altered water balance.³⁴ CD151 has been involved in the assembly and maintenance of the GBM structure and plays a key role in integrin-mediated adhesion of podocytes.^{35,36} Alterations in CD151 are associated with primary glomerular disease in humans and mice.³⁵ Thus, tetraspanin proteins CD37, CD151, and CD63 play different nonredundant roles in the biology of the kidney.

In summary, we report that the B cell protein CD37 controls the formation of IgA-containing immune complexes and the glomerular deposition of IgA. Under normal conditions, therefore, tetraspanin CD37 may be protective against the development of IgAN. The absence of CD37 leads to formation of IgA immune complexes in serum, IgA deposition in glomeruli, and subsequently development of mesangial proliferative renal pathology. Moreover, CD37^{−/−} mice are more susceptible to renal injury as demonstrated in the anti-GBM nephritis model by an increased influx of inflammatory myeloid cells into glomeruli. This study provides novel insights into immunological mechanism underlying increased IgA production and IgA immune complex formation leading to mesangial deposition during IgAN.

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