Molecular Therapy
Original Article

Targeting tissue-resident memory CD8$^+$ T cells in the kidney is a potential therapeutic strategy to ameliorate podocyte injury and glomerulosclerosis

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Although tissue-resident-memory T (T_{RM}) cells, a recently identified non-circulating memory T cell population, play a crucial role in mediating local immune responses and protect against pathogens upon local reinfection, the composition, effector function, and specificity of T_{RM} cells in the kidney and their relevance for chronic kidney disease remain unknown. In this study, we found that renal tissue displayed high abundance of tissue-resident lymphocytes, and the proportion of CD8$^+$ T_{RM} cells was significantly increased in the kidney from patients and mice with focal segmental glomerulosclerosis (FSGS), diabetic kidney disease (DKD), and lupus nephritis (LN). Mechanistically, IL-15 significantly promoted CD8$^+$ T_{RM} cell formation and activation, thereby promoting podocyte injury and glomerulosclerosis. Interestingly, Sparsentan, the dual angiotensin II (Ang II) receptor and endothelin type A receptor antagonist, can also reduce T_{RM} cell responses by intervening IL-15 signaling, exploring its new pharmacological functions. Mechanistically, Sparsentan inhibited Ang II or endothelin-1 (ET-1)-mediated IL-15 signaling, thereby further regulating renal CD8$^+$ T_{RM} cell fates. Collectively, our studies provide direct evidence for the pivotal role of renal CD8$^+$ T_{RM} cells in podocyte injury and further strengthen that targeting T_{RM} cells represents a novel therapeutic strategy for patients with glomerular diseases.

INTRODUCTION

Tissue-resident-memory T (T_{RM}) cells, a recently identified non-circulating memory T cell population mainly located in barrier tissues at interfaces with the environment, play a crucial role in mediating potent local immune responses and provide a long-term localized defense against pathogens.[1–3] The formation and maintenance of T_{RM} cell are influenced by numerous factors, including inflammation, antigen triggering, and tissue-specific cues.[4,5] Emerging evidence has revealed that T_{RM} cells are also present in the kidney and other non-barrier tissues.[6] Recent studies have found a marked increase in CD4$^+$ T_{RM} cells in kidney biopsies from patients with antineutrophil cytoplasmic antibody (ANCA)-dependent glomerulonephritis and further confirmed that infection-induced CD4$^+$ T_{RM} cells may rapidly react to local inflammatory cytokines and aggravate renal autoimmune diseases, supporting a new concept for the predisposing role of microbial infections in aggravating autoimmune diseases.[7] However, most studies so far mainly focus on T_{RM} cells and their relevance for immediate protection against pathogens upon local reinfection; the composition, localization, effector function, and specificity of T_{RM} cells in the kidney and their relevance for chronic kidney disease (CKD) remain unknown.

Podocytes are highly differentiated epithelial cells and are essential for the formation and maintenance of the glomerular filtration barrier.[8] Despite compelling evidence identifying podocyte injury as the key mediator in the pathogenesis of glomerular diseases, such as focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD), diabetic kidney disease (DKD), and lupus nephritis (LN), the delivery of efficient therapies targeting podocytes is still a great challenge. On the other hand, podocyte injuries are closely associated with disruption of immune homeostasis.[9,10] Podocytes share many elements of the innate and adaptive immune system. They not only produce and express complement components and receptors but also express both class I and II major histocompatibility complex (MHC) molecules and co-stimulatory molecules that are involved in local immune responses. Recent studies have reported that antigen presentation by podocytes under inflammatory conditions plays an important role in activating T cell immune responses and facilitating

Received 17 January 2022; accepted 29 April 2022;
https://doi.org/10.1016/j.ymthe.2022.04.024.

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immune-mediated glomerular disease development. Meanwhile, inflammatory cytokines produced by T cells, such as interferon-γ (IFN-γ), can also induce the expression of MHC-I, MHC-II, CD80, and CD86 on podocyte surface, which promotes podocyte present antigen and podocyte apoptosis.[10] Therefore, a comprehensive understanding of the local immune responses associated with the activation and functions of T_{RM} cells in the kidney and their relevance of podocyte injury is necessary for the development of tissue-resident, cell-based immunotherapies for glomerular diseases.

In this study, we found that renal tissue displayed high abundance of tissue-resident lymphocytes, and the proportion of $CD8^+$ T_{RM} cells was significantly increased in the kidney from patients and mice with glomerular diseases. Mechanistically, interleukin-15 (IL-15) significantly promoted $CD8^+$ T_{RM} cell formation and activation, thereby promoting podocyte injury and glomerulosclerosis. Interestingly, Sparsentan, the dual angiotensin II receptor and endothelin type A receptor antagonist, can also reduce T_{RM} cell responses by intervening IL-15 signaling, exploring its new pharmacological functions. Collectively, our studies suggest pharmacological targeting of IL-15-mediated $CD8^+$ T_{RM} cell formation and activation at multiple levels may provide a novel approach for the treatment of glomerular diseases.

RESULTS

The kidney displays high abundance of T_{RM} cells

We performed a 42-antibody panel for mass cytometry to build a composite of human (Figures 1A and S1A) and murine (Figures 1B and S1B) renal leukocytes in the steady state. The immune landscape indicated that T lymphocytes were enriched in the kidney. CD69 expression on T cells is indicative of a tissue-resident phenotype[11] and almost all of renal $CD69^+$ T cells exhibited memory cell phenotypes: $CD45RA^-$ $CCR7^-$ in human (Figure S1C) and $CD44^+$ in mouse (Figure S1D). Next, we examined whether these renal $CD69^+CD44^+$ T cells exhibited tissue-resident features in mouse. *In vivo* labeling assay showed that renal $CD69^+CD44^+$ T cells were protected from intravenous (i.v.) injection antibody labeling ($CD45^-$) and they were *bona fide* resident population (Figure 1C). Phenotypic analysis showed that renal $CD69^+CD44^+$ T cells highly expressed tissue homing chemokine receptor CXCR3 but lowly expressed lymph node homing receptor CD62L (Figure 1D). Furthermore, they produced more effector molecular IFN-γ and perforin than renal $CD69^-CD44^+$ T cells (Figure 1E). Collectively, these data confirmed that the kidney harbored a large number of T_{RM} cells in human and mouse under homeostatic conditions.

The proportion of $CD8^+$ T_{RM} cells is significantly increased in mice and patients with glomerular diseases

We found that the proportion of $CD8^+$ T_{RM} cells was significantly increased in the kidney in independent mouse models for different glomerular diseases, including Adriamycin (ADR)-induced FSGS mice (Figure 2A), *db/db* mice (30 weeks of age, a spontaneous type 2 diabetes mellitus model, Figure 2B), and MRL/*lpr* mice (a mouse model of systemic lupus erythematosus, Figure 2C).[12] Among these animal models, the number of $CD8^+$ T_{RM} cells had no significant difference. Meanwhile, the proportion of $CD4^+$ T_{RM} cells in MRL/*lpr* mice was also increased (Figure S2C), but there were no changes in mice with ADR treatment and *db/db* mice compared with their normal controls (Figures S2A and S2B). We further measured cytokine production in isolated renal $CD8^+$ T_{RM} cells from mice under these pathological conditions and found that $CD8^+$ T_{RM} cells expressed more IFN-γ and perforin (Figures 2D–2F), indicating that T_{RM} cells were activated.

Importantly, immunofluorescent (IF) results showed that the number of $CD8^+$ T_{RM} cells was increased in the tubulointerstitium surrounding the glomeruli in renal biopsies from patients with FSGS, DKD, and LN (Figure 2G). The number of $CD4^+$ T_{RM} cells in renal biopsies from patients with LN was also increased, but there were no changes from patients with FSGS and DKD compared with healthy controls (Figure S2D). Together, our results indicate that $CD8^+$ T_{RM} cells may play a universal role in different glomerular diseases.

The increased $CD8^+$ T_{RM} cells are derived from peripheric $CD69^-$ memory T cells

To identify whether renal T_{RM} cells are derived from circulating $CD69^-$ memory T cells or the local proliferation of T_{RM} cells under pathological conditions, FTY720, a sphingosine-1-phosphate (S1P) inhibitor that prevents lymphocyte circulation,[13] was used for the pretreatment of mice (Figure 3A). There were no more proliferating renal T_{RM} cells in mice with ADR treatment (Figures 3B and 3C). Next, we further determined whether peripheric $CD69^-$ memory T cells differentiated into T_{RM} cells. We sorted $CD8^+CD69^-CD44^+$ T cells from normal mice and then transfused them to normal or ADR-treated nude mice (Figure 3D). Peripheral-infiltrating $CD8^+$ T cells were phenotyped by flow cytometry after 6 weeks of ADR treatment. It was found that more $CD69^+$ T cells were present in the kidney from ADR-treated transfused nude mice (Figure 3E) and highly expressed T_{RM} cells markers, such as CXCR3, and lowly expressed CD62L (Figure 3F). Therefore, we concluded that $CD8^+$ T_{RM} cells are derived from peripheral-infiltrating $CD69^-$ memory T cells under pathological conditions.

IL-15 promotes $CD8^+$ T_{RM} cell formation and activation in the kidney

We found that the level of IL-15 in the kidney was significantly increased in ADR-treated mice (Figure 4A) and positively correlated with the urine-albumin-to-creatinine ratio (UACR, Figure 4B). We further investigated whether IL-15 also regulates $CD8^+$ T_{RM} cell formation and activation as reported.[14] We isolated splenic mononuclear cells and sequential exposure to recombinant IL-15 (rIL-15). It was found that rIL-15 induced $CD69^+$ T cell development. As a negative control, IL-2, one cytokine that shares the β-chain with the receptor for IL-15[15] had no effects on CD69 expression (Figure 4C). Furthermore, IL-15 could directly promote IFN-γ production in $CD8^+$ T_{RM} cells (Figure 4D), indicating that IL-15 can activate renal $CD8^+$ T_{RM} cells.

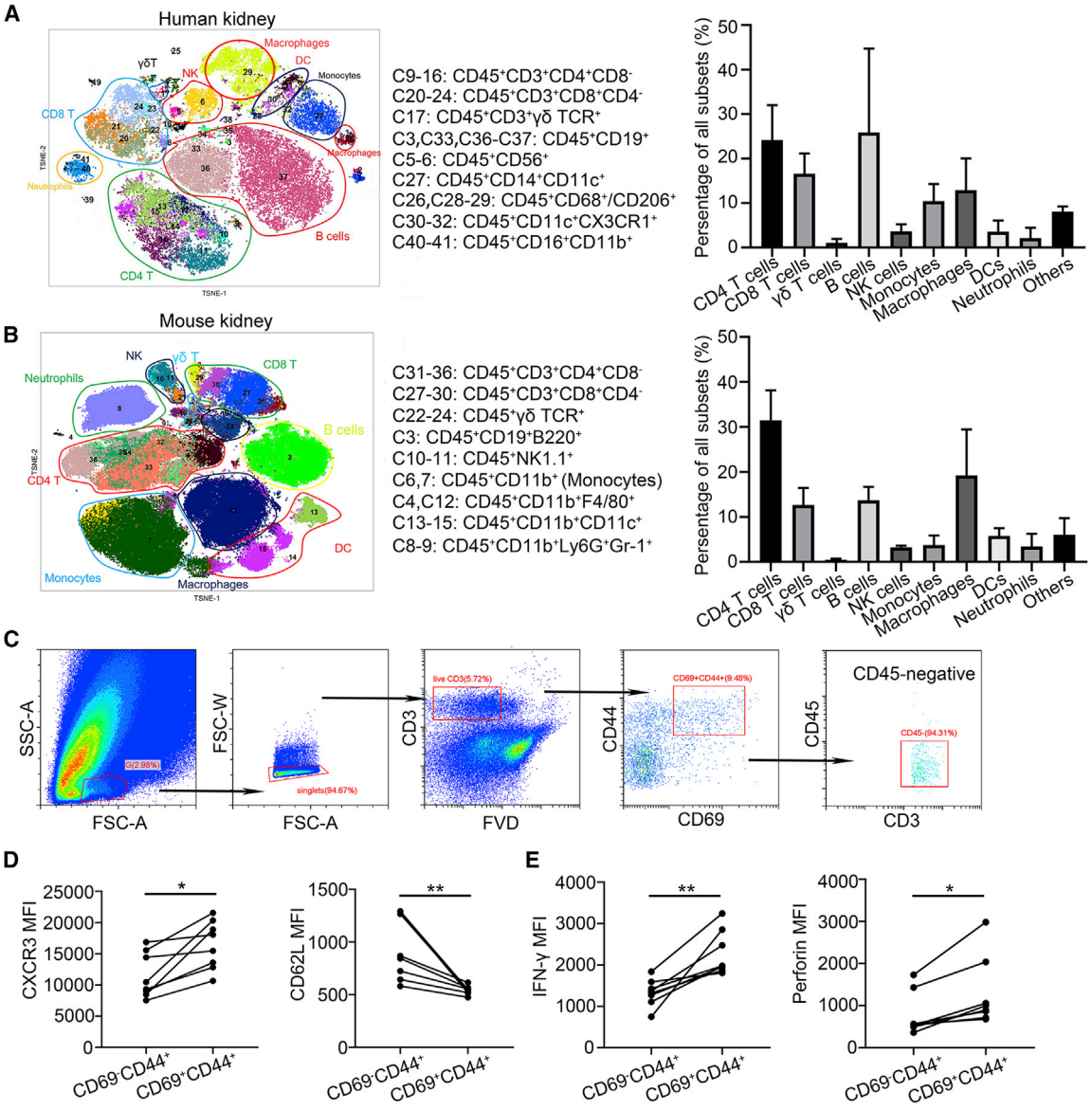

Figure 1. The kidney displays high abundance of T_{RM} cells

See also Figure S1. (A) Unsupervised cell cluster detection and quantification by t-distributed stochastic neighbor embedding (TSNE) and cytometry by time-of-flight (CyTOF) cluster detection algorithm on renal CD45$^+$ cells from normal human kidneys (n = 6). (B) Unsupervised cell cluster detection and quantification by TSNE and CyTOF cluster detection algorithm on renal CD45$^+$ cells from normal mouse kidneys (n = 6). (C) Gating strategy for renal intravascular staining after intravenous (i.v.) injection of allophycocyanin (APC) conjugated CD45 antibody. (D) The median fluorescence intensity (MFI) of CXCR3 and CD62L in renal CD69$^-$CD44$^+$ and CD69$^+$CD44$^+$ T cells individually (n = 8). (E) The MFI of IFN-γ and perforin in renal CD69$^-$CD44$^+$ and CD69$^+$CD44$^+$ T cells individually (n = 8). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01.

IL-15 signaling blockade alleviates podocyte injury and glomerulosclerosis by inhibiting renal CD8$^+$ T_{RM} cell formation and activation

Considering that CD122 is an integral part of the receptor complex for IL-15,[16] CD122 was highly expressed in renal CD8$^+$ T_{RM} cells (Figure S3A), and anti-CD122 antibody inhibited IL-15-induced CD8$^+$ T_{RM} cell formation and activation *in vitro* (Figure S3B), we therefore injected anti-IL-15 or anti-CD122 antibody to ADR-treated nude mice, which were transfused CD8$^+$CD69$^-$CD44$^+$ T cells (Figure 4E). Anti-IL-15 or anti-CD122-antibody treatment significantly inhibited renal CD8$^+$ T_{RM} cell formation (Figure 4F) and activation (Figure 4G). Moreover, anti-IL-15- or anti-CD122-antibody-treated mice showed lower UACR (Figure 4H) and fewer glomerular and podocyte injuries (Figure 4I) compared with their controls. In addition, IL-15 signaling blockade recovered the expressions of nephrin and podocin (Figure 4J).

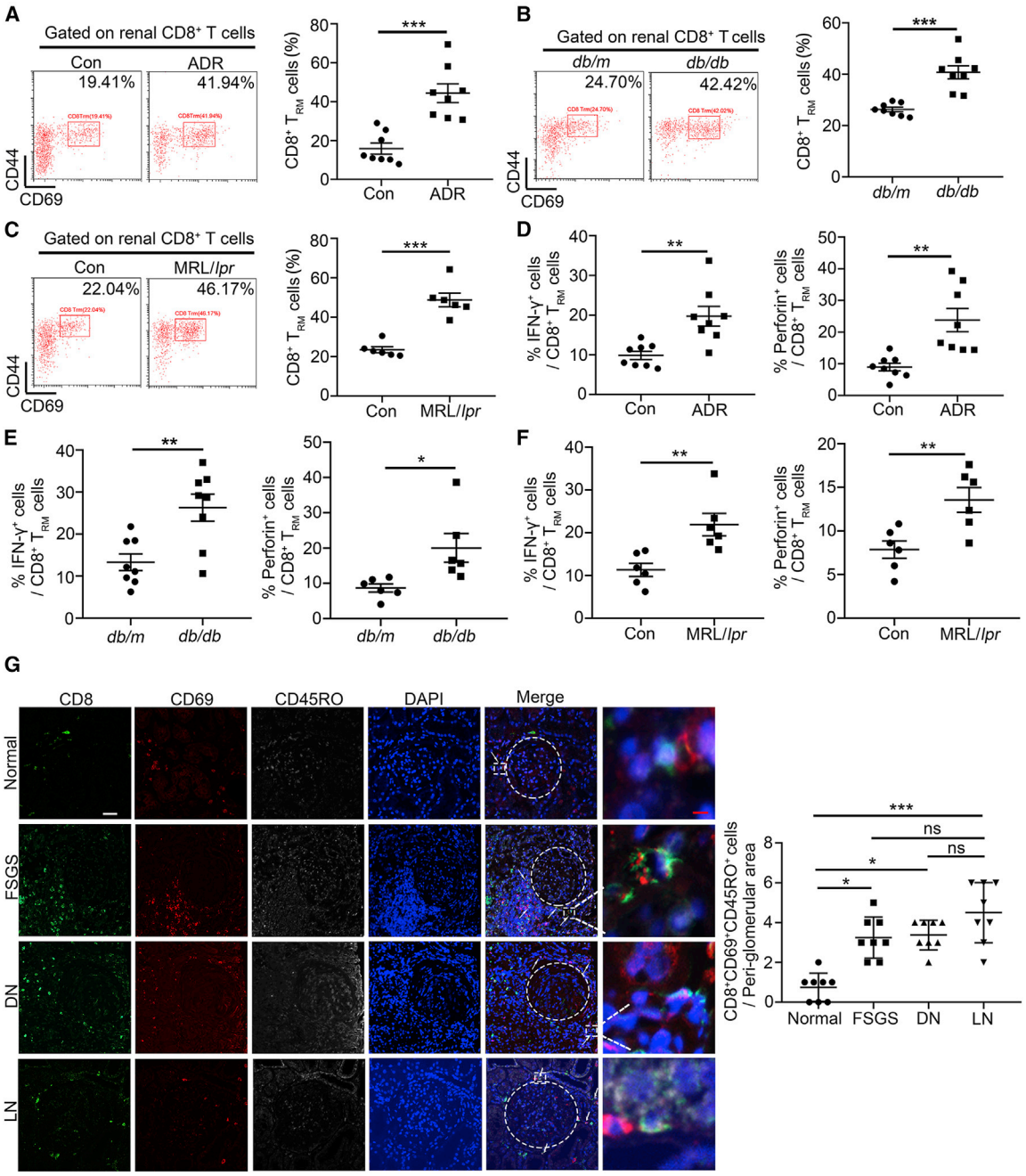

Figure 2. The proportion of CD8⁺ T_{RM} cells is significantly increased in mice and patients with glomerular diseases

See also Figure S2. (A–C) Representative flow cytometric analyses and quantification of renal CD8⁺ T_{RM} ($CD8^+CD69^+CD44^+$) cells in ADR-treated mice (A), *db/db* mice (B), MRL/*lpr* mice, (C) and their controls (n = 8). (D) The frequency of IFN-γ and perforin in renal CD8⁺ T_{RM} cells in control and ADR-treated mice (n = 8). (E) The frequency of IFN-γ (n = 8) and perforin (n = 6) in renal CD8⁺ T_{RM} cells in *db/m* and *db/db* mice. (F) The frequency of IFN-γ and perforin in renal CD8⁺ T_{RM} cells in control and MRL/*lpr* mice (n = 6). (G) Representative immunofluorescence staining for CD8 (green), CD69 (red), and CD45RO (gray) in human renal tissues from normal, subjects with FSGS diabetic nephropathy (DN) and LN. Left: representative images, white arrows highlight CD8⁺ T_{RM} cells ($CD8^+CD69^+CD45RO^+$); scale bars: white, 70 μm; red 5, μm; right: quantification of CD8⁺ T_{RM} cells surrounding the glomeruli per high power field (HPF, n = 8). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ns, no significance.

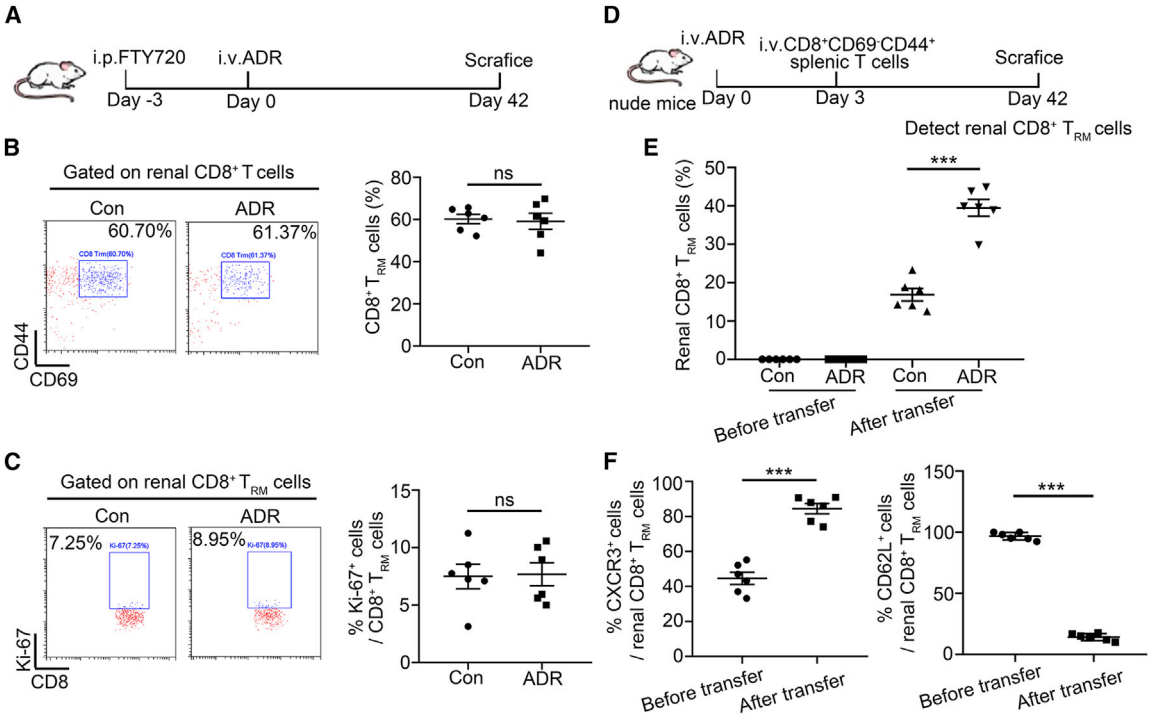

Figure 3. The increased CD8⁺ T_{RM} cells are derived from peripheric CD69⁻ memory T cells

(A) A schematic diagram showing the usage of FTY720 in ADR-treated mice. (B) Representative flow cytometric analyses and quantification of renal CD8⁺ T_{RM} cells (n = 6). (C) Representative flow cytometric analyses and the frequency of Ki-67⁺ cells in renal CD8⁺ T_{RM} cells (n = 6). (D) A schematic diagram showing that nude mice were adoptive transferred with splenic CD8⁺CD69⁻CD44⁺ T cells after ADR injection. Mice were euthanized at day 42. (E) The percentage of renal CD8⁺ T_{RM} cells from control or ADR-treated transfused nude mice (n = 6). (F) The percentage of CXCR3⁺ and CD62L⁺ cells in splenic CD8⁺CD69⁻CD44⁺ T cells (before transfer) and renal CD8⁺ T_{RM} cells (after transfer, n = 6). Data are represented as mean ± SEM. ***$p < 0.001$; ns, no significance.

Furthermore, in *db/db* mice, the level of IL-15 was also increased (Figure 4K) and positively correlated with the proteinuria (Figure 4L). Consistently, in anti-IL-15- or anti-CD122-antibody-treated *db/db* mice (Figure S4A), the formation and activation of renal CD8⁺ T_{RM} cell were markedly inhibited (Figures S4B and S4C). Lower urinary albumin excretion (Figure S4D) and less glomerulosclerosis and podocyte injury were also observed in *db/db* mice after anti-IL-15 or anti-CD122 antibody treatment (Figures S4E and S4F).

As a pleiotropic cytokine, IL-15 can also induce the proliferation of natural killer (NK) cells.[17] To investigate whether blocking of IL-15 signaling could affect NK cells and thereby alleviate podocyte and glomerular injuries, we injected *db/db* mice or ADR-induced mice with anti-NK1.1 antibody for three weeks and found that podocyte and glomerular injuries were not alleviated in mice with deficiency of NK cells, as shown in Figure S5, indicating that blocking of IL-15 signaling protects against podocyte and glomerular injuries, which may not be relevant to NK cells under these pathogenetic conditions.

Sparsentan inhibits CD8⁺ T_{RM} cell formation and activation

Sparsentan is a first-in-class, orally active, selective, and dual antagonist of the angiotensin II type 1 (AT_1) receptor and the endothelin type A (ET_A) receptor.[18,19] Interestingly, we occasionally found that Sparsentan (Figure 5A) could also reduce the number of renal CD8⁺ T_{RM} cells in mice with ADR treatment (Figure 5B). Moreover, Sparsentan reduced the production of IFN-γ (Figure 5C) and perforin (Figure 5D) in isolated renal CD8⁺ T_{RM} cells and alleviated proteinuria (Figure 5E), mesangial matrix expansion (Figure 5F), and podocyte injury (Figure 5G). To further confirm the broad implications of Sparsentan in conferring renal protection, we sought to investigate whether Sparsentan also has beneficial effects in *db/db* mice (Figure 5H). Consistently, Sparsentan inhibited CD8⁺ T_{RM} cell formation (Figure 5I) and activation (Figures 5J and 5K) and alleviated proteinuria (Figure 5L) and podocyte injury (Figures 5M–5N). These data provide direct evidence for the new pharmacological functions of Sparsentan. Targeting CD8⁺ T_{RM} cells may further strengthen therapeutic effects of Sparsentan besides directly acting on podocytes.

Angiotensin II and endothelin-1 induce IL-15 signaling

We found that the levels of angiotensin II (Ang II)[20] and endothelin-1 (ET-1) were enhanced in the kidney from mice with ADR treatment (Figure 6A) and *db/db* mice (Figure 6B), and the levels of Ang II (Figure 6C) and ET-1 (Figure 6D) were positively correlated with the level of IL-15. We further found that primary renal parenchymal cells secreted more IL-15 after Ang II or ET-1 treatment (Figure 6E). In addition, Sparsentan inhibited IL-15 production in the kidney from

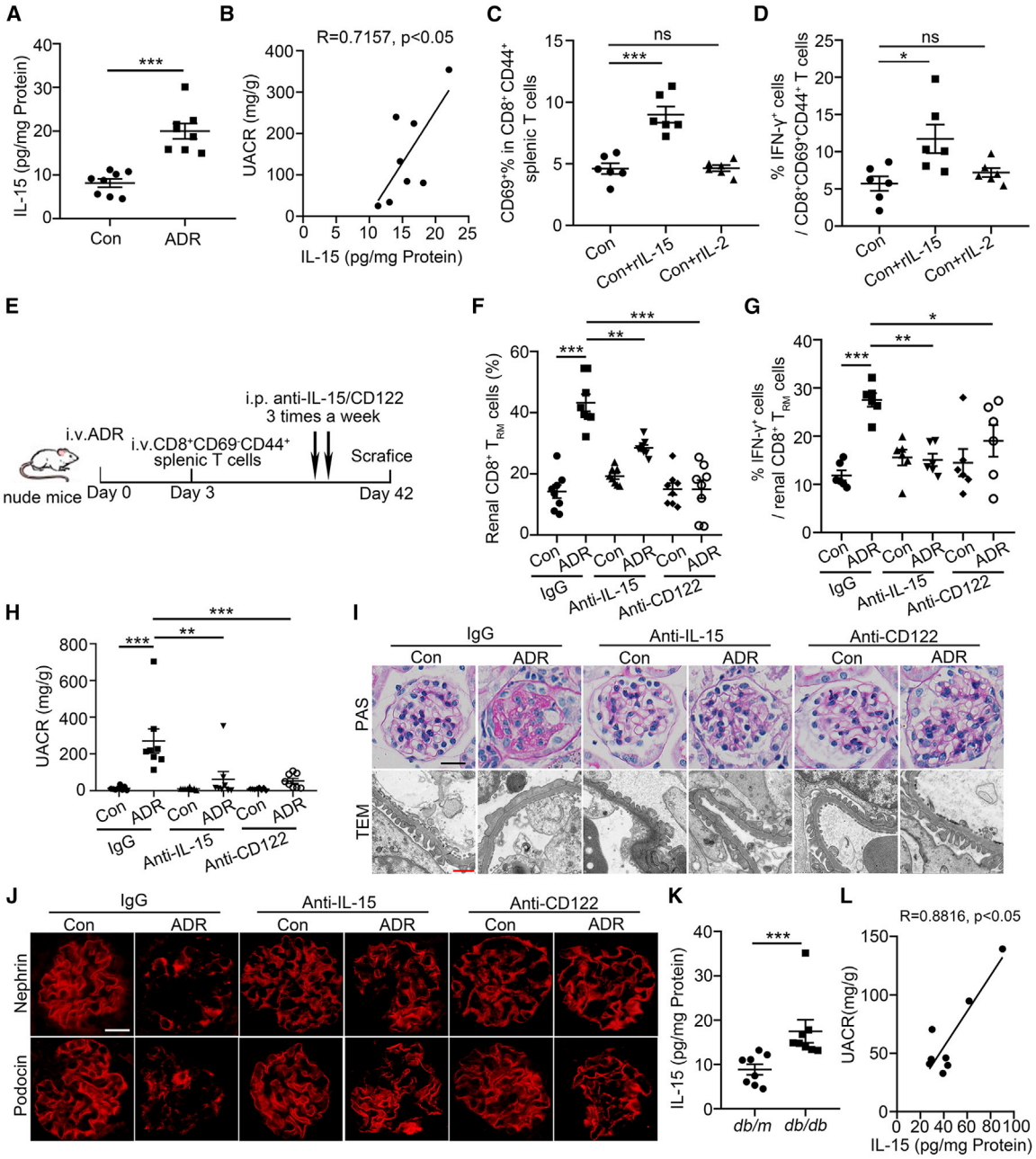

Figure 4. IL-15 signaling blockade alleviates glomerulosclerosis and podocyte injury by inhibiting renal CD8$^+$ T_{RM} cells formation and activation

See also Figures S3–S5. (A) The level of IL-15 in renal cortex of ADR-treated mice (n = 8). (B) Correlation between IL-15 and urine-albumin-to-creatinine ratio (UACR) in ADR-treated mice (n = 8). (C) The percentage of CD69$^+$ in splenic CD8$^+$CD44$^+$ T cells after 3-day culture in the presence of rIL-15 (50 ng/mL) or rIL-2 (50 ng/mL, n = 6). (D) The frequency of IFN-γ in splenic CD8$^+$CD69$^+$CD44$^+$ T cells after 3-day culture in the presence of rIL-15 (50 ng/mL) or rIL-2 (50 ng/mL, n = 6). (E) A schematic diagram showing anti-IL-15/CD122 treatment in transfused nude mice after ADR injection. (F) The percentage of renal CD8$^+$ T_{RM} cells in IgG- or anti-IL-15/CD122-treated control or ADR-treated transfused nude mice (n = 8). (G) The frequency of IFN-γ in renal CD8$^+$ T_{RM} cells from IgG- or anti-IL-15/CD122-treated control or ADR-treated transfused nude mice (n = 6). (H) UACR in mice (n = 8). (I) Morphological examinations of glomerular changes by periodic acid–Schiff (PAS) and transmission electron microscopy (TEM) analyses in mice. Scale bars: black, 20 μm; red, 1 μm. (J) Representative immunofluorescence images about nephrin and podocin expressions in glomeruli from mice. Scale bar, 20 μm. (K) The level of IL-15 in renal cortex of *db/db* mice (n = 8). (L) Correlation between IL-15 and UACR in *db/db* mice. R, Pearson correlation coefficient (n = 8). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

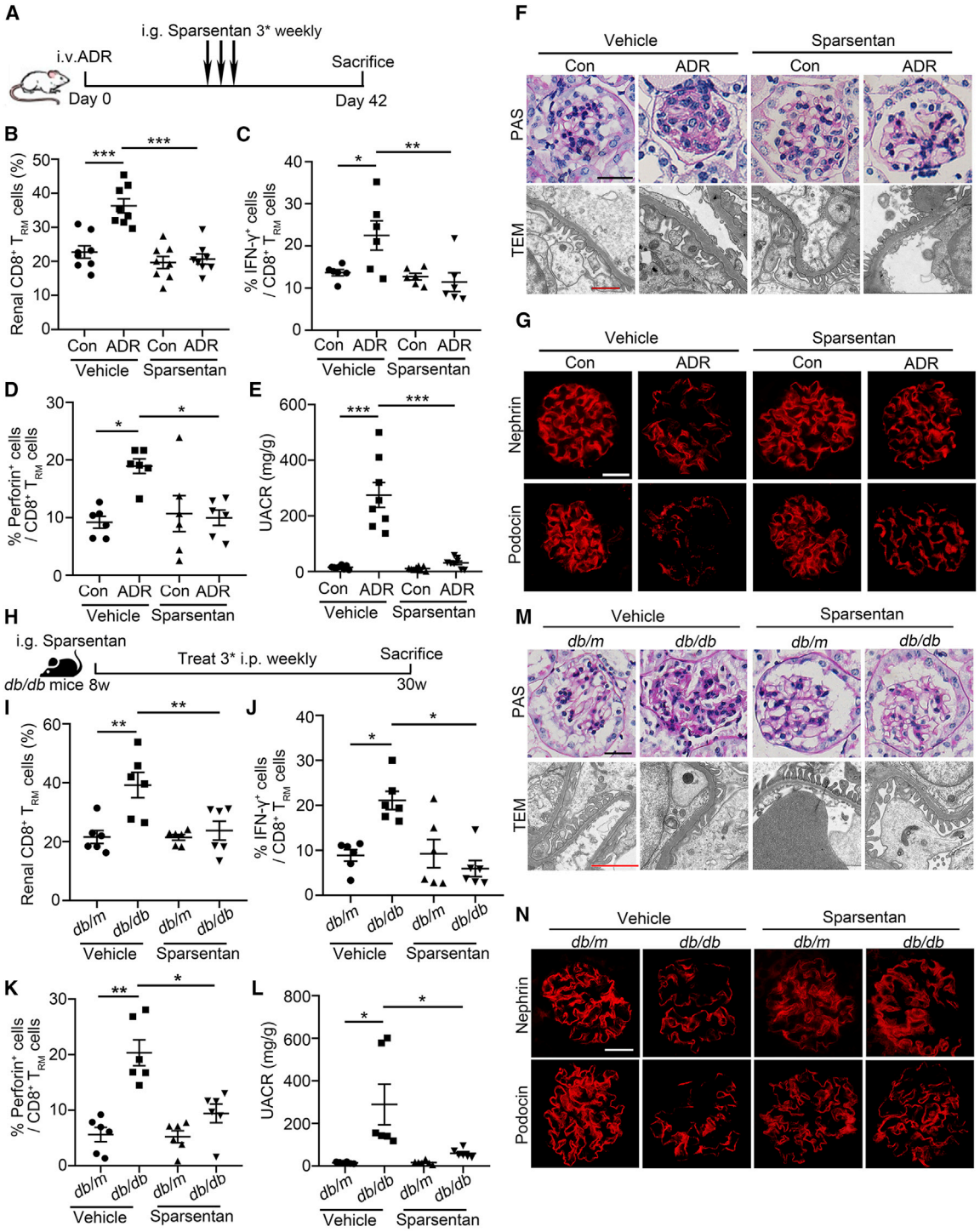

Figure 5. Sparsentan inhibits CD8$^+$ T_{RM} cell formation and activation and alleviates podocyte injury in ADR-treated mice and *db/db* mice

(A) A schematic diagram showing the treatment of Sparsentan in ADR-treated mice. (B) The percentage of renal CD8$^+$ T_{RM} cells in mice (n = 8). (C and D) The frequency of IFN-γ (C) and perforin (D) in renal CD8$^+$ T_{RM} cells (n = 6). (E) Urinary albumin-to-creatinine ratio (UACR) in mice (n = 8). (F) Morphological examinations of glomerular changes by periodic acid–Schiff (PAS) and transmission electron microscopy (TEM) analyses in mice. Scale bars: black 20, μm; red, 1 μm. (G) Representative immunofluorescence

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mice with ADR treatment (Figure 6F) and *db/db* mice (Figure 6G). Our results suggest Sparsentan may inhibit IL-15 production, thereby further regulating renal $CD8^+$ T_{RM} cells fate under pathological conditions.

Activated renal $CD8^+$ T_{RM} cells trigger podocyte injury

To address the effects of renal $CD8^+$ T_{RM} cells on the regulation of podocyte function and fate,[10] we sorted renal $CD8^+CD69^-CD44^+$ T cells and $CD8^+$ T_{RM} cells from ADR-treated mice and then transferred them to normal nude mice. It was found that only $CD8^+$ T_{RM} cells triggered podocyte apoptosis (Figure 7A). *In vitro*, we isolated different types of renal memory T cells and co-cultured with murine podocytes. Similarly, only renal $CD8^+$ T_{RM} cells from ADR-treated mice had cytotoxic effects on podocytes, including enhanced apoptosis (Figure 7B), reduced nephrin and podocin expression (Figure 7C), induced actin cytoskeleton derangement, and mitochondria damage (Figure 7D). However, activated $CD8^+$ T_{RM} cells with pretreatment with anti-IL-15 or anti-CD122 antibody had less cytotoxicity (Figure 7E) and recovered the expressions of podocin in podocytes (Figure 7F). To evaluate the effects of $CD8^+$ T_{RM} cells on other renal parenchymal cells, we then co-cultured the activated renal $CD8^+$ T_{RM} cells and glomerular cells, such as mesangial cells or glomerular endothelial cells. It was found that $CD8^+$ T_{RM} cells had no significant effect on the fates of mesangial cells and endothelial cells (Figure S6). Consistently, Sparsentan alleviated podocyte injury, as evidenced by reduced apoptosis (Figure 7G), recovered podocin expression (Figure 7H), attenuated cytoskeleton, and mitochondria injury (Figure 7I), when we sorted renal $CD8^+$ T_{RM} cells from Sparsentan-treated ADR-treated mice and co-cultured with podocytes.

DISCUSSION

Podocyte dysfunction is central to the underlying pathophysiology of many common glomerular diseases, including DKD, FSGS, LN, and genetic forms of nephrotic syndrome.[8] By detection of immune landscape of human and mice kidney, we found that tissue-resident lymphocytes were present in the kidney. Although the function of renal T_{RM} cells under physiological conditions is still not very clear, previous studies have proposed that T_{RM} cells could be the consequence of a subclinical renal inflammation or infection. Renal T_{RM} cells might be involved in control of urogenital bacterial infection ascending to the kidney. It is also possible that T_{RM} cells are required to control latent chronic infections (e.g., by polyoma viruses).[21] More importantly, under pathogenetic conditions, the proportion of $CD8^+$ T_{RM} cells was substantially increased in the kidney from patients with glomerular diseases, such as FSGS, DKD, and LN, compared with healthy controls, as well as the kidney from experimental mouse models for these glomerular diseases. Meanwhile, we found that the number of $CD4^+$ T_{RM} cells was also increased in the kidney from mice and patients with LN, but not in FSGS and DKD, reflecting that $CD8^+$ T_{RM} cells but not $CD4^+$ T_{RM} cells may be a key and universal regulator contributing to glomerular injury. Therefore, considering that $CD8^+$ T_{RM} cells are undoubtedly attractive therapeutic targets with their distinct functions,[22–24] we further explored their regulatory mechanisms in glomerular diseases, including FSGS and DKD.

In this study, we demonstrated for the first time that $CD8^+$ T_{RM} cells in the kidney were mainly derived from $CD69^-$ memory T cells and further explored a previously unappreciated role of renal $CD8^+$ T_{RM} cells in podocyte injury through their cytotoxic effects on podocytes. The principal hallmark of *bona fide* $CD8^+$ T_{RM} cells is their long-term persistence in non-lymphoid tissues without recirculation in the blood. Differentiation of T_{RM} is controlled by various factors.[25] Our data support that targeting IL-15 signaling pathways is a potential strategy to clear autoreactive memory cells from the kidney and protects against podocyte injury in mice. Mechanistically, IL-15 promoted $CD8^+$ T_{RM} cell formation and activation. IL-15 is a pluripotent cytokine from the IL-2 family that possesses many functions involved in regulating both adaptive and innate immune systems. IL-15 has been investigated for its therapeutic potential for the induction and maintenance of T cell responses because of its unique properties, such as wide expression, tightly regulated secretion, and trans-presentation.[26] Studies have demonstrated that IL-15 is important for the generation of T_{RM} in viral infections and in cutaneous lymphomas,[27] and targeting IL-15 provides a durable treatment strategy for vitiligo.[16] In this study, we found that IL-15 was highly expressed in the kidney and was required for the formation and activation of $CD8^+$ T_{RM} cell under pathological conditions. Furthermore, we demonstrated that blocking of IL-15 signaling alleviated podocyte and glomerular injuries by inhibiting renal $CD8^+$ T_{RM} cells rather than NK cells, which was consistent with previous studies showing that NK cells cannot mediate renal injury in mice with ADR nephropathy.[28] Therefore, we suggest that inhibiting renal $CD8^+$ T_{RM} cells by targeting IL-15 signaling may provide a novel and durable treatment strategy for glomerular diseases.

In this study, another important finding is that Sparsentan can reduce T_{RM} cell responses by intervening IL-15 signaling, exploring its new pharmacological functions. In FSGS, current treatment with corticosteroids or other immunosuppressive drugs is aimed at reducing proteinuria.[29] These agents are routinely combined with renin-angiotensin system (RAS) inhibitors. Due to a spectrum of serious side effects of immunomodulating drugs, the availability of effective, safe, and well-tolerated drugs to protect renal function and reduce proteinuria is an unmet medical need in FSGS. Compared with RAS inhibitors, ET_A receptor antagonists have shown a wide range of beneficial hemodynamic, anti-inflammatory, anti-fibrotic, and podocyte-protective effects in glomerular diseases.[30,31] In particular, the combination of RAS inhibitors and ET_A receptor antagonists has additional benefits in experimental models of kidney diseases and in

images about nephrin and podocin expressions in glomeruli from mice. Scale bar, 20 μm. (H) A schematic diagram showing the treatment of Sparsentan in *db/db* mice. (I) The percentage of renal $CD8^+$ T_{RM} cells in mice (n = 6). (J and K) The frequency of IFN-γ (J) and perforin (K) in renal $CD8^+$ T_{RM} cells (n = 6). (L) UACR in mice (n = 6). (M) Morphological examinations of glomerular changes by PAS and TEM analyses in mice. Scale bars: black, 20 μm; red, 1 μm. (N) Representative immunofluorescence images about nephrin and podocin expressions in glomeruli from mice. Scale bar, 20 μm. Data are represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

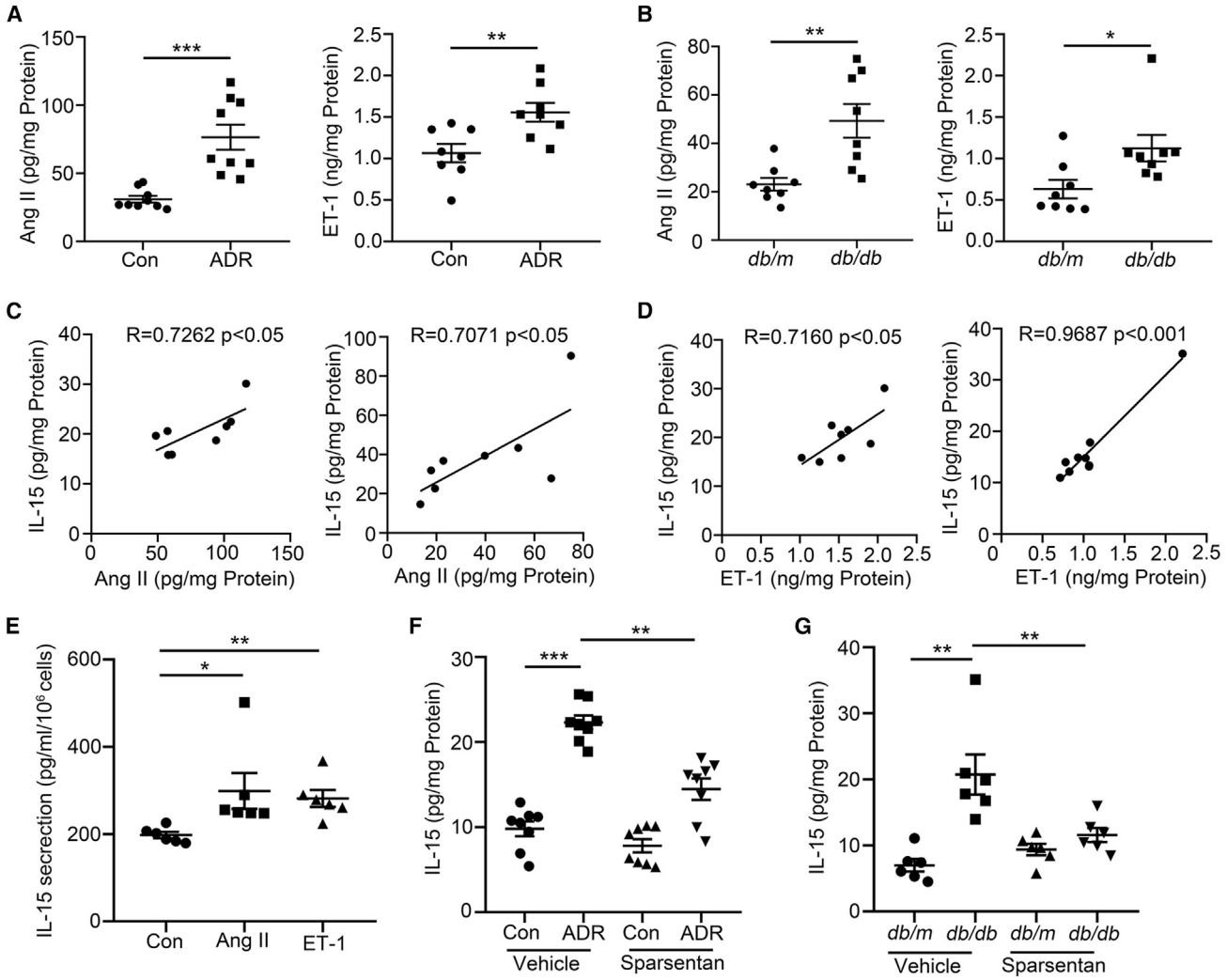

Figure 6. Angiotensin II and endothelin-1 induce IL-15 signaling

(A and B) The levels of angiotensin II (Ang II) and endothelin-1 (ET-1) in the renal cortex from ADR-treated mice (A, n = 9) and *db/db* mice (B, n = 8).

(C) Correlation between Ang II and IL-15 in renal cortex of ADR-treated and *db/db* mice (n = 8). R, Pearson correlation coefficient. (D) Correlation between ET-1 and IL-15 in renal cortex of ADR-treated and *db/db* mice (n = 8). R, Pearson correlation coefficient. (E) The secretion of IL-15 in primary renal parenchymal cells after Ang II (10^{-6} mol/L) or ET-1 (10^{-6} mol/L) treatment (n = 6).

(F and G) The level of IL-15 in renal cortex of ADR-treated mice (F, n = 8) and *db/db* mice (G, n = 6). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

patients with CKD.[32] Sparsentan is a first-in-class, orally active, selective, and dual antagonist of the AT_1 receptor and the ET_A receptor. Since 2020, a multicenter, international, phase 3, randomized, double-blind, active-controlled study of sparsentan in patients with FSGS (DUPLEX; NCT0349368528) trial has been initiated to evaluate the long-term nephroprotective effects and safety of Sparsentan in patients with primary FSGS.[33] In this study, we found that the levels of ET-1 and Ang II were significantly elevated and positively correlated with the level of IL-15 in the kidney from mice with FSGS or DKD. We further explored an unexpected role that Sparsentan can improve podocyte injury by inhibiting IL-15-mediated $CD8^+$ T_{RM} cell formation and activation, despite that we cannot exclude that Sparsentan may directly act on podocytes because Ang II and ET-1 have direct effect on podocyte function. Therefore, we propose that $CD8^+T_{RM}$-cell-dependent or -independent mechanisms may synergize together to protect against podocyte injury by Sparsentan. On the other hand, a feedforward mechanism may exist because the reduced proteinuria in glomerular diseases by Sparsentan may further decrease the $CD8^+$ T_{RM} cell formation and activation by the reduction of renal toxic local antigen.[34,35] Further studies are needed to address this issue.

Although in this study we demonstrate a contribution of $CD8^+$ T_{RM} cells to podocyte injury, further studies are required to elucidate why podocytes are more susceptible to the pathogenicity of renal $CD8^+$

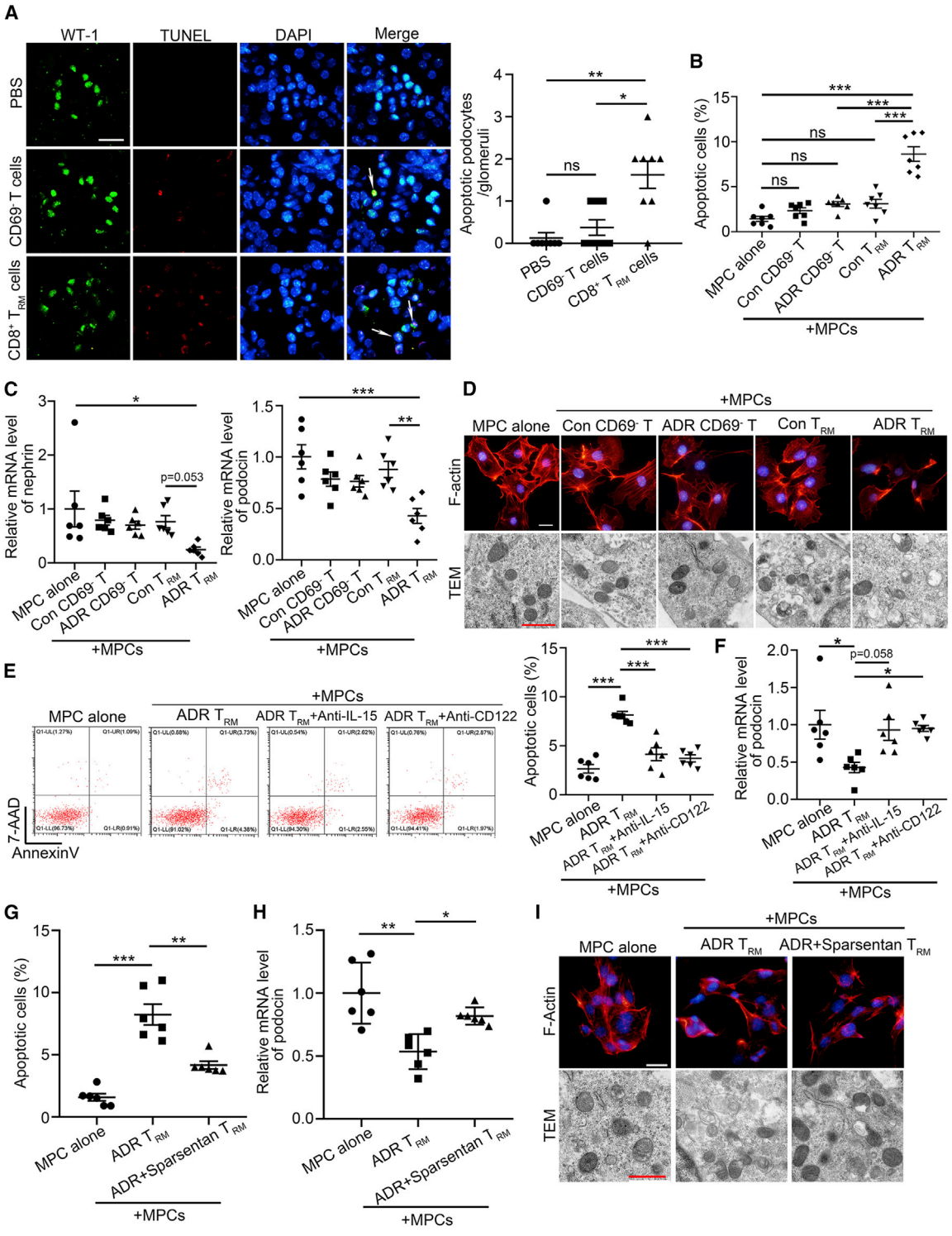

Figure 7. Activated renal CD8$^+$ T_{RM} cells trigger podocyte injury

See also Figure S6. (A) Representative immunofluorescence images and quantifications of apoptotic podocytes per glomeruli by TUNEL assay (10 areas per mouse were analyzed); white arrows highlight the apoptotic podocytes. Scale bars: black, 20 μm; red, 1 μm (n = 8). (B) The percentage of apoptotic murine podocytes (MPCs, n = 7). (C) Relative mRNA levels of nephrin and podocin in MPCs (n = 6). (D) Morphological examinations of cytoskeleton and mitochondria injury by F-actin staining and transmission

(legend continued on next page)

T_{RM} cells than other cell types in the kidney. Furthermore, $CD8^+$ T_{RM} cells are mostly accumulated surrounding the glomeruli; how cytotoxic $CD8^+$ T_{RM} cells influence podocytes remains unclear. Normally, podocytes are not accessible to $CD8^+$ T cells. However, under some pathogenetic conditions, such as crescentic glomerulonephritis or the late stage of DKD, breaches in Bowman's capsule can allow access of $CD8^+$ T cells to the glomerular tuft and podocytes, resulting in their destruction.[36] However, the breached Bowman's capsule is not observed in FSGS.[37] Therefore, the implication of periglomerular $CD8^+$ T_{RM} cells in podocyte injury is closely associated with different glomerular diseases or different stage of diseases. In this study, it is possible that IFN-γ produced by $CD8^+$ T_{RM} cells may potentiate robust local expression of chemokines and rapidly recruit other circulating inflammatory immune cells into glomeruli, which could amplify immune responses and result in podocyte injury. On the other hand, based on the results from *in vitro* studies by using a transwell co-culture system, renal $CD8^+$ T_{RM} cells from ADR-treated mice had cytotoxic effects on podocytes. Therefore, it is also possible that $CD8^+$ T_{RM} cells might release inflammatory cytokines, which get into glomeruli to injure podocytes directly. Of course, we cannot exclude that under some certain pathogenetic conditions, breaches in Bowman's capsule allow access of $CD8^+$ T_{RM} cells to the glomerular tuft and podocytes, resulting in podocyte injury. Therefore, further studies for addressing this issue are also of great interest.

In conclusion, our studies for the first time provide direct evidence for the pivotal role of renal $CD8^+$ T_{RM} cells, suggesting that targeting T_{RM} cells may represent a novel therapeutic strategy for patients with glomerular diseases.

MATERIALS AND METHODS

Human renal biopsy samples

Renal biopsies had been performed as part of routine clinical diagnostic investigation. The samples of renal biopsies were obtained from Department of Pathology, Shandong University School of Basic Medical Sciences. Normal controls were obtained from the healthy kidney poles of individuals who underwent tumor nephrectomies without other kidney diseases. The investigations were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the Research Ethics Committee of Shandong University after informed consent was obtained from the patients.

Animal studies

All experimental protocols for animal studies were approved by the Institutional Animal Care and Use Committee of the School of Basic Medical Sciences, Shandong University (document no. ECSBM SSDU2018-2-074) and conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Different groups were allocated in a randomized manner and investigators were blinded to the allocation of different groups when doing surgeries and doing outcome evaluations. The mouse models used in this study were described in the supplemental methods.

Cells isolation and sorting

The kidney was enzymatically digested with 400 μg/mL collagenase D (Roche, Mannheim, Germany) and 10 U/mL DNase I (Roche, Mannheim, Germany) for 45 min at 37°C. Subsequently, leukocytes were isolated by density gradient centrifugation using 40% Percoll (Merck Millipore, Darmstadt, Germany). $CD8^+CD69^-CD44^+$ T cells or renal $CD8^+$ T_{RM} cells were purified with fluorescence-activated cell sorting (MoFlo Astrios EQ, Beckman Coulter).

Adoptive transfer

Splenic $CD8^+CD69^-CD44^+$ cells were adoptively transferred to nude mice by a single tail-vein injection (106/head). Renal $CD8^+$ $CD69^-CD44^+$ T cells, and $CD8^+$ T_{RM} cells from ADR-treated mice were adoptively transferred to nude mice by a single tail-vein injection (106/head).

In vivo labeling assay

Intravascular injection of anti-CD45 mAb (clone: 30-F11, 2.5 μg per mouse) was administered to mice 5 min before sacrifice.[38,39]

Mass cytometry

The detailed methods are described in the supplemental method. A summary of the antibodies/clones used in the mass cytometry analysis is presented in the Tables S1 and S2.

Flow cytometry

Surface IF staining was performed at 4°C for 30 min. For intracellular (IC) cytokine staining, cells were stimulated with 50 ng/mL phorbol-12-myristate-13-acetate (PMA, MultiSciences), 1 μg/mL ionomycin (MultiSciences), and brefeldin A (5 μg/mL) for 5 h. Cells were fixed with IC fixation buffer (Invitrogen) after surface staining, permeabilized with permeabilization buffer (Invitrogen), and stained with IC antibodies cocktail for 30 min at 4°C. A summary of the antibodies/clones used in the flow cytometry analysis is presented in the Table S3. Cells were then washed and resuspended in phosphate buffer saline. Acquisition was performed on a CytoFLEX S Flow Cytometer, and data were analyzed using CytExpert software (both from Beckman Coulter).

Cell culture

The detailed cell preparations and cell culture conditions of murine podocyte cell line (MPC), glomerular mesangial cell line (MC), and

electron microscopy (TEM) analyses in MPCs. Scale bars: black, 20 μm; red, 1 μm. (E) Representative flow cytometric analyze and quantification of apoptotic MPCs (n = 6). (F) Relative mRNA levels of podocin in MPCs (n = 6). (G) The percentage of apoptotic MPCs (n = 6).
(H) Relative mRNA levels of podocin in MPCs (n = 6). (I) Morphological examinations of cytoskeleton and mitochondria injury by F-actin staining and TEM analyses in MPCs. Scale bars: black, 20 μm; red, 1 μm. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

glomerular endothelial cell line (GENC) were briefly described in the supplemental methods.

Transwell migration assay

In a transwell co-culture system, MPCs, MCs, or GENCs (5×10^4, lower) were seeded on a 0.8 mm Transwell insert (Corning, Corning, NY, USA) with medium and co-cultured with renal $CD8^+$ memory T cells (5×10^3, upper) from normal or ADR-treated mice.

Enzyme-linked immunoabsorbent assay

According to the manufacturer's instructions, renal cortex homogenates or cells supernatant samples were analyzed by mouse IL-15 (MultiSciences), ET-1 (OmnimAbs, CA, USA), and Ang II (Omni mAbs, CA, USA) ELISA kit.

Antibody and drug treatment

Anti-mouse IL-15 antibody (300 μg/head; Bio X Cell, clone AIO.3), anti-mouse CD122 antibody (100 μg/head; Bio X Cell, clone TM-beta 1), anti-mouse NK1.1 antibody (200 μg/head; BioLegend, clone PK136), or isotype controls (Bio X Cell, clone C1.18.4) were administered intraperitoneally three times weekly. The current concentrations of IL-15,[40] CD122,[16] and NK1.1[40] neutralizing antibodies were used, as previous studies indicated. Sparsentan (30 μmol/kg, MCE) was administered orally three times weekly. FTY720 (240 μg/kg, MCE) was administered intraperitoneally before ADR treatment.

Transmission electron microscopy

Electron microscopic sample handling and detection were performed by the electron microscopic core lab of Shandong University. Tissues and cells were collected and fixed with 2.5% glutaraldehyde at 4°C. Sections were washed 15 min for 3 times in 0.1 mol/L PBS and post-fixed in 1% osmium tetroxide at room temperature for 2 h. Specimens were then dehydrated using 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol and 100% acetone in series. After dehydration, the sections were embedded in Pon 812 resin overnight at 37°C using acetone as a transitional solvent. The ultra-thin sections were cut and post-stained with 2% saturated uranyl acetate and lead citrate.

Multiplex immunohistochemistry staining

The detailed multiplex immunohistochemistry stain method descriptions are available in the supplemental methods. Details of antibodies are described in Table S4.

Immunofluorescence staining

Tissues were transferred to 4% paraformaldehyde (PFA) and fixed at 4°C overnight, followed by paraffin embedded and cross-sectioned (3 μm) for IF staining by using a modified protocol, as previously described.[41] Details of antibodies are described in Table S4.

TUNEL assay

Cell death in the kidney section was detected by TUNEL assay following the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

RNA extraction and real-time quantitative PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). The mRNA expression levels were determined by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA). The sequences of specific primers are listed in Table S5.

Statistics

Data are expressed as mean ± SEM. Statistical analyses were performed with GraphPad Prism (v.8.0, GraphPad Software, San Diego, CA). Normality assumption of the data distribution was assessed using Kolmogorov-Smirnov test. Comparisons between two groups were performed using two-tailed Student's t test for normally distributed data and Mann-Whitney rank-sum test for non-normally distributed data. Differences between multiple groups with one variable were determined using one-way ANOVA followed by *post hoc* Tukey's test. To compare multiple groups with more than one variable, two-way ANOVA followed by *post hoc* Tukey's test was used. For correlation analysis, Pearson correlation coefficient was applied as appropriate.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2022.04.024.

ACKNOWLEDGMENTS

We thank Translational Medicine Core Facility of Shandong University for consultation and instrument availability that supported this work. This study was supported by the National Nature Science Foundation of China (91949202, 82090020, 82090024, 81970580, 81800645, 81800643, 81873614, and 81900659); National Key R&D Program of China (2020YFC2005000); and Shandong Provincial Natural Science Foundation, China (ZR2019ZD40 and ZR2019BH030).

AUTHOR CONTRIBUTIONS

L.L., W.T., Y.Z., and M.J. conducted experiments, performed data analysis, and helped write the manuscript. L.W. helped analyze flow cytometry data. Q.L., Q.H., and X.P. contributed with cells isolation from animals. Y.Z. and J.W. performed *in vivo* animal studies. W.T. and Y.X. helped design experiments. Z.W., J.Z., X.W., M.L., Y.S., and C.Z. analyzed human renal biopsy samples. F.Y. designed the experiment, interpreted the data, wrote the manuscript, and approved the final version of the manuscript for publication.

DECLARATION OF INTERESTS

All authors declare no conflict of interest.

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