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RGC-32 mediates proinflammatory and profibrotic pathways in immune-mediated kidney disease

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ABSTRACT

Systemic lupus erythematosus is an autoimmune disease that results in immune-mediated damage to kidneys and other organs. We investigated the role of response gene to complement-32 (RGC-32), a proinflammatory and profibrotic mediator induced by TGFβ and C5b-9, in nephrotoxic nephritis (NTN), an experimental model that mimics human lupus nephritis. Proteinuria, loss of renal function and kidney histopathology were attenuated in RGC-32 KO NTN mice. RGC-32 KO NTN mice displayed downregulation of the CCL20/CCR6 and CXCL9/CXCR3 ligand/receptor pairs resulting in decreased renal recruitment of IL-17$^+$ and IFNγ$^+$ cells and subsequent decrease in the influx of innate immune cells. RGC-32 deficiency attenuated renal fibrosis as demonstrated by decreased deposition of collagen I, III and fibronectin. Thus, RGC-32 is a unique mediator shared by the Th17 and Th1 dependent proinflammatory and profibrotic pathways and a potential novel therapeutic target in the treatment of immune complex mediated glomerulonephritis such as lupus nephritis.

1. Introduction

RGC-32 (response gene to complement 32) is an intracytoplasmic protein widely expressed in normal tissues including brain, kidney, spleen, thymus, multiple tumors and in a variety of cell lines. It was originally identified and characterized by our group as a differentially expressed gene in response to sublytic complement activation [1]. Upon upregulation by complement, growth factors, hormones and cytokines, RGC-32 translocates to the nucleus and acts as or interacts with transcription factors, regulating the expression of multiple genes involved in cell growth, viability and tissue-specific differentiation [2–8]. Initially characterized as a cell cycle regulator it has been shown to modulate a number of other cellular processes including cell migration, cellular differentiation, reproductive cycle and immune regulation [4,9–12]. RGC-32 has been reported to impact a variety of pathological processes such as carcinogenesis, metabolic disorders, angiogenesis, atherosclerosis, and autoimmunity [13–22].

As a downstream target of TGFβ, RGC-32 plays a role in TGFβ dependent profibrotic pathways. In animal models, RGC-32 has been characterized as a profibrotic mediator in organs such as the lung, skin and kidney [23,24]. In humans, RGC-32 was upregulated in tubular cells of children with IgA nephropathy where it correlated with α-smooth muscle actin (SMA), TGFβ and with the degree of renal pathological lesions [25] while in vitro, in human PTEC (proximal tubular epithelial cells), RGC-32 promoted TGFβ induced epithelial-mesenchymal transition and extracellular matrix deposition of fibronection (FN) and collagen I [6].

Our group has shown that RGC-32 exerts a proinflammatory role by promoting Th17 responses in vitro and enhances Th-17 mediated autoimmune diseases such as experimental autoimmune encephalomyelitis in vivo [10]. Th17 pathway has been linked to human lupus including lupus glomerulonephritis (GN) and mouse models of this disease [26–30]. We have previously shown that RGC-32 expression is increased in PBMC from lupus patients and in the glomeruli and tubulointerstitium of patients with lupus nephritis ([22] and manuscript in preparation). Serum levels of RGC-32 were elevated in the serum of

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children with SLE [20].

Given the proinflammatory and profibrotic functions of RGC-32, suggesting a potential role in the end-organ pathology that characterizes LN, we set out to characterize the local role of RGC-32 in a murine model of immune complex GN. To this end we used the well characterized model of nephrotoxic nephritis (NTN), a Th-17 dependent, immune complex GN and analyzed the effect of RGC-32 gene ablation on disease phenotype at a step downstream of antibody (Ab) production.

Our findings demonstrate that RGC-32 deficiency attenuated kidney disease measured by proteinuria and histopathology and loss of renal function. RGC-32 enhanced NTN development by exerting a dual role: a) a proinflammatory role mediated by upregulation of chemokine mediated renal recruitment of IL-17 and IFNγ secreting cells followed by the Th17 and Th1 dependent infiltration with neutrophils and monocytes and b) a profibrotic role. These findings suggest that RGC-32 is a potential therapeutic target for the treatment of IC mediated GN.

2. Methods

2.1. Mice

All mice were on C57BL/6 background, used at 10–12 weeks of age and housed in specific pathogen-free conditions. RGC-32$^{-/-}$ mice have been described previously [31]. Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory. All procedures were approved by the University of Maryland School of Medicine Office of Animal Welfare Assurance.

2.2. Induction of NTN

Accelerated nephrotoxic serum nephritis was induced as described [32]. Briefly, 10–12 week old WT and RGC-32 KO mice were injected with 250 μl of 1:1 sheep IgG (Pel Freeze Biologicals, Rogers, Az) in complete Freund's adjuvant (Sigma Aldrich, St Louis MO) via subcutaneous injection (day −3) and then received an i.v. injection of 50 μl sheep anti-GBM nephrotoxic serum (NTS) (generous gift from Dr. Shu Man Fu) or PBS. All time points described are reported in reference to the date of NTS challenge which is considered day 0.

2.3. Assessment of proteinuria and renal damage

Mice were monitored for proteinuria development via Albustix test strips (Siemens Healthcare Diagnostic, Tarrytown, NY). Spot urine samples were collected at 3, 7, 10 and 14 days after NTS serum injection. Results expressed as mg/dl are based upon color change. Color changes between categories were assigned the midrange proteinuria value. Blood urea nitrogen (BUN) levels in blood obtained at the same timepoints, were measured using Azostix test strips (Siemens).

Murine sera from highly positive NTN mice were used as a standard and results were converted to arbitrary units. Serum from nonimmunized mice were used as normal controls.

2.4. Renal histopathology

Fourteen days after NTS injection kidneys from euthanized mice were harvested for histologic analysis. Kidney sections were deparaffinized, stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) and Masson's trichrome then scored by two experienced nephropathologists (J.P. and C.D.) who were blinded to the experimental groups. Histology scores were determined as described [33]. Briefly, sections were assessed for glomerular deposits, endocapillary proliferation, crescent formation, interstitial inflammation and tubular casts, atrophy and dilatation. Each category was assigned a score of 0–4, where 4 is severe disease and 0 is normal appearing histology. The proportion of crescentic glomeruli was assessed in at least 50 glomeruli per section. Scores for glomerular deposits, endocapillary proliferation, glomerular crescent formation were averaged to obtain a glomerular score. Scores for interstitial inflammation and tubular casts and dilatation were averaged to obtain a score for tubular histology.

2.5. Renal IgG and C3 deposition

5 μm frozen sections were fixed in acetone and blocked in 1% BSA in PBS then incubated with FITC goat anti- mouse IgG (Fcy specific) (Jackson ImmunoResearch Labs, West Grove, PA) and anti-mouse C3 Ab (Cedarlane Laboratories, Burlington, Ca). For quantitative analysis, the intensity of glomerular immunoglobulin and C3 deposition was scored blindly on a scale of 0 (absent deposition) to 4 (heavy glomerular staining). The mean intensity of 20 random glomeruli for each sample was determined.

2.6. Immunohistochemical staining

Blocks of formalin fixed paraffin embedded kidneys were sectioned at 3 μm and stained for RGC-32 and FN as previously described [34] The sections were washed in PBS, and endogenous peroxidase was quenched with 0.3% hydrogen peroxide in PBS for 10 min. The slides were then incubated overnight at 4 °C with mouse IgG anti-fibronectin (Thermo Fisher Scientific, Waltham, MA) or rabbit anti RGC-32 Ab (Bioss Inc., Woburn, Ma), then washed with PBS and processed with an R.T.U. Vectastain Universal Quick Kit (Vector Labs, Burlingame, CA). The reactions were developed using Nova RED (Vector Labs) as the chromogen substrate, and the sections were then washed in distilled water and counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO), dehydrated, mounted, and scored by two observers in a blinded fashion. Control sections were prepared by immunostaining without the primary antibody. RGC-32, trichrome and Fibronectin stainings were assessed on a scale of 0 (no staining) to 4 (intense) staining.

2.7. RNA isolation and real-time PCR

Total RNA was isolated from cortical kidneys and from isolated kidney infiltrating cells by homogenization in Trizol (Life technology, Carsbad CA). By flow cytometry, the fraction of CD45^{+} hematopoetic cells among isolated kidney mononuclear cells ranged between 9 and 18%. 1 μg of total RNA was reverse-transcribed and real time PCR was performed in a StepOnePlus Real Time PCR System (Applied Biosystems) using a FastStart Universal SYBR Green Master Mix (Roche applied Science, Indianapolis, IN) as previously described [10]. Forward and reverse primers were provided by IDT (Coralville, IA) (Supplemental table I). Values were normalized to 18S.

2.8. Isolation of spleen cells and kidney-infiltrating cells for flow cytometry

RBC-lysed, single-cell suspensions from spleen were surface-stained with fluorochrome-conjugated B220 (RA3-6B2), CD138 (281–2), CD4 (L3T4) CD62L (Ly-22), CD44 (IM7), CD25 antibodies (BD Pharmingen, Franklin Lakes, NJ) and analyzed by FACS. Intracellular staining for IL-17 A (BD Pharmingen, Franklin Lakes, NJ) and Foxp3 (eBioscience, San Diego, CA) were performed per the manufacturer's instructions. Suspension of kidneys were generated by 30 min incubation of sliced kidneys in 2 mg/ml collagenase D (Roche applied Science, Indianapolis, IN) at 37 °C followed by Percoll gradient centrifugation. CD45+ cells were isolated using PE labelled anti CD45 ab (BioLegend, San Diego, CA) followed by the mouse PE positive selection kit (STEM cell Technologies, Vancouver, Ca) per manufacturer's recommendations. Cells were surface stained with anti-CD11b, CD11c, Ly6g, (BioLegend, San Diego, CA), Ly6c, F4/80 (eBioscience, San Diego, CA), CCR6, CD4 Abs (BD Pharmingen). For intracellular cytokine staining, splenocytes or renal cells were stimulated with PMA and ionomycin (Calbiochem, La Jolla, CA) in the presence of Monensin (BD PharMingen) for 4 h. Cells were then

stained intracellularly for IL-17 A and IFNγ (BD Pharmingen, Franklin Lakes, NJ) using the BD intracellular staining kit (BD PharMingen) per the manufacturer's instructions. Fox P3 staining was performed with a kit from eBioscience. Gating strategy is shown in Supplementary Fig. 1. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.9. Western blotting

Western blotting was performed as previously described [34]. Renal cortex was homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors and processed by 10% SDS-PAGE. Rabbit IgG anti-collagen I (Rockland, Pottstown, PA), rabbit IgG anti-collagens III (Santa Cruz Biotechnology, Dallas, TX) and rabbit anti-RGC-32 polyclonal IgG (Novus biologicals, Centennial, CO) were used as primary antibody, followed by incubation with goat anti-rabbit IgG antibody (Santa Cruz Biotechnology). B-actin was used as loading control.

2.10. Mouse anti-sheep IgG ELISA

Serum was collected on days 0, 7, 10 and 14 and mouse anti-sheep IgG Ab (Sigma Chemical Co.), IgG1, IgG2a/c and IgG2b antibodies were measured by ELISA as described previously [35].

2.11. Statistical analysis

Student's *t*-test (for parametric data), Mann Whitney test (for nonparametric data) and one-way analysis of variance were used to analyze differences in data. A p value <0.05 was considered statistically significant. All values are shown as means± SEM and are representative of three experiments unless otherwise noted. All data were evaluated and plotted using GraphPad Prism 10.0 (San Diego, CA).

3. Results

3.1. RGC-32 is upregulated in kidneys of NTN mice

We assessed the expression of RGC-32 in the kidneys of WT mice with NTN. By quantitative RT-PCR, RGC-32 mRNA was upregulated by 2 fold in the renal cortex of WT NTN mice on day 7 and further increased by 2.6 fold on day 14 ($p < 0.05$ for both time points vs untreated controls), before decreasing to 1.5 fold by day 21 (Fig. 1A). RGC-32 mRNA was also upregulated by 2.5 fold in renal infiltrating cells of WT NTN mice and peaked on day 7 after NTN induction (Fig. 1B). RGC-32 transcript upregulation was confirmed at protein level by Western blot analysis of kidney lysates (Fig. 1C). While RGC-32 was not detectable in kidney

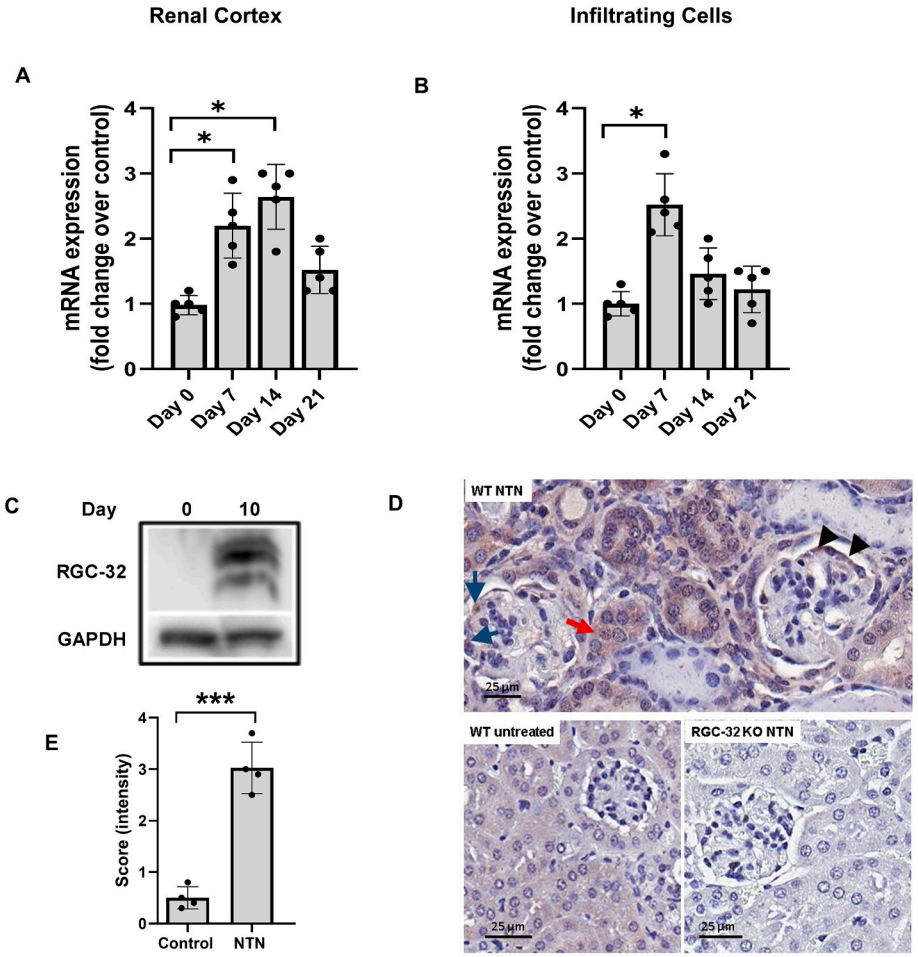

Fig. 1. RGC-32 expression is upregulated in NTN. NTN was induced in WT and RGC-32 KO mice as described in Material and Methods. RGC-32 expression in kidneys was examined by RT-PCR, WB and immunostaining. A. RGC-32 mRNA expression in renal cortex at the specified time-points. Results are expressed as fold change over non-nephritic WT controls. B. RGC-32 mRNA expression in renal infiltrating cells in WT mice at 7, 14 and 21 days after induction of NTN. C. RGC-32 protein expression in whole kidney lysates measured by western blotting on day 10. Representative image is shown. D. Representative immunohistochemistry staining for RGC-32. WT nephritic mice (upper panel) show strong tubular (brown) staining (red arrow), staining in podocytes (black arrowheads) and mild focal staining in rare endocapillary cells (blue arrows). Low tubular expression of RGC-32 in untreated WT mice noted in lower left panel. Negative staining in RGC-32 KO controls is shown in right lower panel. Scale bars, 25 µm. E. RGC-32 staining intensity score. Data are mean ± SEM of 4–5 five mice per group (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lysates of untreated mice it increased dramatically by day 10 in kidney lysates of mice with NTN. As previously described, by IHC staining, RGC-32 exhibits low expression in tubular cells of untreated, control mice (Fig. 1D lower left panel) [23,31] and was strongly upregulated in the tubules of NTN mice (Fig. 1D red arrow). In addition, RGC-32 was also expressed in glomeruli, mostly in podocytes (Fig. 1D blue arrow). In addition, mild focal staining was detected in a few glomerular cells (Fig. 1D arrowhead). Thus, RGC-32 is upregulated in NTN mice in resident tissue cells including tubuli, podocytes, glomerular cells and in renal infiltrating mononuclear cells.

3.2. RGC-32 deficiency decreases proteinuria and renal damage in NTN

To gain insight into the functional role of RGC-32 in the kidney during immune –complex mediated nephritis, we induced NTN in WT and RGC-32 KO mice. Examination of H&E and PAS-stained kidney sections on day 14 after NTS injection revealed severe glomerular alterations i.e., glomerular hypercellularity, deposition of PAS stained material and crescent formation in RGC-32 sufficient mice (Fig. 2A). In contrast, RGC-32 KO mice displayed only slight mesangial hypercellularity. Quantification of glomerular damage showed significantly decreased glomerular score (Fig. 2B) and proportion of glomerular crescent formation (Fig. 2C) in RGC-32 KO compared to WT mice. In addition, RGC-32 KO mice displayed a trend for lower tubular damage score compared to WT mice but this did not reach statistical significance (Fig. 2D).

NTS-treated WT mice developed significant proteinuria that was detected starting on day 4 and peaked on day 10. In contrast, RGC-32 KO mice exhibited significantly decreased proteinuria at day 7, 10 and 14 after the induction of nephritis (Fig. 2E). Functionally, NTS-treated RGC-32 KO mice showed a trend for lower levels of BUN, as compared to NTS-treated WT mice, although this did not reach statistical significance (Fig. 2F). These results suggest that RGC-32 promotes renal damage and impaired renal function during NTN.

3.3. Systemic T and B cell immune response were unaltered in the absence of RGC-32

To evaluate whether RGC-32 deficiency induces alterations in the systemic humoral and cellular immune responses after NTS treatment, we performed detailed phenotypic characterization of splenic T and B cells in WT and RGC-32 KO mice. The systemic T cell response assessed by flow cytometry of spleen cells on day 7 after NTN induction showed comparable frequency of effector $CD4^+$ T cells ($CD4^+CD62L^{lo}CD44^{hi}$), Tregs ($CD4^+CD25^+Foxp3^+$) and IL-17 A and IFNγ expressing $CD4^+$ T cells in WT and RGC-32 KO mice (Fig. 3A-F). Serum levels of IL-17 A and IFNγ were also similar in WT and RGC-32 KO mice (not shown). Similarly, the systemic B cell response was not impaired in RGC-32 KO mice with NTN as the percentage of splenic $B220^{lo}CD138^{hi}$ plasma cells did not differ between RGC-32 KO and WT mice (Fig. 4A). Furthermore, serum titers of antigen-specific total mouse anti-sheep IgG measured by ELISA at 7 days after NTN induction were comparable in both groups (Fig. 4B). Additionally, there were no significant differences in the relative representation of the various antigen-specific IgG isotypes IgG1, IgG2a/c and IgG2b (Fig. 4B). Next, we assessed kidney deposition of mouse anti-sheep Ab. By immunofluorescence staining of frozen sections, both WT and RGC-32 KO nephritic mice showed linear deposits of mouse IgG along the glomerular basement membrane (Fig. 4C). Semi-quantitative scoring of glomerular deposition of mouse anti-sheep IgG revealed no difference between nephritic WT and RGC-32 KO mice. Furthermore, C3 complement component deposition observed in glomerular capillary loops did not differ in WT vs. KO mice (Fig. 4C).

Overall, these data indicate that systemic B and T cells responses were largely unaffected in the absence of RGC-32 after NTS administration. Thus, the differences in kidney pathology were not due to RGC-32 interfering with the induction phase of NTN suggesting a local role for RGC-32 in kidney pathology.

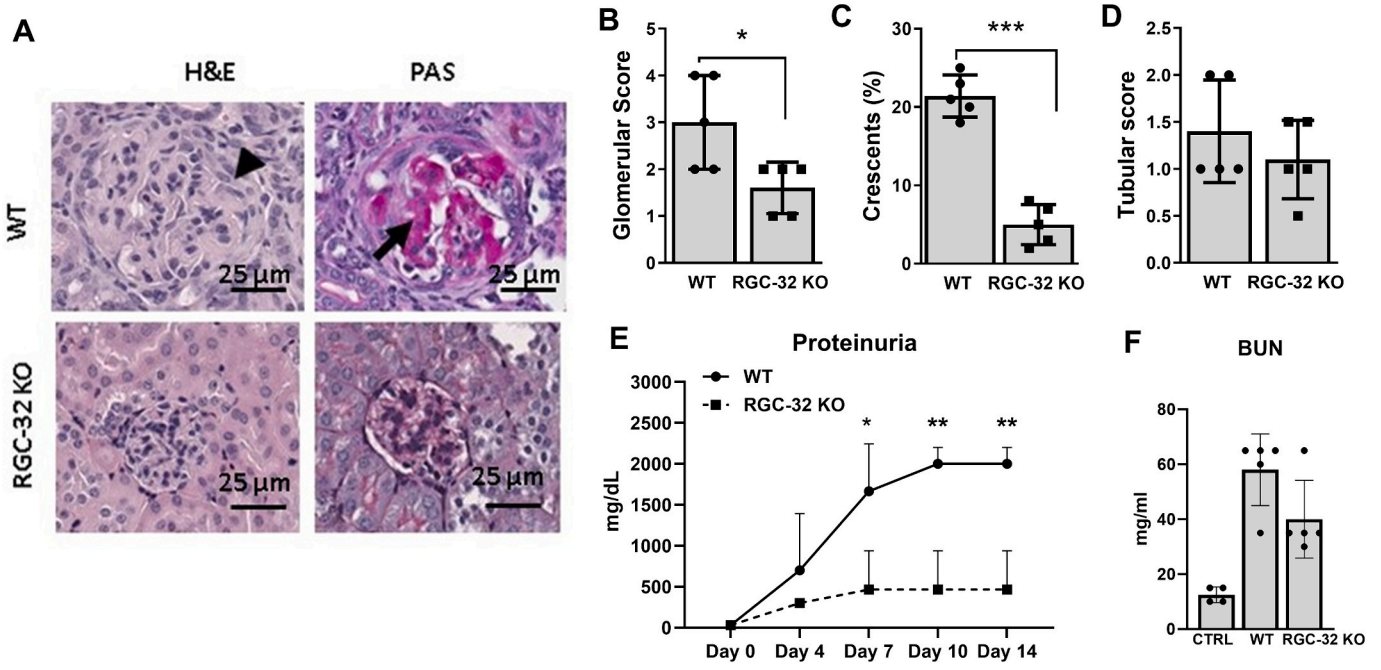

Fig. 2. RGC-32 deficiency attenuates NTN. NTN was induced in WT and RGC-32 KO mice as described in Material and Methods. A. Representative H&E and PAS staining on day 14 after induction of NTN showing glomerular hypercellularity, glomerular deposition of PAS stained material (arrow) and crescent formation (arrowhead) in WT mice while RGC-32 KO mice display only slight mesangial hypercellularity. Scale bars, 25 µm. B–C. Quantification of glomerular score and crescent formation. D. Tubulointerstitial score. E. Proteinuria. F. Serum BUN. Data are mean ± SEM of five mice per group (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

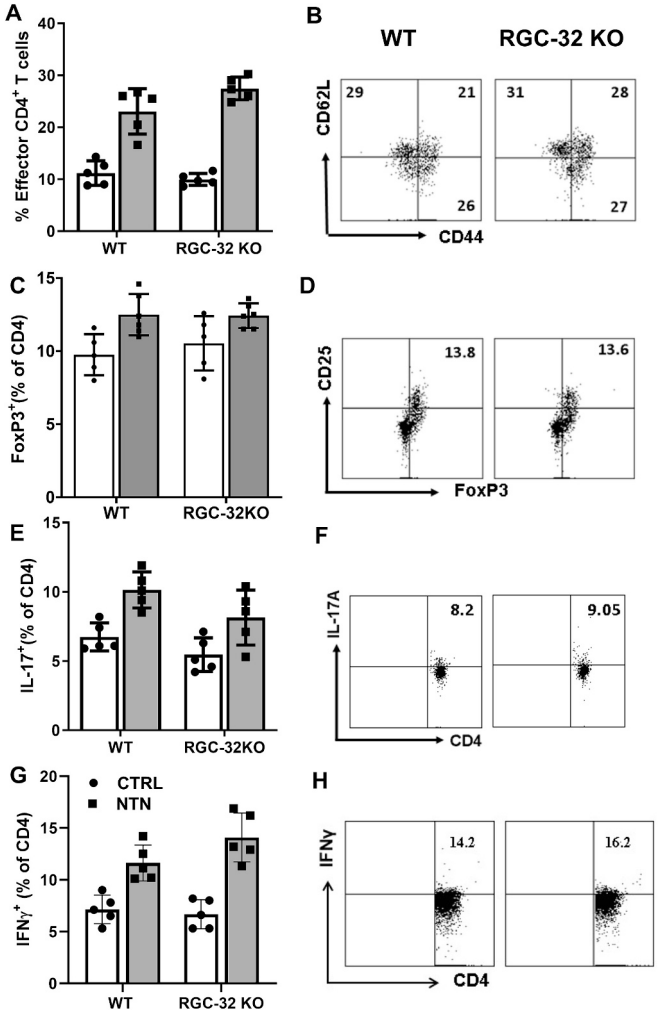

Fig. 3. Systemic T cell responses are not altered in RGC-32 KO mice. NTN was induced in WT and RGC-32 KO mice as described in Material and Methods. Flow cytometric analyses of splenic cells were performed at day 7 to determine the frequency of effector $CD4^+$ T cells ($CD4^+CD62L^{lo}CD44^{hi}$) (A–B), Tregs ($CD4^+CD25^+Foxp3^+$) (C–D) IL-17 A (*E*-F) and IFNγ expressing $CD4^+$ T cells (G-H). Percentage positive cells and representative flow cytometric staining profile of NTN mice are shown. Number in the dot plot represent percentage of cells. Data are mean ± SEM of five mice per group.

3.4. *RGC-32 deficiency limits recruitment of IL-17 and IFNγ producing $CD4^+$ T cells through the CCL20/CCR6 and CXCL9/CXCR3 axis*

Multiple studies have implicated IL-17 as an essential, early driver of NTN pathology [29,30,36–38]. As nephritic WT and RGC-2 KO mice displayed equally efficient systemic immune response in terms of IL-17 expression by splenocytes (Fig. 3), we investigated whether local recruitment of Th17 cells is altered in the absence of RGC-32. To this end, we measured transcript levels and frequency of IL-17 A producing $CD4^+$ T cells in isolated infiltrating renal cells. Expression of IL-17 mRNA paralleled the expression of RGC-32 in infiltrating cells and peaked on day 7 after NTN induction in WT mice then declined by day 14 (Fig. 5A). By comparison, RGC-32 KO NTN mice display significantly decreased transcript levels of IL-17 at day 7. In concordance with the mRNA levels, the frequency of IL17A producing cells in $CD45^+$ purified infiltrating renal cells was significantly lower in RGC-32 KO mice compared to WT controls (Fig. 5B). In concordance with the decreased number of IL-17 producing cells, RGC-32 KO mice also displayed significantly lower transcript levels of the Th17 cell receptor CCR6 and decreased frequency of infiltrating $CCR6^+$ cells (Fig. 5C-D).

The interaction between CCR6 and its ligand CCL20 is critical for the recruitment of Th17 cells. Thus, we determined whether the decreased migration of Th17 cells in the absence of RGC-32 could be due to altered CCL20 expression. Consistent with previous reports [38,39] we found that the mRNA expression of CCL20 was upregulated in renal infiltrating cells and the renal cortex of WT NTN mice (Fig. 5E). In contrast, RGC-32 KO mice displayed significantly decreased mRNA expression of CCL20 in both infiltrating and kidney resident cells (Fig. 5E-F).

CCR6 is also expressed on and recruits regulatory T cells. Thus, we assessed whether the number of intrarenal Treg cells is altered in RGC-32 KO NTN mice. In contrast to IL-17 secreting cells, the percentage and absolute number of intrarenal $FoxP3^+CD4^+$Tregs did not differ between the groups (Supplemental Fig. 2).

In later stages of the disease, $CXCR3^+$ Th1 cells are recruited in the kidney in response to CXCL9 produced by renal dendritic cells and tubular epithelial cells [37,40,41]. We assessed whether RGC-32 deficiency alters the recruitment of IFNγ producing $CD4^+$ T cells. Consistent with previous observations, IFNγ transcripts peaked later, on day 21 after disease induction in WT NTN mice [38]. IFNγ transcripts were significantly decreased at this time point in RGC-32 KO mice (Fig. 6A). By flow cytometry, RGC-32 KO mice showed significant decrease in the absolute numbers of CD4 + IFNγ+ renal infiltrating cells and nearly significant decrease in the frequency of $IFN\gamma^+CD4^+$ cells (Fig. 6B-C).

The interaction between Th1 cell receptor CXCR3 and its ligand CXCL9 is critical for the recruitment of Th1 cells. Consistent with the decreased number of IFNγ producing cells, mRNA expression of CXCR3 was significantly decreased in RGC-32 KO nephritic mice (Fig. 6E-F). Furthermore, CXCL9 transcript was also significantly decreased in RGC-32 KO compared to WT nephritic mice (Fig. 6F) suggesting that RGC-32 deficiency limits recruitment of IFNγ producing $CD4^+$ T cells through the CXCL9/CXCR3 axis.

Collectively, these data suggest that RGC-32 promotes the renal recruitment of IL-17 A and IFNγ producing cells in NTN by upregulating their ligands, CCL-20 and respectively CXCL9.

3.5. *RGC-32 deficiency decreases renal inflammation*

Next we evaluated the contribution of RGC-32 to the local inflammatory process. Previous studies have shown that early in the course of the disease nephritogenic Th17 cells recruit neutrophils and promote tubulointerstitial inflammation and subsequent renal damage [29,38,42]. Thus, we first assessed the number of $Ly6^+g$ $Ly6c^+$ neutrophils in kidneys of WT and RGC-32 KO nephritic mice. Compared to untreated controls, we detected increased frequency and number of $Ly6^+g$ $Ly6c^+$ neutrophils in kidneys of WT NTN mice at day 7 after NTN induction. In contrast, a significantly lower percentage and absolute number of $Ly6g^+$ $Ly6c^+$ cells were detected in perfused kidneys of nephritic RGC-32 KO mice (Fig. 7A-C).

In concordance with the decreased neutrophil recruitment of RGC-32 KO NTN mice, the mRNA expression of the Th17 dependent neutrophil attracting chemokine CXCL5 was significantly decreased in in cortical renal tissue in RGC-32 KO mice at 7 days after NTN induction (Fig. 7 D). Interestingly, transcripts of the T-cell independent neutrophil attracting chemokines, CXCL1 and CXCL2, were also downregulated in the absence of RGC-32 suggesting a role for RGC-32 in IL-17 dependent and independent neutrophil recruitement.

Th1 cells play a major role in recruiting pro-inflammatory cells such as inflammatory monocytes. Concordant with the decreased number of IFNγ producing cells, the influx of inflammatory monocytes as determined by the percentage and absolute numbers of $CD11^bF4/80^{lo}$ renal infiltrating cells was significantly decreased in RGC-32 KO nephritic mice (Fig. 7G-H). In addition, the number of $CD11^bF4/80^{hi}$ resident macrophages was also decreased in RGC-32 KO mice (Fig. 7I-J) suggesting that proliferation or survival of tissue resident macrophages could also be affected in the absence of RGC-32.

Thus, these data suggest that the ameliorated nephritis in RGC-32 KO

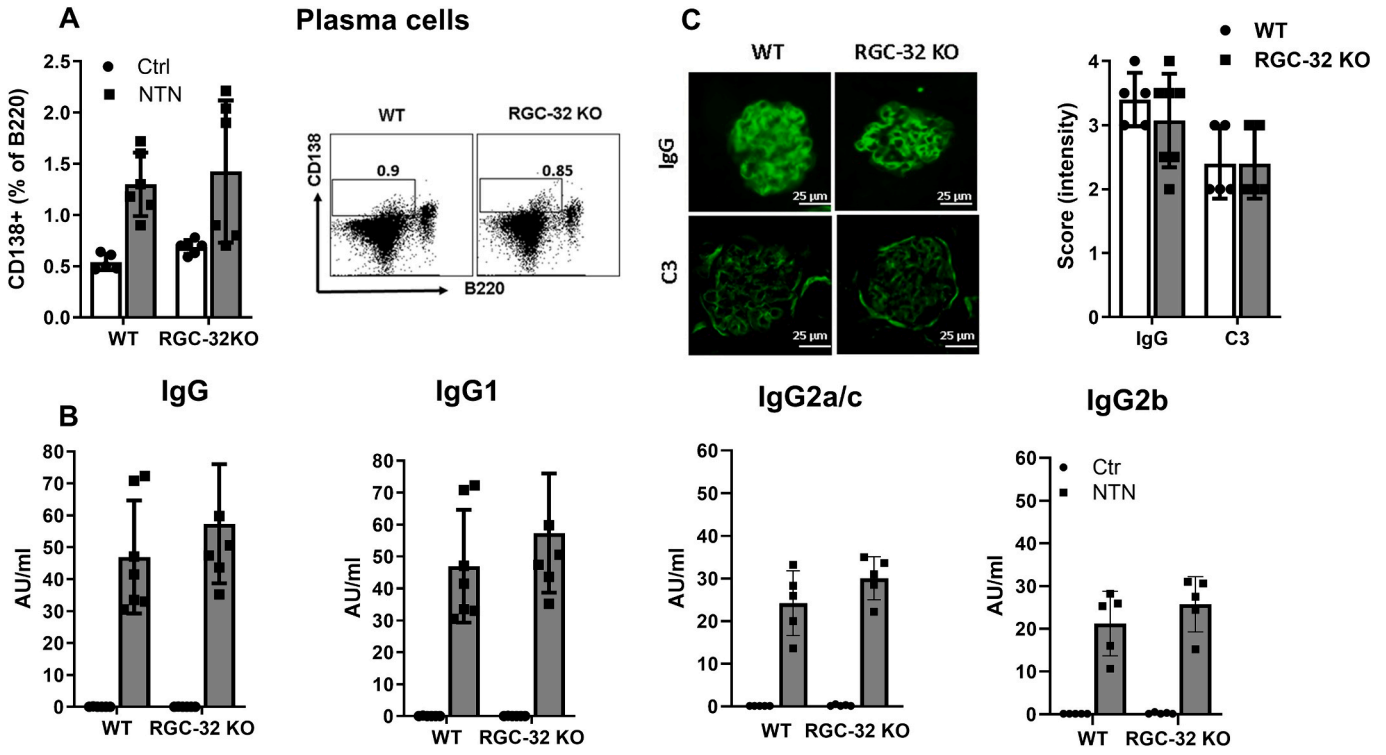

Fig. 4. Systemic B cell responses are not altered in RGC-32 KO mice.
A. Flow cytometric analysis of spleen cells from WT and RGC-32 KO mice was performed at day 14 to determine the frequency of plasma cells ($B220^{lo}CD138^{hi}$). Percentage positive cells and representative flow cytometric staining profile of NTN mice are shown. B. At day 7 serum titers of mouse anti-sheep total IgG, IgG1, 2a/c and 2b, were measured by ELISA in WT and RGC-32 KO nephritic mice. C. Immunofluorescence staining and scores of mouse IgG and C3 deposition in the kidneys of WT and RGC-32 KO NTN mice. Similar staining intensity and quantification scores of IgG and C3 deposition were observed. Data are mean ± SEM of five mice per group. Scale bars, 25 µm.

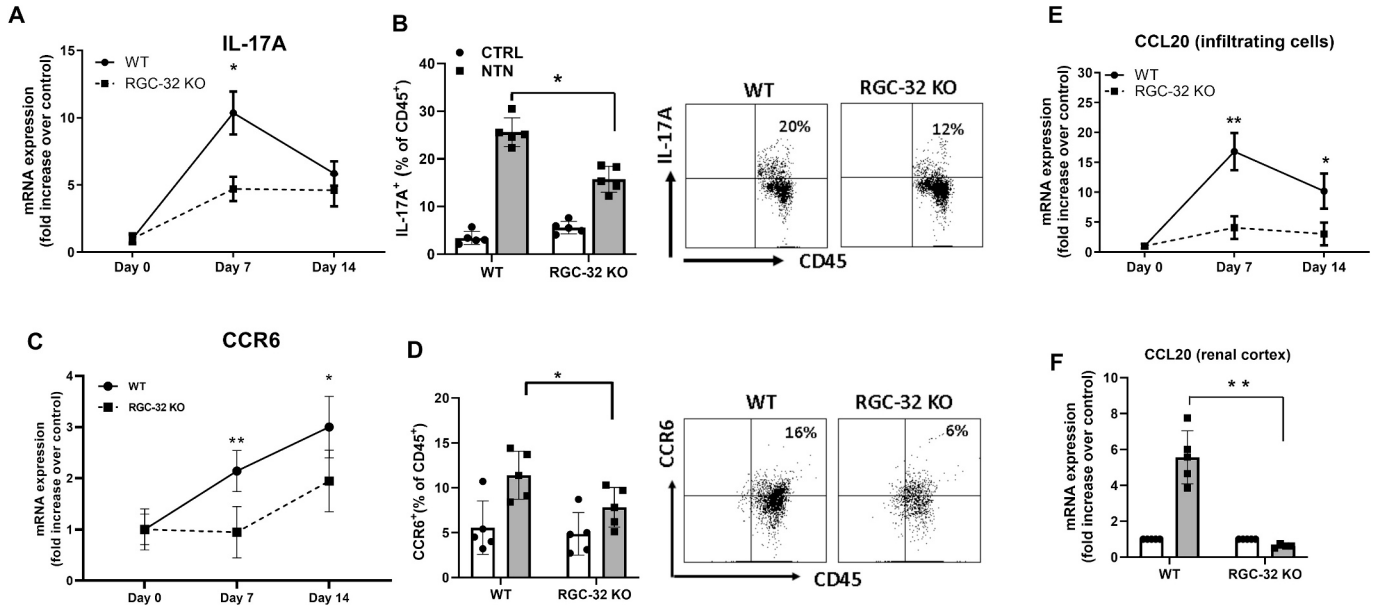

Fig. 5. Intrarenal IL-17 producing cells are decreased in RGC-32 KO mice. A. Expression of IL-17 A mRNA in renal infiltrating cells of nephritic WT and RGC-32-32 KO mice on days 7 and 14 after induction. B. Percentage and representative flow cytometric staining profile of IL-17 A producing $CD45^{+}CD4^{+}$ T cells in kidneys at day 7 after injection of NTS. C. Expression of CCR6 mRNA in infiltrated cells from kidneys of nephritic RGC-32 sufficient and deficient mice. D. Percentage and representative flow cytometric staining profile of $CCR6^{+}$ $CD4^{+}$ T cells in kidneys at day 7 after injection of NTS. E. Expression of CCL20 mRNA in renal infiltrating cells. F. Expression of CCL20 mRNA in renal cortex. Data are mean ± SEM of five mice per group (* = $p < 0.05$; ** = $p < 0.01$).

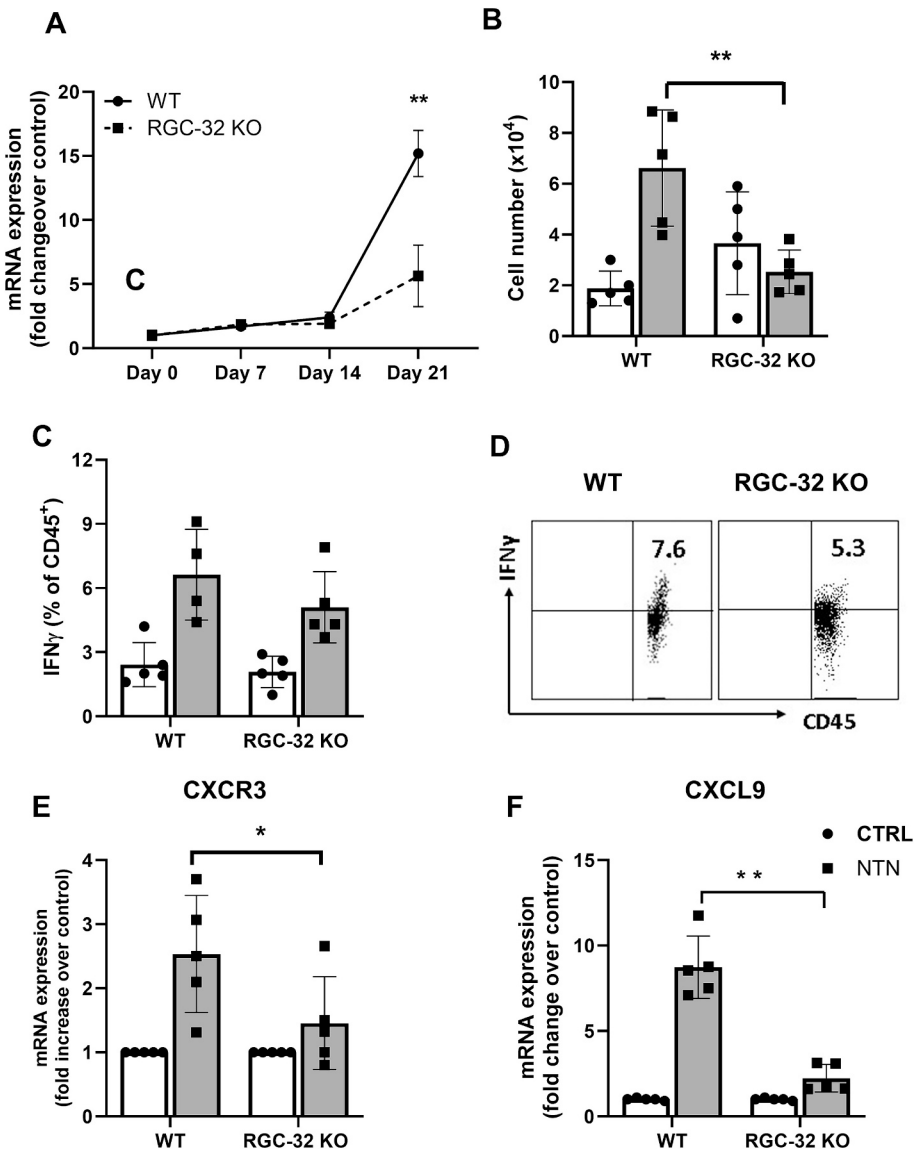

Fig. 6. Intrarenal IFNγ producing cells are decreased in RGC-32 KO mice. A. Expression of IFNγ mRNA in renal infiltrating cells of nephritic WT and RGC-32 KO mice. B–D. Absolute number, percentage and representative flow cytometric staining profile of IFNγ producing $CD4^+$ T cells in kidneys at day 21 after injection of NTS. E-F. Expression of CXCL9 and CXCR3 mRNA in renal infiltrated cells of RGC-32 sufficient and deficient mice.

mice is partially due to the impaired renal trafficking of effector Th17 and Th1 cells and the subsequently decreased influx of innate immune cells.

3.6. *RGC-32 deficiency attenuates fibrosis in RGC-32KO mice*

The persistent inflammatory response in NTN results in glomerular sclerosis and tubulointestitial fibrosis [43,44]. As RGC-32 is a downstream target of TGFβ in the profibrotic pathway [2,6,23,24,34] we asked whether RGC-32 deficiency alters the renal fibrotic process in this model. To this end, we assessed mRNA expression of Col1a1, Col3a1 and Fn1 in WT and RGC-32 KO NTN mice. As previously reported, mRNA expression of these genes was significantly upregulated in WT mice at day 21 after disease induction [45]. In contrast, RGC-32 KO mice displayed minimal or no upregulation of these genes (Fig. 8A-C). Upregulation of Collagen I and III protein in WT but not in RGC-32 KO nephritic mice was confirmed by Western blot (Fig. 8D-E). Furthermore, trichrome and IHC staining for FN confirmed decreased deposition of collagen and FN, respectively in the glomerular and interstitial areas in RGC-32 KO compared to WT mice (Fig. 8F-H). Taken together these data show that the abrogation of RGC-32 reduces both the inflammatory and the fibrogenic process in NTN.

4. Discussion

This study provides the first detailed characterization of the local role of RGC-32 in a well established model of experimental glomerulonephritis. We provide evidence that RGC-32 is an important driver of the disease in this inducible model of immune complex mediated glomerulonephritis. RGC-32 KO mice with NTN displayed an attenuated disease phenotype as demonstrated by decreased proteinuria, attenuated renal dysfunction and renal histopathologic changes. Furthermore, our data show that functionally, RGC-32 promotes the local recruitment of pathogenic IL-17 and IFNγ producing cells by enhancing the expression of the CCL20/CCR6 and CXCL9/CXCR3 ligand-receptor pairs, thus augmenting the influx of Th17 and Th1 dependent innate immune cells. In addition, we demonstrate that RGC-32 also promotes renal fibrosis, in line with the known profibrotic role of RGC-32 in other models [6,23,24].

In a first step we analyzed the renal expression and distribution of

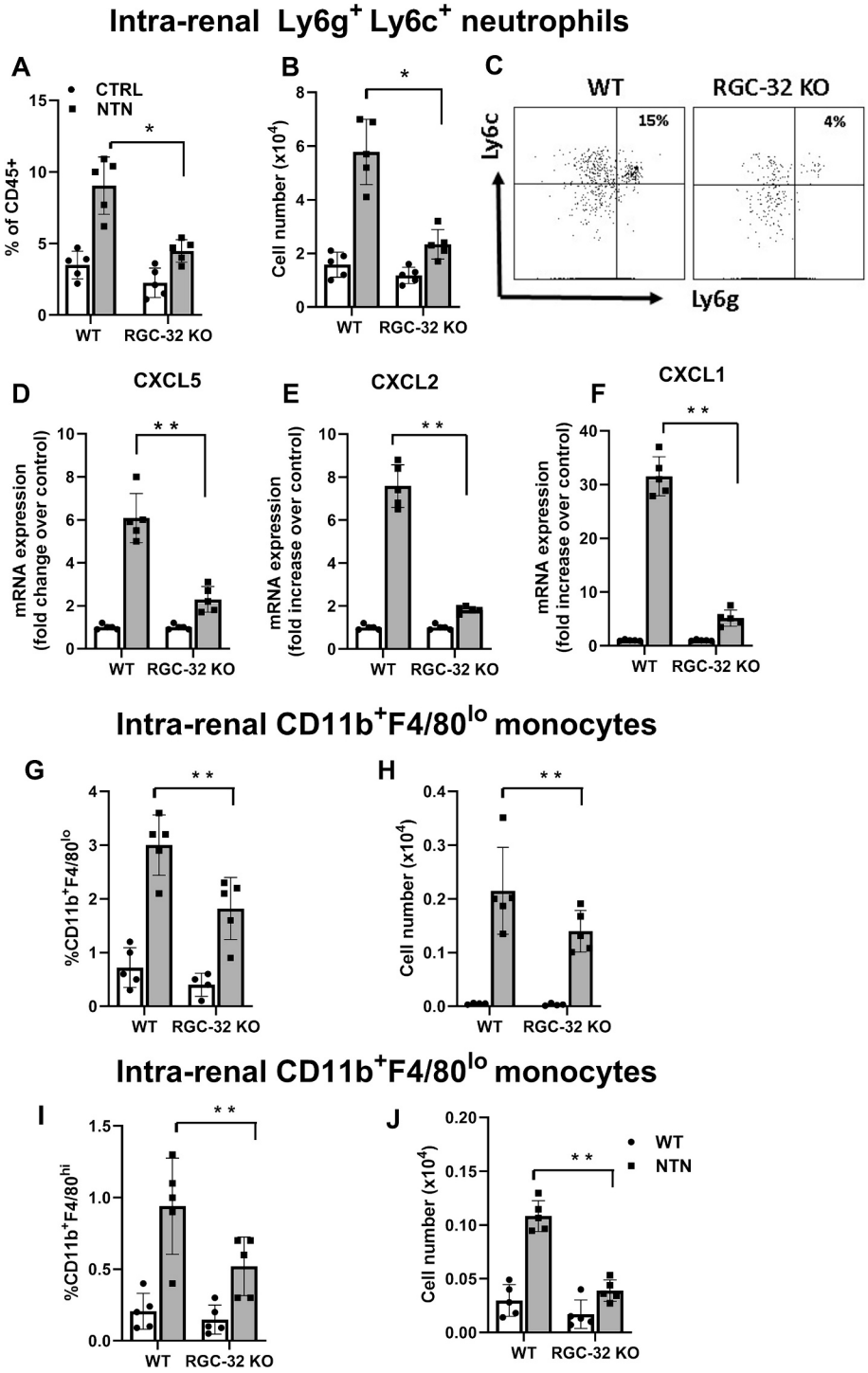

Fig. 7. Neutrophil and monocyte infiltration is decreased in RGC-32 KO NTN mice. Renal infiltrating cells from kidneys of WT and RGC-32 KO nephritic mice and controls were prepared and analyzed by flow cytometry for $Ly6G^+$ $Ly6c^+$ neutrophils, $CD11b^+F4/80^{lo}$ inflammatory monocytes and $CD11b^+/F4/80^{hi}$ macrophages. A-B. Percentage and absolute number of infiltrating $Ly6G^+$ $Ly6c^+$ neutrophils cells in kidneys of WT and RGC-32 KO mice. C. Representative flow cytometric analysis of infiltrating $Ly6G^+$ $Ly6c^+$ neutrophils in purified $CD45^+$ infiltrating cells. D. Expression of CXCL5 mRNA in renal cortex on day 10 after NTN induction. E-F. Expression of CXCL1 and CXCL2 mRNA in renal cortex on day 3 after NTN induction.
G-H. Percentage and absolute number of $CD11b^+F4/80^{lo}$ monocytes. I-J. Percentage and absolute number of infiltrating $CD11b^+/F4/80^{hi}$ macrophages. Data are mean ± SEM of five mice per group (* = $p < 0.05$; ** = $p < 0.01$).

RGC-32 in WT NTN mice. A search for RGC-32 in the Genotype-Tissue Expression (GTEx) database indicated that the bulk basal gene expression of RGC-32 in the kidney is relatively low [46]. Prior studies have indeed shown a low baseline protein expression of RGC-32 in renal endothelial, tubular and interstitial cells [6,23,31,47]. However, upregulated expression was reported in tubules and glomerular mesangial cells in experimental models of unilateral ureteral obstruction and mesangioproliferative GN [23,48], in the tubules, interstitium and glomeruli of patients with lupus nephritis [22] and in tubules of patients with IgA nephropathy [25]. In WT mice with NTN, we found a

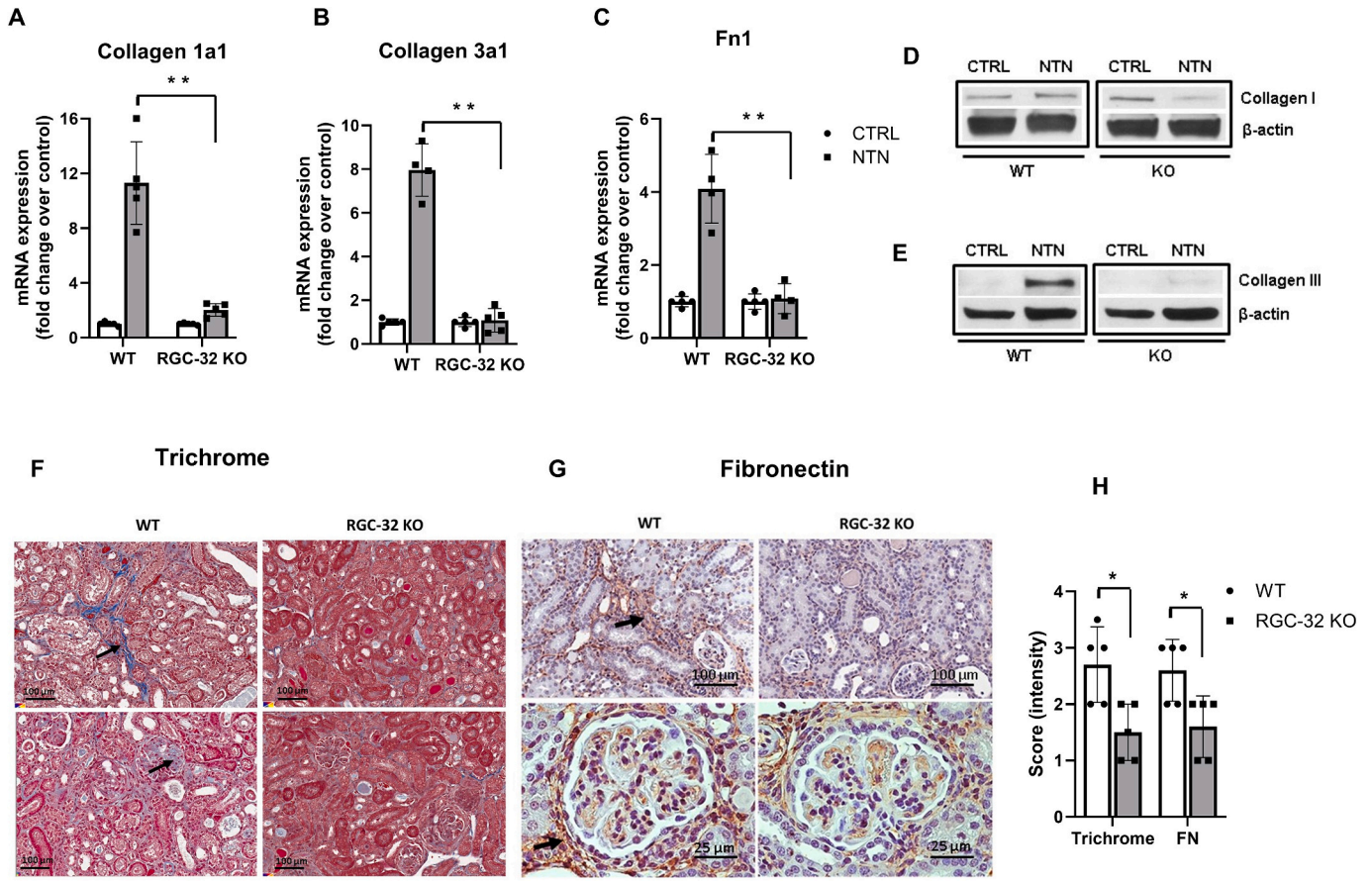

Fig. 8. RGC-32 deficiency attenuates fibrosis in RGC-32 KO NTN mice.
A-C. RT-PCR for Col1a1, Col3a1 and Fn1 from kidneys of WT and RGC-32 KO nephritic mice and controls at 21 days after disease induction. D-E. Western blot for Collagen I and III. F. Sections from WT and RGC32 KO kidneys were stained with Masson's-modified trichrome. Top and bottom left side panels show fibrous strands in the interstitium and glomerular deposits respectively (black arrows), while in RGC-32 KO mice there is no evidence of interstitial or glomerular sclerosis Scale bars 100 µm. F. Staining for Trichrome and Fibronectin showing blue and brown fibrillar interstitial deposits (top panel) and glomerular deposition (bottom panel) in WT but not RGC-32 KO mice. Scale bars, 100 µm for upper panels and 25 µm for lower panels. H. Intensity staining for trichome and FN. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significant upregulation of RGC-32 transcripts in the kidney cortex, confirmed by Western blot analysis from kidney lysates, and in kidney-infiltrating cells. By immunohistochemistry, RGC-32 was mainly localized to tubular cells and in a few glomerular cells, including podocytes. To obtain further insight into other cell types that could express RGC-32, we analyzed publicly available omics data. A search utilizing the CELLxGENE platform-based analysis of single cell RNA-Seq data from healthy adult mouse kidneys [49], revealed that RGC-32 is predominantly expressed by dendritic and endothelial cells and had the highest expression level in interstitial fibroblasts. Although detected in a lower proportion of cells, RGC-32 transcripts were also found in mononuclear phagocytes, hematopoetic cells, lymphocytes and podocytes, in agreement with our data showing RGC-32 expression in both resident and renal infiltrating cells. Taken together, these data suggest multiple potential local targets of RGC-32 including PTEC, podocytes, infiltrating innate and/or immune cells and other resident cells. Further investigations are required to assess the potential contribution of RGC-32 expression in parenchymal vs. hematopoietic cells to the pathogenesis of the disease.

Humoral and cell-mediated immune mechanisms mediate kidney disease in NTN [38,44,50]. We investigated whether the attenuated renal disease in RGC-32 KO mice was related to a decrease in the systemic B or T cell response. Nephritic WT and RGC-32 KO mice showed comparable responses in terms of serum mouse anti-sheep total IgG and IgG1, 2a, 2b/c titers as well as glomerular mouse IgG and C3 deposition. In addition, the cellular immune response as assessed by the frequency of splenic Th1, Th17 and Treg cell subsets was similar in RGC-32 sufficient and deficient mice. Thus, RGC-32 blockade did not interfere with the induction of the disease and the attenuated nephritis in RGC-32 KO NTN mice was not due to the inability to mount an efficient humoral or cellular immune response.

The recruitment and infiltration of effector T cells into the kidney initiates and perpetuates the glomerular and tubulointerstitial tissue injury. Th17 cells are the first wave of pathogenic T helper cells that infiltrate the renal interstitium and glomeruli in NTN [29,30,36–38,51]. At later stages, Th1 cells are the prevailing mediators of renal tissue injury [38,40]. In our study, the kinetics of IL-17 A^+ $CD4^+$ cells that peaked on day 7 and that of IFNγ secreting cells that peaked later on day 21, is consistent with these previous reports [38]. A number of studies showed that IL-17 deficiency is protective at early time points while IFNγ deficiency is protective at later timepoints in the disease course [38,52]. Our data showed that RGC-32 deficiency decreased the frequency and absolute number of both the IL-17 A and the IFNγ producing $CD4^+$ cells, in parallel with the decreased expression of their surface receptors, CCR6 and CXCR3 respectively, suggesting that RGC-32 promotes the infiltration of both T effector cell subsets during the early as well as the late phase of NTN.

It is well documented that T regs are recruited to renal lymph nodes and to the kidneys of NTN mice in an attempt to limit renal inflammation [39,53–57]. A number of studies using multiple approaches

including adoptive transfer, overexpression or depletion/ablation of FoxP3 have demonstrated the ability of Tregs to downregulate either the Th1, the Th17 immune responses, or both in the kidney in NTN mice and attenuate the NTN disease phenotype [55,58]. We found similar percentage and numbers of FoxP3 Tregs in the kidneys of WT and RGC-32 KO NTN mice. Furthermore, we have previously shown that Treg suppression activity is not altered in the absence of RGC-32 [10]. Thus the attenuated disease phenotype and the decrease in renal IFNγ and IL-17 secreting cells in our study is not due to altered numbers or function of T regs.

CCL20/CCR6 and CXCL9/CXCR3 ligand-receptor pairs are critical for the trafficking of Th17 and Th1 cells into the kidney. Our data showing decreased mRNA expression of CCL20 and CXCL9 chemokines in RGC-32 KO NTN mice along with decreased disease severity parallel those obtained in CXCL9 and CXCR3 deficient mice [41,59]. The decreased mRNA expression of CCL20 and CXCL9 chemokines in RGC-32 KO NTN mice along with the unaltered systemic Th17 and Th1 response, suggest that the observed decrease in the number of infiltrating IL-17 and IFNγ secreting cells is due to the decreased recruitment of these effector T cells. These data underscore an important function of RGC-32 in regulating the trafficking of Th17 and Th1 cells in the kidney.

Conflicting data have been reported regarding the cross-regulation between Th1 and Th17 effector cytokines. An intrarenal counter-regulatory effect of IL-17 on the Th1 response was reported by Odobasic et al. [52]. In contrast, Paust et al. reported that IL-17 is required for the Th1 response while Th1 cells limit the renal Th17 responses through the intrarenal regulation of the CXCL9/CXCR3 and CCL20/CCR6 axes [38]. Specifically, IL-17 producing cells triggered the expression of CXCL9 and CCL-20, while IFNγ induced CXCL9 but inhibited CCL20 expression. The concordant decrease in the intrarenal frequency of IL-17 A and IFNγ secreting cells in our study would be in line with a role for IL-17 in triggering a full Th1 response but not with a counter-regulatory loop between Th17 and Th1 cells, further supporting the role of RGC-32 in the trafficking of Th17 and Th1 cells through the downregulation of CCL20 and CXCL9 chemokines.

Th17 cells exert a proinflammatory role by recruiting pathogenic neutrophils while at later stages, Th1 cells recruit more pro-inflammatory cells such as monocytes [29,38,40–42]. In concordance with the decreased frequency of IL-17 and IFNγ expressing cells, RGC-32 KO mice exhibit decreased infiltration of neutrophils and monocytes suggesting that the attenuated NTN phenotype in RGC-32 KO mice is partly due to the decreased recruitment of Th17 and Th1 dependent inflammatory cells.

The immune mediated renal injury in the NTN model results in fibrosis in both the glomerular and interstitial compartments [37,45,60]. A large body of evidence indicates a role for RGC-32 in profibrotic pathways. As a downstream target of TGFβ, RGC-32 was shown to mediate extracellular matrix (ECM) deposition by murine astrocytes and human PTEC in vitro [6,23,34] and in the mouse model of obstructive uropathy in vivo. In addition, RGC-32 was shown to play a role in the ECM deposition by mesangial cells in response to sublytic C5b-9 [48]. A potential role in human disease was suggested by a study in IgA nephropathy showing a correlation between the upregulated tubular expression of RGC-32 and expression of TGFβ and αSMA [25]. Consistent with these reports, our data show that RGC-32 deficiency attenuates renal fibrosis in NTN. Our data parallel those reported by Sun et al. in the acute bleomycin model of systemic sclerosis [24]. In this model, RGC-32 deficiency decreased the deposition of collagen in skin and lung tissues. By contrast, these results differ from our data in the noninflammatory, chronic model of bleomycin induced pulmonary fibrosis where RGC-32 plays a protective role against lung fibrosis in vivo [61]. In line with this protective role, silencing RGC-32 in vitro in a rat kidney cell line increased expression of α-SMA and FN [5]. It is possible that RGC-32 deficiency protects from inflammation driven fibrosis by primarily interfering with the immune mediated inflammatory process. In support of this idea, similar to our NTN model, in the acute bleomycin model, RGC-32 deficiency attenuated the inflammatory process, and in particular the accumulation and polarization of macrophages [24]. Thus, RGC-32 may exert pro and/or anti-fibrotic actions which may depend on the cell type, tissue and local inflammatory context.

In conclusion, our study demonstrates an important role for RGC-32 in the development of an immune complex mediated GN that mimics aspects of human disease. RGC promoted the Th17 and Th1 dependent proinflammatory responses and the subsequent, organ specific pathways of renal fibrosis. Thus, RGC-32 blockade is a potential novel therapeutic strategy directed at the proinflammatory and profibrotic program in immune complex mediated GN.

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Compliance with ethical standards

All Institutional and National Guidelines for the care and use of animals were followed.

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CRediT authorship contribution statement

Alexandru Tatomir: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sonia Vlaicu:** Writing – review & editing, Methodology, Data curation. **Vinh Nguyen:** Supervision, Methodology, Formal analysis, Data curation. **Irina G. Luzina:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Sergei P. Atamas:** Writing – review & editing, Software, Formal analysis, Data curation. **Cinthia Drachenberg:** Writing – review & editing, Methodology, Formal analysis, Data curation. **John Papadimitriou:** Methodology, Formal analysis, Data curation. **Tudor C. Badea:** Writing – review & editing, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Horea G. Rus:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Violeta Rus:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None of the authors has any financial conflict of interest to disclose.

Data availability

Data is available from the corresponding author upon request.

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