

# Lymphocytes are dispensable for glomerulonephritis but required for renal interstitial fibrosis in matrix defect-induced Alport renal disease

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One current theory for the emergence of glomerular nephritis implicates Th1-type cellular responses associated with delayed-type hypersensitivity, involving T cells and macrophages. Using a mouse model for progressive glomerulonephritis, we investigate the role of B and T cells in the pathogenesis of glomerular inflammation. Deletion of  $\alpha 3$  chain of type IV collagen in mice ( $\alpha 3$ (IV) collagen null mice) results in GBM defects, glomerulonephritis and tubulointerstitial inflammation, fibrosis and significant immune infiltration including activated B- and T-lymphocytes. To evaluate the contribution of lymphocytes to the pathogenesis of glomerulonephritis and renal fibrosis, we generated mice that are deficient in both the  $\alpha 3$ (IV) collagen and Rag-1 ( $\alpha 3$ /Rag-1 DKO). Lymphocyte deficiency significantly reduces fibrosis in the renal interstitium, but ultrastructural GBM defects persist. Interestingly, glomerulonephritis in the double null mice persists at a similar level with comparable proteinuria. Here we demonstrate that despite the presence of B-cell and T-cells in the inflamed glomeruli, their deletion does not impede the emergence of glomerulonephritis but has a negative impact on the progression of renal interstitial fibrosis.

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Glomerulonephritis and renal fibrosis is an indicator of the severity of kidney disease in human kidney biopsies and is associated with poor prognosis.<sup>1–4</sup> Unless spontaneously resolved, some form of glomerulonephritis progresses to more advanced fibro-cellular and fibrous crescents, irreversibly compressing and scarring the glomeruli and disabling its filtration property. A better understanding of the pathogenesis of glomerulonephritis may lead to the development of new therapeutic strategies. Nephrotoxic nephritis rodent models, in which the animals are given heterologous antisera to the glomerular basement membrane (GBM), are most commonly studied for the emergence of glomerulonephritis. In these models, the mechanisms for glomerulonephritis implicates a CD3+ T cell-driven Th1-type responses with delayed-type hypersensitivity.<sup>5–13</sup> In contrast, Ophascharoensuk *et al*<sup>14</sup> report that in their model of murine nephritis, proliferating glomerular epithelial cells are the main cellular components of the crescent, with early crescents devoid of macrophages and T cells. Furthermore, a growing body of

evidence has suggested that parietal epithelial cells are involved in crescent formation, with transdifferentiation of parietal epithelial cells and acquisition of new epitopes, shared by macrophages.<sup>15–23</sup> These results indicate that origins of glomerulonephritis still remain ambiguous, and mechanisms may differ according to the underlying renal disease.

In this study, we investigate the roles of B and T cells in the pathogenesis of glomerulonephritis in the type IV collagen  $\alpha 3$  chain null mice. Deletion of the type IV collagen  $\alpha 3$  chain ( $\alpha 3$ KO mice) impairs the obligate type IV collagen  $\alpha 3/\alpha 4/\alpha 5$  protomer formation in the GBM.<sup>24</sup> The  $\alpha 3$ (IV) collagen null mouse is one of the animal models used to study the pathophysiology of Alport Syndrome.<sup>25,26</sup> These mice are deficient in producing type IV collagen  $\alpha 3$  chain and develop progressive glomerulonephritis, tubulo-interstitial disease, and thinning and focal multilaminated thickening of the GBM.<sup>24,26,27</sup> The background strain of mouse strongly affects the disease progression and survival of the  $\alpha 3$ (IV)

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collagen null mice. This modifier effect implies that GBM make up and the immune response towards the damaged GBM is likely influenced by inherent genotypic differences.<sup>28,29</sup> The  $\alpha 3(\text{IV})$  collagen null mice on C57Bl/6J background exhibit detectable proteinuria starting at 12 weeks of age and die of end stage renal failure between 22–27 weeks of age.<sup>24</sup>

It is proposed that a robust lymphocytic infiltration in the damaged kidneys is triggered by the GBM structural defects and miscommunications resulting from aberrant cell-matrix interaction in the glomeruli. It is assumed that such infiltration of B-cells and T-cells contribute significantly to the emergence of glomerulonephritis and tubulo-interstitial nephritis leading to tissue fibrosis. To address the specific role of the T- and B-lymphocytes in this process, we crossed the  $\alpha 3(\text{IV})$  collagen null mice with the Rag-1 null mice and analyzed the disease progression in these double null mice ( $\alpha 3/\text{Rag-1}$  DKO mice). The deletion of the Rag-1 gene results in a blockade of lymphocyte differentiation by disabling V(D)J recombination.<sup>30</sup> While mature B- and T-cells are absent in the Rag-1 null mice, NK cells, neutrophils and macrophages are present in these mice.

Our results demonstrate that B- and T-lymphocytes are not required for the glomerulonephritis but are essential components of tubulointerstitial inflammation and fibrosis. Collectively, our study highlights that the pathogenesis of glomerulonephritis does not involve B and T-lymphocytes in the  $\alpha 3(\text{IV})$  collagen null mice.

## MATERIALS AND METHODS

### Mice

The generation and disease progression in the  $\alpha 3\text{KO}$  mice was described previously.<sup>24,26</sup> The mice were backcrossed (10 generations) into the C57BL/6J background. The Rag-1 KO (C57BL/6J background) mice were purchased from the Jackson Laboratory. All animals with the Rag-1 gene deletion were housed in autoclaved cages with irradiated murine chow and autoclaved water. The  $\alpha 3\text{KO}$  mice were housed under standard conditions. All animals were housed in the Beth Israel Deaconess Medical Center animal facility. All animal studies were reviewed and approved by the Animal Care and Use Committee of the Beth Israel Deaconess Medical Center.

### Urine Albumin/Creatinine

Urine samples were collected at 8, 12 and 22 weeks of age and kept frozen prior to analyses. Creatinine concentration was measured using the colorimetric assay Quantichrome (DICT-500) from BioAssays (Hayward, CA, USA) according to the manufacturer's directions. Albumin concentrations were measured using the Mouse Albuminuria ELISA (Bethyl laboratory, Montgomery, TX, USA) according to the manufacturer's direction.

### Blood Creatinine

Blood samples (100  $\mu\text{l}$ ) were collected at 8, 12 and 22 weeks of age. Plasma was separated from cellular components by centrifugation (5 min, 6000 r.p.m., RT) and stored frozen prior to analyses. Plasma creatinine was measured using HPLC analysis (Dr Steve Dunn, Thomas Jefferson University, Division of Nephrology Rodent Core Laboratories, Philadelphia, PA, USA).

### Kidney Tissue Analyses

Mice were euthanized using  $\text{CO}_2$  at 8, 12, and 22 weeks of age and kidneys harvested. The kidneys were dissected and fixed accordingly.

### Immunocytochemistry

Sagittal sections were embedded in OCT compound and snap frozen in liquid nitrogen. Thin frozen sections (0.6  $\mu\text{m}$ ) were fixed in ice-cold acetone (20 min) and immunostained against podocin, staining the podocyte (dilution 1:200, a gift from Dr Peter Mundel, Mount Sinai School of Medicine, New York, NY, USA), CD3 (dilution 1:200, eBioscience, 14-0031) and CD19 (dilution 1:250, Serotec MCA1439). TRITC- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch) were used at a 1:200 dilution. The slides were mounted with Vectashield Mounting Medium with DAPI (Vectashield; H1200) and glass coverslip, and analyzed using the Axioskop 2 fluorescent microscope, AxioCam HRC camera and the Axiovision 4.3 software.

### Light Microscopy

Kidneys were also fixed in formalin and paraffin sections used for Haematoxylin and Eosin, Periodic Acid Schiff and Masson's Trichrome staining under standard conditions (Histology Core Facility, Beth Israel Deaconess Medical Center, Boston, MA, USA). Morphometric analyses for the histological assessment of renal injury, here glomerular sclerosis and interstitial fibrosis, were performed as described previously.<sup>29</sup> For the analysis of interstitial fibrosis, Masson's Trichrome-stained kidney sections were observed using a counting grid at  $\times 400$  magnification. The number of grid intersections, which coincide with interstitial fibrosis was expressed as a percentage of the total number of grid intersections. Five random views were counted per slide and five slides were observed per experimental group. For the analysis of glomerular sclerosis and crescentic glomeruli, 100 glomeruli were counted per slide and five slides were counted per experimental group. The number of normal glomeruli was expressed as a percentage of the total number of glomeruli counted for glomerular sclerosis and the number of crescentic glomeruli was expressed as a percentage of total glomeruli. Glomerular crescent cellularity was evaluated from 50 glomeruli in five mice per experimental group. The number of cells (nuclei) in the crescent of 10 glomeruli in five mice per experimental group was compared.

## Electron Microscopy

For transmission electron microscopy, 1-mm<sup>3</sup> kidney sections were fixed overnight (in 2% glutaraldehyde in 0.1 M cacodylic acid, pH 7.4). Tissue specimens were post fixed with 1% osmium tetroxide, dehydrated and embedded in Embed 812 (Electron Microscopy Sciences, Hatfield, PA, USA). The blocks were sectioned at 75 nm on a Leica UCT ultramicrotome (Bannockburn, IL, USA) using a Diatome diamond knife (Electron Microscopy Sciences, Hatfield, PA, USA). Images were taken on a Technia G12 Biotwin TEM (FEI, Hillsboro, OR, USA) equipped with an AMT CCD camera (Advanced Microscopy Techniques, Danvers, MA, USA). All processing, sectioning and scope work was done in the Electron Microscopy Center, Department of Anatomy and Cell Biology, at the Indiana University School of Medicine.

## Statistical Analyses

Standard errors were calculated and *t*-test and ANOVA were used to determine statistical differences. A level of  $P < 0.05$  was considered statistically significant.

## RESULTS

At 8 weeks of age, the  $\alpha 3$ KO mice exhibit nearly normal renal function and histology.<sup>26</sup> Twelve-week-old  $\alpha 3$ KO mice present with detectable proteinuria and mild-to-moderate glomerulonephritis and interstitial fibrosis (20% decrease in the number of normal glomeruli). By 22 weeks of age, the mice develop massive proteinuria, severe crescentic glomerulonephritis, glomerulosclerosis, interstitial fibrosis, and ultrastructural defects in the GBM.

### Lack of B- and T-lymphocyte Infiltration in the Injured Glomeruli of the Mice

To confirm a loss of mature B- and T-cell infiltration in the kidneys of 22-week-old  $\alpha 3$ /Rag-1 DKO animals, kidney sections from these mice, the  $\alpha 3$ KO, and the Rag-1 KO littermates were labeled for mature B (CD19) and mature T (CD3) lymphocyte markers. The CD3- or CD19-positive cells were not detected in the Rag-1 KO and the  $\alpha 3$ /Rag-1 DKO mice, whereas significant number of T-cells and B-cells were observed in the  $\alpha 3$ KO animals (Figure 1). T-cells and B-cells were mainly detected in the renal interstitium. Injured glomeruli in the  $\alpha 3$ KO mice exhibit very few cells that are CD3<sup>+</sup> but none that are CD19<sup>+</sup>. CD3<sup>+</sup> and CD19<sup>+</sup> cells are not detected in the inflamed glomeruli of the  $\alpha 3$ /Rag-1 DKO mice.

### Significant Difference is Detected in the Renal Histology Between the $\alpha 3$ KO and $\alpha 3$ /Rag-1 DKO Mice

Light microscopy analysis Haematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS) and Masson's Trichrome staining (MTS) was performed using the kidneys from the  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO mice (Figure 2a). Haematoxylin and Eosin labeling of the renal cortex reveals diffuse glomerular and

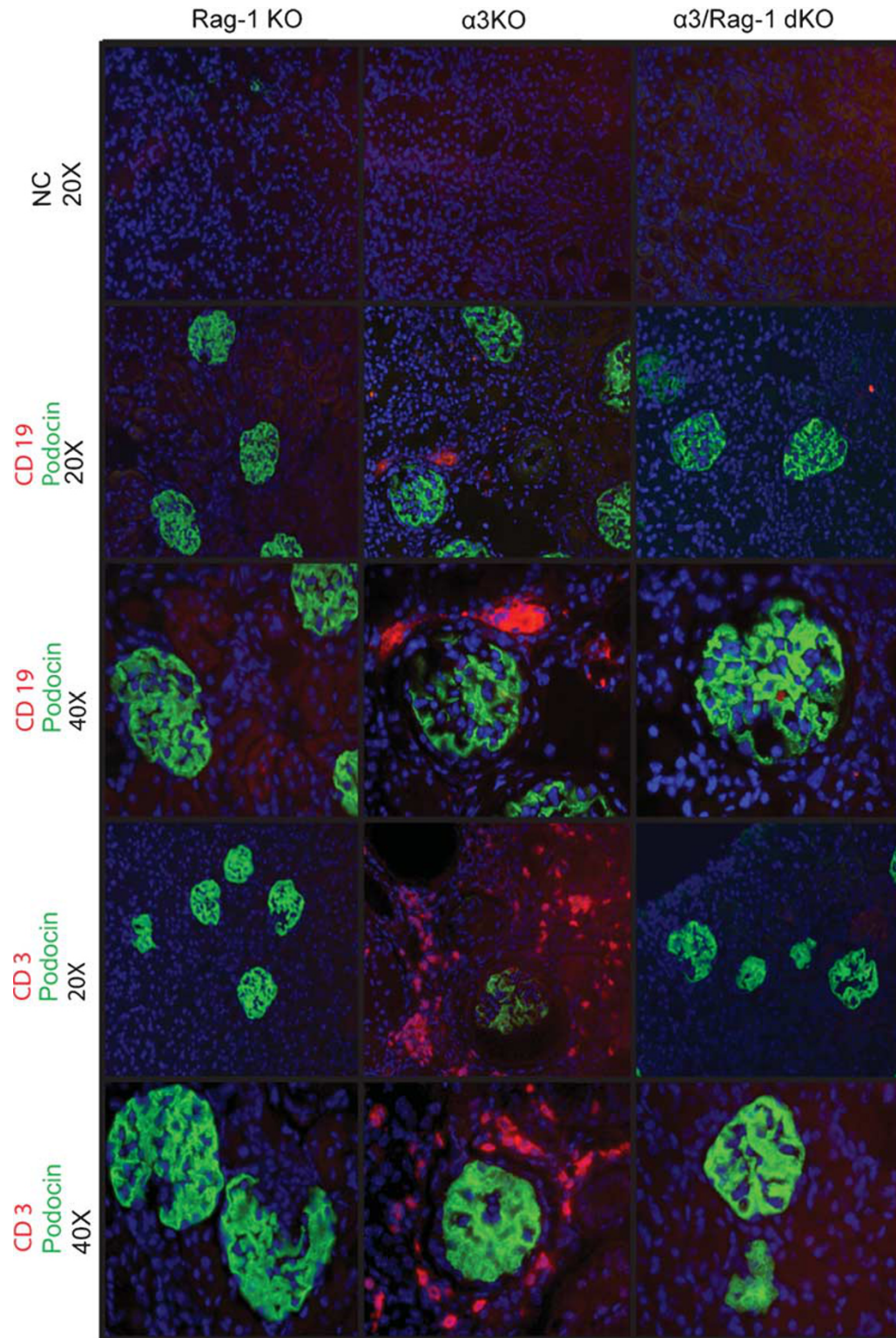
tubular damage, with shrunken and sclerotic glomeruli and tubular casts (Figure 2a, middle panel). Despite mature lymphocyte ablation in the  $\alpha 3$ /Rag-1 DKO mice, leukocyte infiltration (likely macrophages, neutrophils, and NK cells) persists in the interstitium of these mice. Periodic Acid Schiff labeling of glomeruli reveals formation of a fibrous cap with epithelial and mesangial hypercellularity in both the  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO, in contrast to the glomeruli of the Rag-1 KO animals (Figure 2a, lower panel). The  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO animals exhibit tubular atrophy and fibrotic scarring, as determined using Masson's Trichrome labeling of the cortical region of the kidney (Figure 2a, upper panel). Morphometric analysis reveals a significant difference in the glomerulosclerosis between the  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO mice (Figure 2b). Tubular atrophy and interstitial fibrosis is significantly greater in the  $\alpha 3$ KO mice when compared to the  $\alpha 3$ /Rag-1 DKO mice (Figure 2b, lower panel). Morphometric evaluation of kidney sections from 22-week-old  $\alpha 3$ KO mice show ~70% tubular atrophy and ~12% interstitial fibrosis whereas  $\alpha 3$ /Rag-1 DKO mice show a significant reduction in tubular damage, with ~40% tubular atrophy and ~5% interstitial fibrosis. The Rag-1 KO littermates exhibit normal kidney histology. The number of crescentic glomeruli is slightly lower in the  $\alpha 3$ /Rag-1 DKO mice (~15%) when compared to the  $\alpha 3$ KO mice (~22%). Cellularity in the crescent is greater in the  $\alpha 3$ KO mice (~40%) when compared to the  $\alpha 3$ /Rag-1 DKO (~28%) (Figure 2b, lower panel).

### Renal Function in the $\alpha 3$ KO and $\alpha 3$ /Rag-1 DKO Mice

The ratio of urine albumin to urine creatinine was used as a measure of proteinuria and with an increased ratio indicating an increase in glomerular vascular leak via the glomerular pathology. Increased urine protein was observed in the  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO mice at 12 and 22 weeks of age, while the Rag-1 KO mice exhibit normal range of urine protein (Figure 3a). Serum creatinine levels in the  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO mice increase as the disease progresses, while the littermate Rag-1 KO animals show normal range of serum creatinine, comparable to wild type mice (0–0.1 mg/dl, Figure 3b). Serum creatinine concentration at 22 weeks of age is significantly greater in the  $\alpha 3$ KO mice (0.45 mg/dl), when compared to the  $\alpha 3$ /Rag-1 DKO mice (0.2 mg/dl). This suggests a direct improvement in the interstitial disease of  $\alpha 3$ KO mice when lymphocytes are deleted.

### Ultrastructural GBM Defects in the $\alpha 3$ KO and $\alpha 3$ /Rag-1 DKO Mice

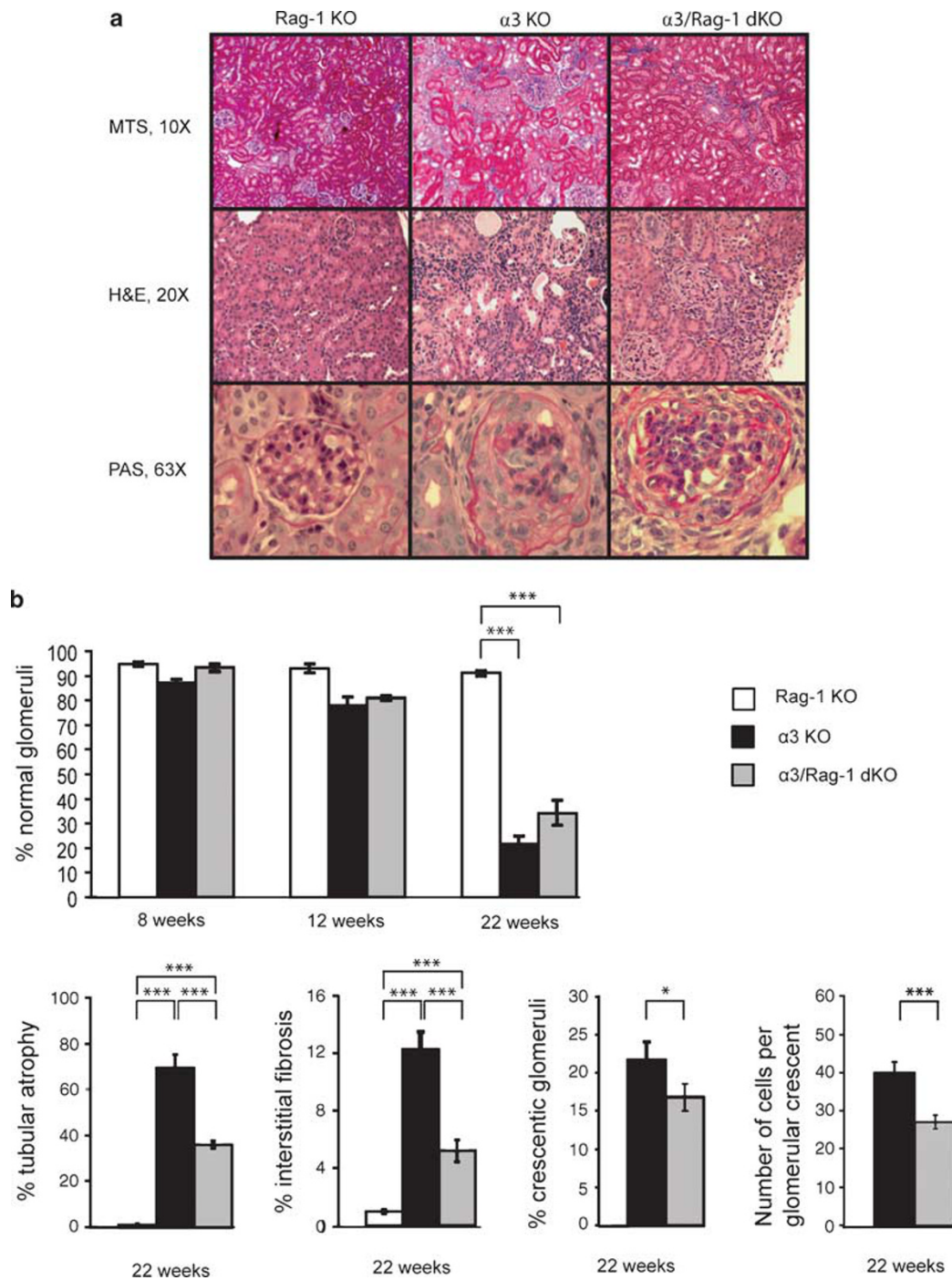
Twelve- and 22-week-old mice from each group were sacrificed and their kidneys harvested and prepared for transmission electron microscope (TEM) analysis (Figure 4). The Rag-1 KO animal presents with a continuous, homogeneous electron dense GBM, similar to the wild-type animals (Figure 4). The GBM in the  $\alpha 3$ KO and  $\alpha 3$ /Rag-1 DKO mice reveal thinning, focal multilaminated thickening,



**Figure 1** Immunocytochemistry labeling for mature B-cells and T-cells: antibodies were used against CD19 and CD3, cell surface markers were used in the kidney tissue sections of 22-week-old Rag-1 KO,  $\alpha$ 3KO, and  $\alpha$ 3/Rag-1 DKO mice. CD3 and CD19 positive cells are shown in red; podocin staining of podocytes in the glomeruli is shown in green.

splitting and basket weave appearance of the GBM with podocyte foot processes effacement, characteristic of renal disease associated with Alport syndrome (Figure 4). These

results suggest that recruitment of lymphocytes within the injured glomeruli do not appear to impact the accumulation of structural defects in the GBM.

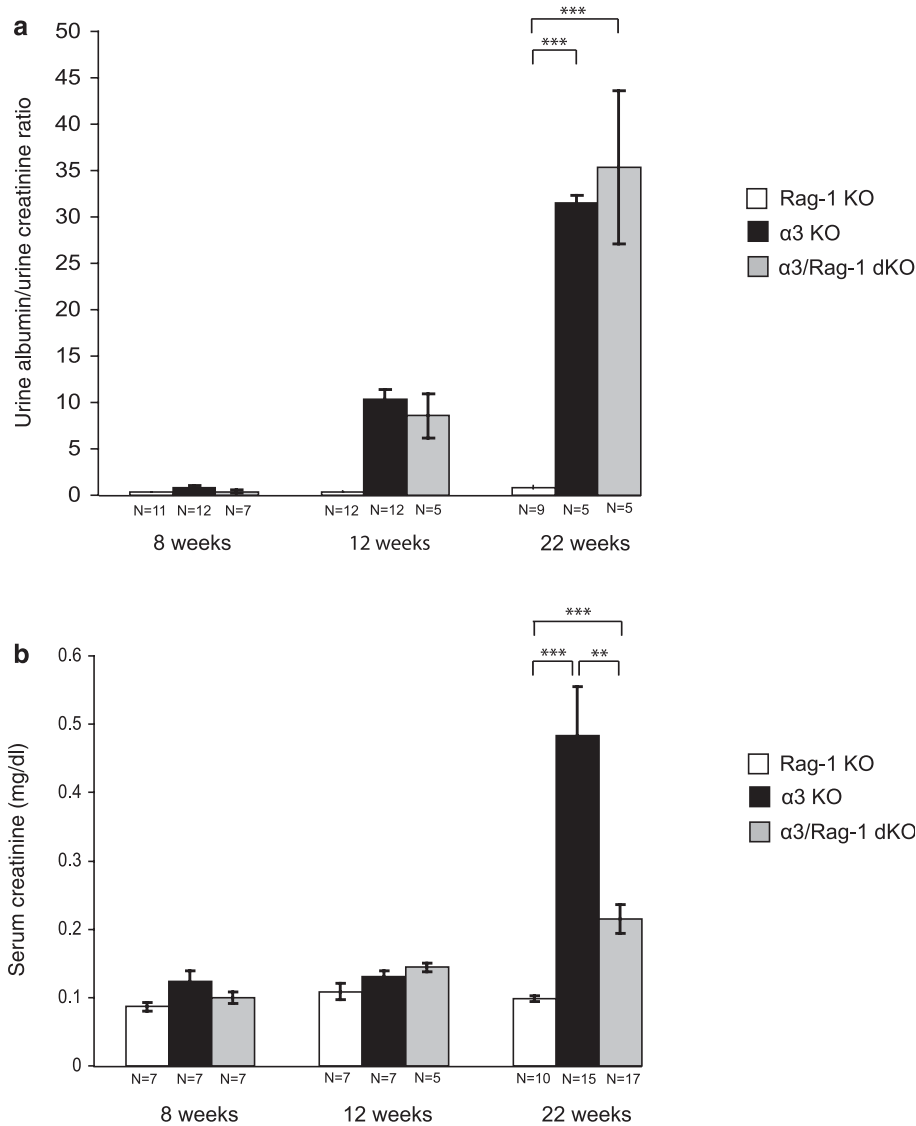


**Figure 2** Light microscopy imaging and morphometric analyses. (a) Haematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS) and Masson’s Trichrome staining (MTS) of kidneys from 22-week-old Rag-1 KO,  $\alpha 3$ KO, and  $\alpha 3$ /Rag-1 DKO mice. Respective magnifications are indicated on the figure. (b) Morphometry of glomerular sclerosis (expressed as % of normal glomeruli) of 8, 12 and 22-week-old Rag-1 KO (N = 5),  $\alpha 3$ KO (N = 5), and  $\alpha 3$ /Rag-1 DKO mice (N = 5), percent tubular atrophy, interstitial fibrosis, crescentic glomeruli and crescent cellularity of 22-week-old Rag-1 KO (N = 5),  $\alpha 3$ KO (N = 5), and  $\alpha 3$ /Rag-1 DKO mice (N = 5). \* $P < 0.05$ , \*\*\* $P < 0.001$  (ANOVA; *t*-test for % crescentic glomeruli and % number of cells per glomerular crescent).

**DISCUSSION**

The present study reveals that deficiency in lymphocytic infiltration does not prevent glomerulonephritis in the  $\alpha 3$ KO mice. Our results demonstrate that lymphocytes are not

required for the emergence of glomerulonephritis, but may influence the rate of glomerular disease. Based on unbiased statistical analyses of renal function tests and morphometric measurements of renal histology, glomerulonephritis, and

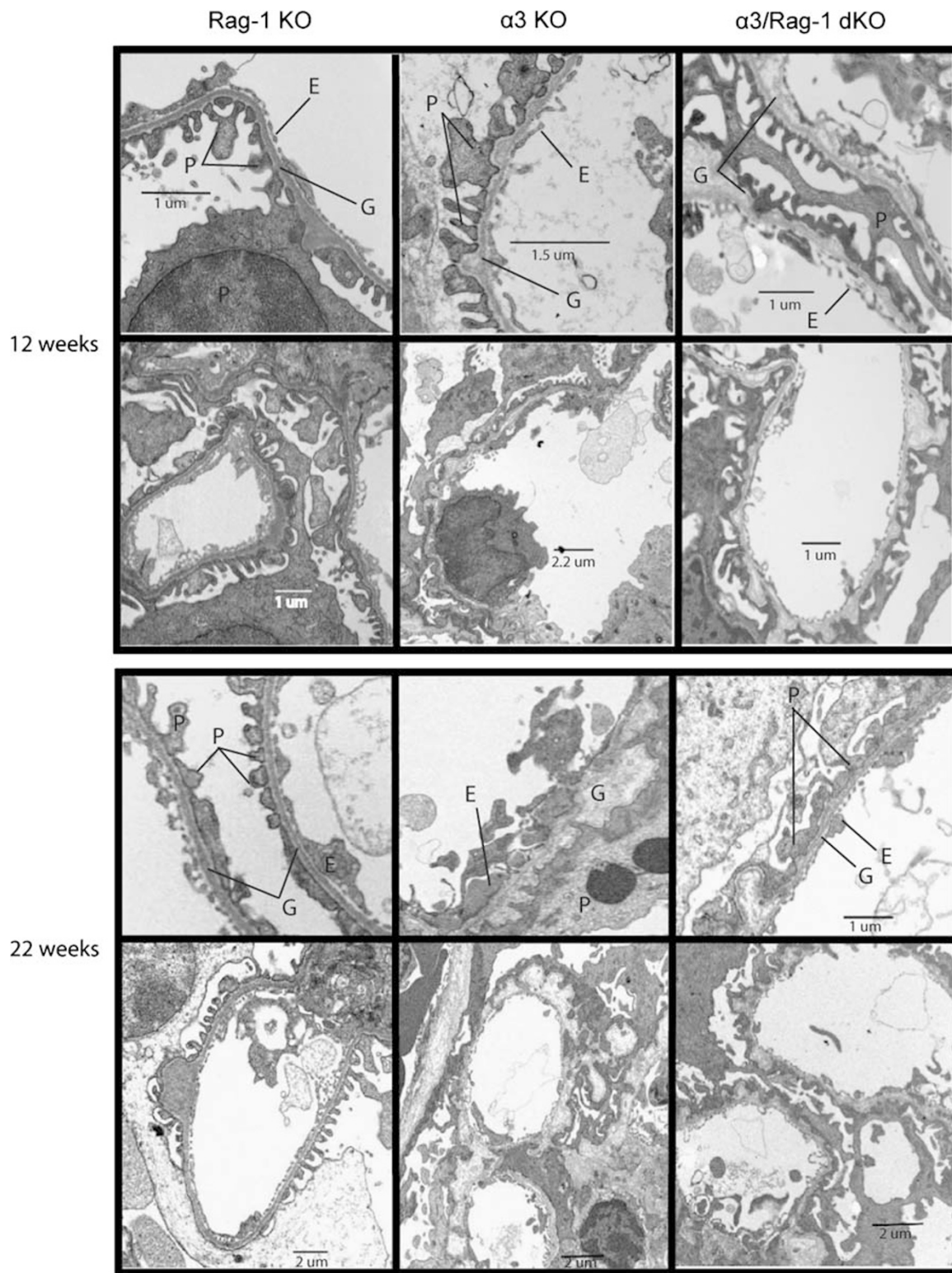


**Figure 3** Glomerular functions. Rag-1 KO,  $\alpha$ 3KO, and  $\alpha$ 3/Rag-1 DKO mice at 8, 12, and 22 weeks of age were analyzed. Urine albumin/urine creatinine ratio (a) and serum creatinine concentration in mg/dl (b) indicate normal glomerular function for the Rag-1 KO animals over time, which was significantly different than for the  $\alpha$ 3KO and  $\alpha$ 3/Rag-1 DKO animals, showing similar progression in loss of glomerular filtration properties. There were no significant differences in urine albumin/urine creatinine ratio between the  $\alpha$ 3KO and  $\alpha$ 3/Rag-1 DKO groups. A significant difference in serum creatinine was found between the two groups at 22 weeks of age. *N* = number of animals per group, \*\**P* < 0.01, \*\*\**P* < 0.001 (ANOVA).

proteinuria was determined to persist at a similar level in both  $\alpha$ 3/Rag-1 DKO and  $\alpha$ 3KO experimental groups, whereas depletion of active lymphocytes in the DKO animal reduced the injury in the renal interstitium. While the initial insult in the  $\alpha$ 3KO mice is targeted deletion of a GBM collagen to induce a structural defect, the ensuing injury involves massive influx of immune infiltrate. Our study shows that in the  $\alpha$ 3KO glomeruli, lymphocytes are not detected, but the renal interstitium contains many CD3<sup>+</sup> and CD19<sup>+</sup> lymphocytes. The GBM structural defect likely results in abnormal endothelium–GBM–podocyte communications. The loss of podocyte attachment to the defective GBM may result in de-differentiation and subsequent proliferation of the visceral epithelium, in addition to spillage of

inflammatory mediators into the Bowman's space. Proliferation of the visceral and parietal epithelium could contribute to crescent formation in the  $\alpha$ 3KO mice. Once the glomerular disease initiates, it progressively leads to glomerulonephritis.<sup>16,31</sup> Eventually, the glomerular damage spreads to the renal interstitium with tubular damage, atrophy, and fibrosis. Proteinuria is first detected in association with glomerular disease, subsequently serum creatinine levels go up to coincide with renal tubular damage and interstitial fibrosis.

Glomerulonephritis develops despite the ablation of lymphocytes. These results strongly argue that mature T-cells and B-cells are not required for the glomerular disease in the  $\alpha$ 3KO mice and that adaptive immunity may not play a



**Figure 4** Ultrastructural analyses of the Glomerular Basement Membrane. Transmission Electron Microscopy of 12- and 22-week-old Rag-1 KO,  $\alpha 3$ KO, and  $\alpha 3$ /Rag-1 DKO mice. P: podocyte foot processes, E: fenestrated endothelium, G: GBM.

role in the emergence of glomerulonephritis, but influences the rate of crescent evolution. While the number of glomerular crescents and inflammation is slightly lower in

the  $\alpha 3$ /Rag-1 DKO mice, glomerular function as evidenced by proteinuria, is unaltered in these mice when compared to the  $\alpha 3$ KO mice.

Specific autoimmunity towards glomerular antigens in the nephrotoxic nephritis mouse models indicate a possible role for T-cells in glomerulonephritis in this setting.<sup>5–9,11–13</sup> In contrast, our study demonstrates that T-cell depletion in our mouse model of progressive glomerular nephropathy does not impact the emergence of glomerulonephritis. The cellular composition of glomerular crescents in the  $\alpha 3$ KO mouse model does not appear to contain CD3<sup>+</sup> and CD19<sup>+</sup> lymphocytes, but is rather composed of proliferating visceral and parietal glomerular epithelial cells, and possibly macrophages, although their identification in glomerular crescents is challenging.<sup>15–17,20–22</sup>

Overall, deletion of B-cell and T-cells in  $\alpha 3$ KO mice does not result in a protection from glomerular disease and loss of renal function. Proteinuria remains the same in the  $\alpha 3$ KO mice and the  $\alpha 3$ /Rag-1 DKO mice. Interestingly, ultra-structural analysis by electron microscopy does not reveal any significant difference between the  $\alpha 3$ KO mice and the  $\alpha 3$ /rag-1 DKO mice. In contrast, absence of lymphocytes leads to significant protection from tubulo-interstitial inflammation. Despite similar leukocyte (macrophages, NK cells, and neutrophils) infiltration in the renal interstitium of both  $\alpha 3$ KO mice and the  $\alpha 3$ /rag-1 DKO mice, absence of mature B and T lymphocytes significantly reduced the injury to the renal interstitium. Nephrogenic T-cell response and T-cell infiltration in interstitial nephritis initiate, amplify, and perpetuate local inflammatory response which contributes to tubular atrophy and interstitial fibrosis.<sup>34,36–38</sup> However, the emergence of tubular interstitial fibrosis in our  $\alpha 3$ /Rag-1 DKO mice, as suggested by Ninichuk *et al*,<sup>32</sup> could result from macrophage recruitment and activation via MCP-1 expression.<sup>33–35</sup> Lower serum creatinine levels in the double null mice indicate that ablating active lymphocytes decreases the injury to the renal interstitium. Tubular damage and atrophy is significantly attenuated, resulting in reduced level of fibrosis. This report provides compelling evidence for the role of adaptive immunity in mediating tubular damage and facilitating fibrosis with insignificant impact on the glomerular disease and function.

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