Group 1 innate lymphoid cells are involved in the progression of experimental anti-glomerular basement membrane glomerulonephritis and are regulated by peroxisome proliferator-activated receptor α

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Innate lymphoid cells play an important role in the early effector cytokine-mediated response. In Wistar Kyoto rats, $CD8^+$ non-T lymphocytes ($CD8^+$Lym) infiltrate into glomeruli during the development of anti-glomerular basement membrane (anti-GBM) glomerulonephritis. Here, we examined the profiles and roles of $CD8^+$Lym in anti-GBM glomerulonephritis. The regulation of $CD8^+$Lym by peroxisome proliferator-activated receptor (PPAR)-α in anti-GBM glomerulonephritis was also evaluated. Glomerular infiltrating $CD8^+$Lym were lineage-negative cells that showed markedly high expression of *IFN-γ* and *T-bet* mRNAs but not *Eomes*, indicating these cells are group 1 innate lymphoid cells. In anti-GBM glomerulonephritis, the glomerular mRNAs of innate lymphoid cell-related cytokines (*IFN-γ* and *TNF-α*) and chemokines (*CXCL9*, *CXCL10*, and *CXCL11*) are significantly increased. Treatment with a PPARα agonist ameliorated renal injury, with reduced expression of these mRNAs. *In vitro*, enhanced IFN-γ production from innate lymphoid cells upon IL-12 and IL-18 stimulation was reduced by the PPARα agonist. Moreover, *CXCL9* mRNA in glomerular endothelial cells and *CXCL9*, *CXCL10*, and *CXCL11* mRNAs in podocytes and macrophages were upregulated by IFN-γ, whereas the PPARα agonist downregulated their expression. We also detected the infiltration of innate lymphoid cells into glomeruli in human anti-GBM glomerulonephritis. Thus, innate lymphoid cells are involved in the progression of anti-GBM glomerulonephritis and regulated directly or indirectly by PPARα. Our findings suggest that innate lymphoid cells could serve as novel therapeutic targets for anti-GBM glomerulonephritis.

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Translational Statement

Recently identified innate lymphoid cells (ILC1s) regulate early immune responses as innate immune cells prior to the development of adaptive immunity; they also exhibit phenotypic and functional characteristics that resemble those of T helper cell 1 cells. The present study revealed the involvement of ILC1s in the progression of experimental rat anti-glomerular basement membrane glomerulonephritis and the direct and indirect effects of a proliferator-activated receptor–α agonist on ILC1 regulation. Furthermore, in human anti-glomerular basement membrane glomerulonephritis, ILC1s were detected in glomeruli, particularly prior to the development of crescent formation, suggesting that ILC1s are involved in the early phase of inflammation. These results constitute evidence that ILC1s could serve as a novel therapeutic target; moreover, based on their ability to regulate ILC1s, proliferator-activated receptor–α agonists might be a suitable treatment for patients with anti-glomerular basement membrane glomerulonephritis.

Innate lymphoid cells (ILCs) are a novel group of immune cells. ILCs are classified into group 1, 2, and 3 ILCs based on their phenotypic and functional characteristics of T helper cell 1 (Th1), Th2, and Th17 cells, respectively. Hence, ILC1s, including natural killer (NK) cells, are characterized by the production of interferon-γ (IFN-γ) and the expression of transcription factor T-box factor expressed in T cells (T-bet). However, unlike T and B cells, ILCs do not express adaptive antigen recognition receptors or phenotypic markers of myeloid or dendritic cells. Instead, their functions are mediated by cytokine signals from myeloid and non-hematopoietic cells.[1–3] ILC1s regulate early immune responses against the type of encountered pathogen or tissue damage, and their migration is regulated by chemokine ligand 9 (CXCL9), CXCL10, and CXCL11, because ILC1s express CXC chemokine receptor 3, which is activated by the above-mentioned chemokines.[4] High proportions of ILC1s have been found in patients with several diseases, suggesting an association between ILC1s and the pathogenesis of inflammatory diseases.[5–8]

In a rat model of anti-glomerular basement membrane (anti-GBM) glomerulonephritis (GN), crescentic GN develops with infiltration of $CD8^+$Lym and activated macrophages (Mφ).[9–14] During adaptive immunity, infiltrating Mφ regulated by T helper cell subsets (Th1, Th2, and Th17 cells) directly induced glomerular injury, including crescent formation.[15–19] Experimental anti-GBM GN is attenuated by inhibiting the function of or changing the phenotype of Mφ.[9,10] In rat models of anti-GBM GN, $CD8^+$Lym infiltrate into glomeruli from an early phase of inflammation, and the depletion of $CD8^+$Lym markedly reduces the amount of Mφ infiltration into glomeruli, resulting in an improvement of crescentic GN with the suppression of glomerular IFN-γ expression.[11,12] In contrast, migration inhibition of Mφ have no effect on the number of $CD8^+$Lym that infiltrate into glomeruli, despite improvements in anti-GBM GN.[13] These results indicate that glomerular injury directly induced by Mφ is regulated by $CD8^+$Lym. However, little is known regarding the profile of $CD8^+$Lym in the development of anti-GBM GN.

PPARα belongs to the nuclear receptor family of ligand-activated transcription factors that regulates gene expression involved in lipid metabolism, atherosclerosis, neovascularization, and inflammation.[20–22] PPARα is expressed in various tissues and cells, including the kidney, lymphocytes, and Mφ.[22,23] Therefore, in rodent models of inflammatory and autoimmune diseases, PPARα signaling is widely used to regulate immune responses.[21,24–31] Although several studies have been published on the anti-inflammatory effect of PPARα via the regulation of inflammatory cytokines and chemokines, only a few have focused on the effects and mechanism of action of PPARα on the regulation of cytokines and chemokines in anti-GBM GN.[24–27]

In this study, we investigated the profiles and roles of glomerular-infiltrating $CD8^+$Lym and the therapeutic effects of PPARα on the regulation of $CD8^+$Lym in the development of anti-GBM GN in Wistar Kyoto rats.

RESULTS

Identification of the $CD8^+$Lym profile

Severe necrotizing and crescentic GN was induced by a single injection of anti-rat GBM antibody (Figure 1a). As previously reported, a large number of Mφ and $CD8^+$Lym infiltrated into glomeruli on day 7 (Figure 1b and c).[9,10,32] Immunofluorescence microscopy showed that most of the glomerular-infiltrating $CD8^+$Lym did not express CD3, CD68, CD103, or CD161 (Figure 1d and e, Supplementary Table S1A). Similarly, flow cytometric analysis revealed that the majority of the glomerular-infiltrating $CD8^+$ cells were lineage marker (CD3, CD4, CD14, CD45R, CD103, CD161, and granulocyte marker)-negative (Lin^-) (Figure 1f and g, Supplementary Table S1B and C); nearly all of the glomerular-infiltrating Lin^- cells expressed CD8 (Supplementary Figure S1, Supplementary Table S1D). Approximately 5% to 10% of leukocytes that infiltrated into glomeruli were $CD8^+$Lym. To identify the functional characteristics of $CD8^+$Lym, we further examined the mRNA expression of $CD8^+$Lym. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed that *IFN-γ* and *T-bet* mRNAs were markedly high in $CD8^+$Lym, compared with Mφ. The expression of *tumor necrosis factor-α* (*TNF-α*) mRNA in $CD8^+$Lym was not different from that in Mφ (Figure 1h, Supplementary Table S1E). Moreover, $CD8^+$Lym displayed significantly lower levels of *eomesodermin* (*Eomes*) mRNA, compared with conventional NK cells (Lin^-CD161^+ cells) (Supplementary Figure S2A and B, Supplementary Table S1F). To detect the major source of IFN-γ in the early phase of anti-GBM GN, we next investigated the IFN-γ–producing cells in glomeruli. Flow cytometric analysis revealed that $CD8^+$Lym comprised the majority of cells expressing IFN-γ in the early phase of anti-GBM GN (Figure 1i and j). These data indicate that the phenotype of glomerular-infiltrating $CD8^+$Lym is identical to that of ILC1s; moreover, $CD8^+$Lym are the major IFN-γ–producing cells in the early phase of anti-GBM GN.

Glomerular ILC1-related cytokines and chemokines expression in anti-GBM GN rats

We next examined whether the glomerular expression of ILC1-related genes was upregulated in anti-GBM GN rats. The qRT-PCR analysis revealed that, in anti-GBM GN, there were significant increases in the glomerular expression of ILC1-related cytokines (*IFN-γ* and *TNF-α*) and chemokines (*CXCL9*, *CXCL10*, and *CXCL11*) (Figure 2a and b, Supplementary Table S1G), as well as that of *interleukin* (*IL*)*-1β*, *inducible nitric oxide synthase* (*iNOS*), and *C-C motif chemokine ligand 2* (*CCL2*), all of which are associated with Mφ (Figure 2c, Supplementary Table S1G). Likewise, the ILC1-related transcription factor *T-bet*, and *IL-12p40*, which activate ILC1, were significantly upregulated (Figure 2a and d, Supplementary Table S1G). However, the expression of *IL-18* mRNA, which is associated with the activation of immune cells, including ILC1 and Th1 cells, was not upregulated in anti-GBM GN (data not shown).

PPARα activation attenuated anti-GBM GN

Because PPARα agonists regulate inflammatory cytokines and chemokines, including IFN-γ, CXCL9, CXCL10, and CXCL11, we examined the expression of PPARα in anti-GBM GN.[24–27] Nearly all glomerular-infiltrating $CD8^+$Lym expressed PPARα (Figure 3a and b, Supplementary Table S1H); both $CD8^+$Lym and Mφ equally expressed mRNA for *PPARα* and *enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase* (*Ehhadh*, a target gene of PPARα) (Figure 3c, Supplementary Table S1I). On induction of anti-GBM GN, the expression levels of *PPARα* and *Ehhadh* mRNAs were significantly downregulated (Figure 3d, Supplementary Table S1J). These results suggested that glomeruli disruption leads to downregulation of PPARα; moreover, PPARα may regulate the activation of $CD8^+$Lym and Mφ. We next examined the effects of PPARα on anti-GBM GN. In vehicle-treated control rats (control group), the levels of serum creatinine gradually increased in a time-dependent manner and significantly improved in anti-GBM GN rats treated with a PPARα agonist (PPARα group)

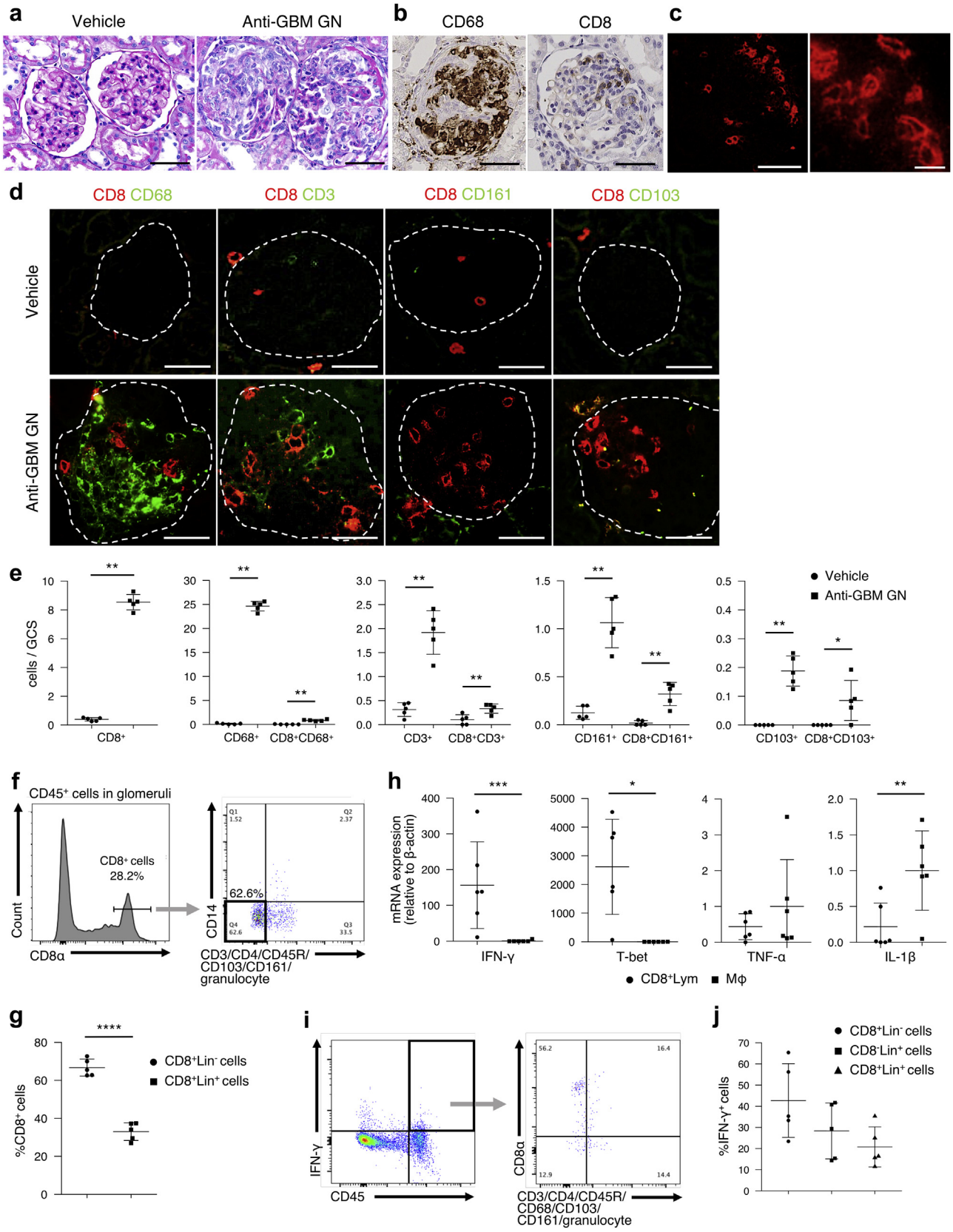

Figure 1 | The characteristics of CD8$^+$ non-T lymphocytes (CD8$^+$Lym) were consistent with those of group 1 innate lymphoid cells. (**a**) Representative light microscopic findings in vehicle and anti-glomerular basement membrane (anti-GBM) glomerulonephritis (GN) (periodic acid–Schiff stain). (**b**) Representative immunohistochemical staining for CD68 and CD8 in anti-GBM GN. (**c**) Representative immunofluorescence staining for CD8 (red) in anti-GBM GN. (**d**) Representative immunofluorescence staining for (continued)

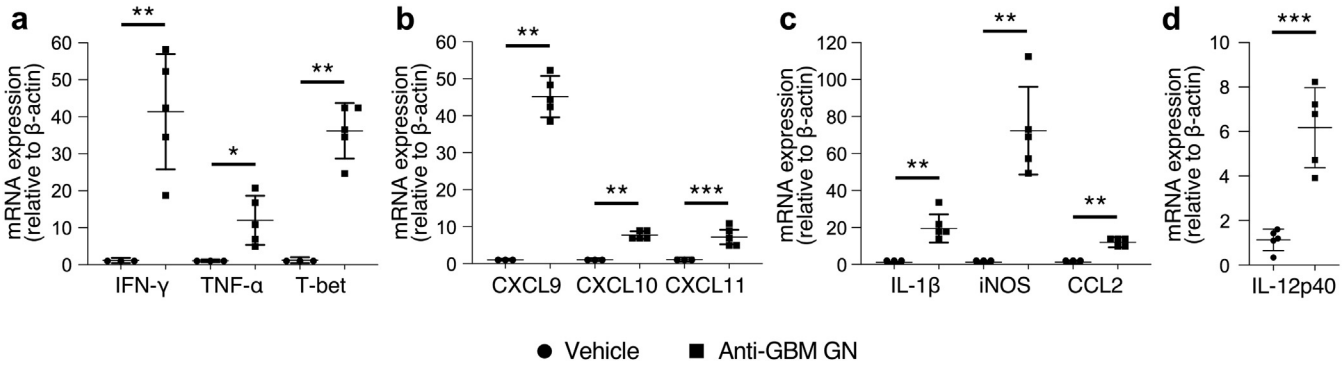

Figure 2 | Expression of group 1 innate lymphoid cell–related cytokine and chemokine mRNAs was significantly upregulated in the glomeruli of anti-glomerular basement membrane (anti-GBM) glomerulonephritis (GN) rats. Quantitative real-time polymerase chain reaction analysis of mRNAs for group 1 innate lymphoid cell–related cytokines and transcription factors, including *interferon-γ* (*IFN-γ*), *tumor necrosis factor-α* (*TNF-α*), and *T-box factor expressed in T cells* (*T-bet*) (**a**); group 1 innate lymphoid cell–related chemokines, including *CXC chemokine ligand 9* (*CXCL9*), *CXCL10*, and *CXCL11* (**b**); macrophage-related genes, including *interleukin-1 β* (*IL-1β*), *inducible nitric oxide synthase* (*iNOS*), and *C-C motif chemokine ligand 2* (*CCL2*) (**c**); and *IL-12p40* (**d**), in isolated glomeruli. Results are normalized to *β-actin* and are presented as relative expression compared with that of the vehicle group (set as 1). Values represent the mean ± SD of evaluations from each group ($n = 5$ per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$.

(Figure 3e, Supplementary Table S1K). The large amount of albuminuria detected in control group on day 7 showed a decreasing trend in the PPARα group (Figure 3f, Supplementary Table S1K). Similarly, necrotizing and crescentic glomeruli were significantly reduced in the PPARα group on day 7 (Figure 3g and h, Supplementary Table S1K). In the control group, the number of glomerular-infiltrating $CD68^+$ Mφ gradually increased from days 3 to 7 (Figure 3i and l, Supplementary Table S1K). In contrast, the number of glomerular-infiltrating $CD8^+$Lym markedly increased from days 5 to 7 (Figure 3j and m, Supplementary Table S1K). On days 3 and 5, very few $CD3^+$ T cells were detected in glomeruli (Figure 3k and n, Supplementary Table S1K). By day 7, the number of glomerular-infiltrating $CD3^+$ T cells had slightly increased. Administration of the PPARα agonist significantly reduced the glomerular infiltration of Mφ, $CD8^+$ Lym, and $CD3^+$ T cells on day 7 (Figure 3i–n, Supplementary Table S1K).

PPARα signaling suppressed glomerular expression of ILC1-related cytokines and chemokines *in vivo*

To elucidate the mechanism of PPARα agonist activity in anti-GBM GN, we next examined the glomerular cytokine and chemokine environment. The qRT-PCR analysis revealed that the glomerular mRNAs of ILC1-related cytokines and chemokines were significantly suppressed on days 5 and 7 in the PPARα group (Figure 4a, b, and d–f; Supplementary Table S1L); however, the PPARα agonist caused no reductions in either the number of glomerular-infiltrating $CD8^+$Lym or the expression of *T-bet* mRNA on day 5 (Figures 3f and h and 4c, Supplementary Table S1K and L). The expression of *IL-12p40* mRNA was not downregulated by administration of the PPARα agonist (Figure 4j, Supplementary Table S1L). These findings suggest that the PPARα agonist inhibited ILC1 accumulation into glomeruli and directly suppressed ILC1 activation. Treatment with the PPARα agonist also significantly decreased the expression of Mφ-related cytokine and chemokine mRNAs (Figure 4g–I, Supplementary Table S1L). In contrast, upregulation of the mRNAs for *intercellular adhesion molecule-1* (*ICAM-1*) and *vascular cell adhesion molecule-1* (*VCAM-1*), which are mainly expressed on glomerular endothelial cells (GEnCs), *Eomes*, and *T-cell receptor β*, which is mainly expressed on T cells and NK T cells, was not suppressed in the PPARα group (Figure 4k and l, Supplementary Figure S3A, and Supplementary Table S1L and M). Downregulation of the

Figure 1 | (continued) CD8 (red), CD68 (green), CD3 (green), CD161 (green), and CD103 (green) in vehicle and anti-GBM GN. Original magnification ×400 (**a, b, c [left], d**) and ×600 (**c [right]**). Bars = 50 μm (**a, b, c [left], d**) and 15 μm (**c [right]**). (**e**) Numbers of glomerular-infiltrating single- and dual-stained cells were counted in vehicle or anti-GBM GN groups. Cell infiltration was quantified by the mean numbers of single- and dual-stained cells per glomerulus in 30 glomerular cross sections (GCSs), respectively. (**f**) Representative flow cytometric analyses of glomeruli isolated from Wistar Kyoto rats on day 7 following the induction of anti-GBM GN. The left panel shows the expression of CD8α on $CD45^+$ glomerular-infiltrating leukocytes. $CD8\alpha^+$ lineage marker $(Lin)^-$ ($CD3^-CD4^-CD14^-CD45R^-CD103^-CD161^-$granulocyte$^-$) glomerular-infiltrating leukocytes are shown in the right panel (black outlined area). (**g**) Frequencies of $CD8^+Lin^-$ cells and $CD8^+Lin^+$ cells in glomerular-infiltrating $CD8^+$ cells. (**h**) Quantitative real-time polymerase chain reaction analysis of mRNA for *interferon-γ* (*IFN-γ*), *T-box factor expressed in T cells* (*T-bet*), *tumor necrosis factor-α* (*TNF-α*), and *interleukin-1 β* (*IL-1β*) in isolated glomerular-infiltrating $CD8^+$Lym and macrophages (Mφ), defined as $CD8^+Lin^-$ cells and $CD14^+$ cells, respectively. Results are normalized to *β-actin* and are presented as relative expression compared with that of Mφ (set as 1). (**i**) Representation of IFN-γ expression in isolated glomeruli obtained from anti-GBM GN rats. (**j**) Frequencies of $CD8^+Lin^-$ cells, $CD8^-Lin^+$ cells, and $CD8^+Lin^+$ cells in glomerular-infiltrating $CD45^+IFN\text{-}\gamma^+$ cells. Values represent the mean ± SD of evaluations from each group (**e, g, i**: $n = 5$ per group; **h**: $n = 6$ per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, and ****$P < 0.001$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

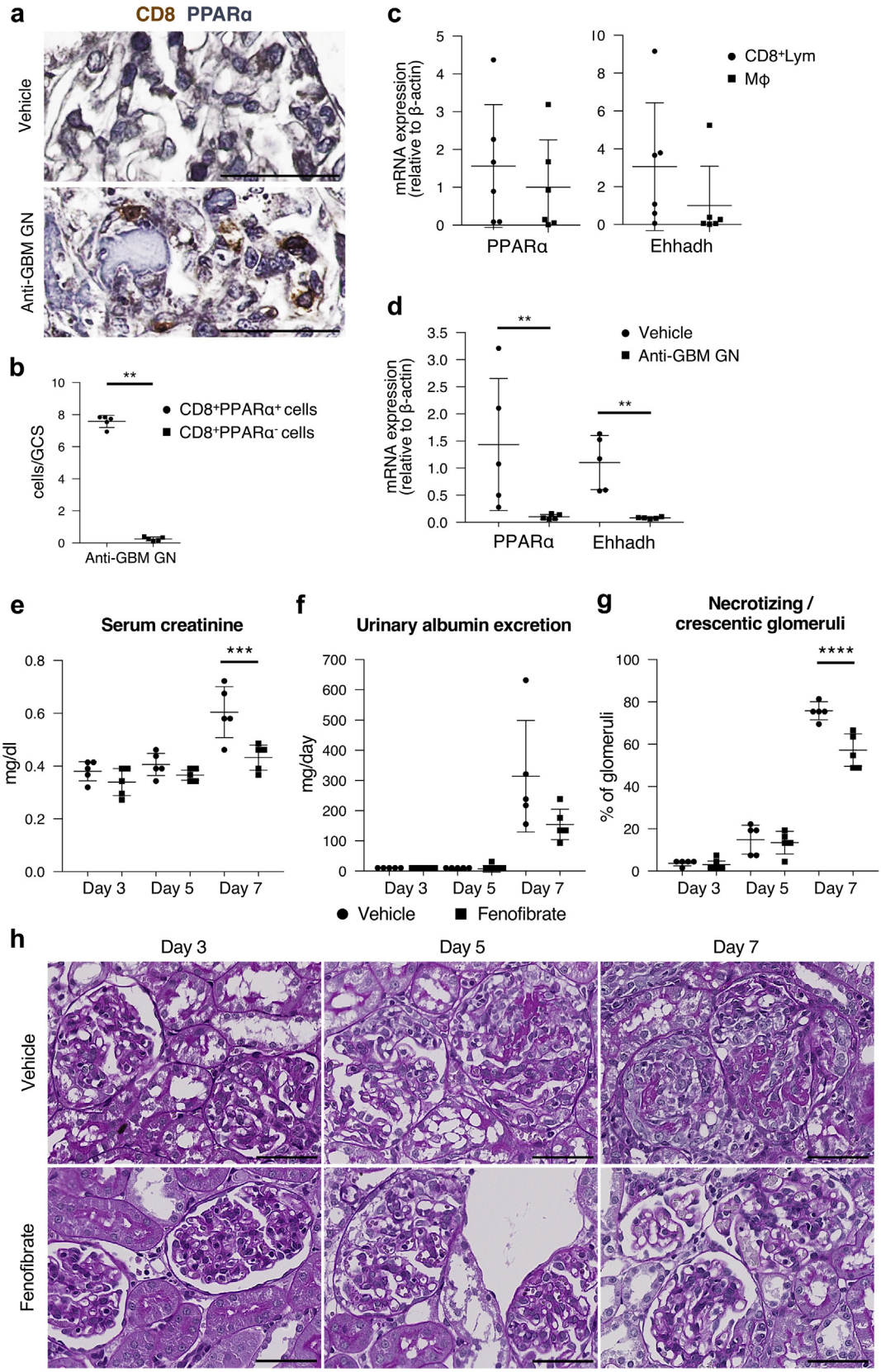

Figure 3 | Peroxisome proliferator-activated receptor-α (PPARα) activation attenuated glomerular injury in anti-glomerular basement membrane (anti-GBM) glomerulonephritis (GN) by inhibiting group 1 innate lymphoid cells and macrophage (Mφ) migration into glomeruli. (**a**) Representative immunohistochemical staining for CD8 and PPARα in vehicle and anti-GBM GN. (**b**) Numbers of glomerular-infiltrating $CD8^{+}PPAR\alpha^{+}$ non-T lymphocytes (Lym) and $CD8^{+}PPAR\alpha^{-}$Lym were evaluated in anti-GBM GN groups at 7 days (continued)

expression levels of *PPARα* and *Ehhadh* mRNA, induced in anti-GBM GN, were significantly inhibited by the PPARα agonist (Figure 4m and n, Supplementary Table S1L).

PPARα directly inhibited the activation of ILC1s and Mφ *in vitro*

We next examined the suppressive effect of PPARα on ILC1 activation *in vitro*. Sorted $CD8^+$ILC1s were stimulated with both IL-12 and IL-18 in the presence or absence of the PPARα agonist (Figure 5a–d). IFN-γ production from ILC1s was significantly enhanced on IL-12 and IL-18 stimulation. This enhancement of ILC1 activation was significantly reduced in the presence of the PPARα agonist (Figure 5c and d, Supplementary Table S1N). Similarly, we examined the direct effect of PPARα on Mφ. The qRT-PCR analyses revealed that the upregulation of *iNOS*, *CXCL9*, *CXCL10*, and *CXCL11* by IFN-γ stimulation was significantly suppressed by PPARα activation, as indicated by upregulation of *PPARα* and *Ehhadh* mRNA (Figure 5e, Supplementary Table S1O). These results suggested that PPARα directly inhibited the activation of not only ILC1s but also Mφ.

PPARα agonist inhibited the expression of ILC1-related chemokines in GEnCs and podocytes

We next explored the sources of ILC1-related chemokine production. GEnC and podocyte injuries are involved in the progression of anti-GBM GN, and various chemokines are produced from GEnCs and podocytes following glomerular injury.[32–36] In GEnCs, *CXCL9* mRNA, but not *CXCL10* or *CXCL11* mRNA, was significantly upregulated by stimulation of IFN-γ (Figure 6a, Supplementary Table S1P). In contrast, podocytes exhibited significantly increased mRNA expression of *CXCL9*, *CXCL10*, and *CXCL11* following stimulation of IFN-γ (Figure 6b, Supplementary Table S1Q). The upregulation of these chemokines was significantly inhibited in the presence of PPARα agonist in both GEnCs and podocytes (Figure 6a and b, Supplementary Table S1P and Q). However, in GEnCs, the upregulation of *ICAM-1* and *VCAM-1* induced by TNF-α was not suppressed by treatment with the PPARα agonist (Supplementary Figure S3B, Supplementary Table S1R), consistent with the findings from analyses of isolated glomeruli from anti-GBM GN rats (Supplementary Figure S3A, Supplementary Table S1M). These data indicate that PPARα signaling suppresses the production of ILC1-related chemokines from activated GEnCs, podocytes, and Mφ, thereby resulting in the inhibition of ILC1 migration.

ILC1s infiltrated into glomeruli in human anti-GBM GN

To confirm the involvement of ILC1s in the progression of human anti-GBM GN, we analyzed human renal biopsy specimens obtained from patients with anti-GBM GN and compared them with renal tissues from patients who underwent nephrectomy due to renal cell carcinoma (renal control tissues). Human ILC1s were defined as lineage (CD3, CD16, CD19, CD20, CD56, and CD68)-negative and -positive for CD127 and T-bet.[1–3] In human anti-GBM GN samples, approximately 90% of glomeruli showed crescent formation (Figure 7a). Although no ILC1s were detected in the renal control tissues, a considerable number of ILC1s were detected in glomeruli of patients with anti-GBM GN, especially in glomeruli without crescent formation (Figure 7b and c, Supplementary Table S1S). Indeed, the number of glomerular-infiltrating ILC1s was significantly greater in glomeruli without crescents than in those with crescents (Figure 7d, Supplementary Table S1T). These data indicate that ILC1s are involved in the progression of anti-GBM GN, especially in the early phase of inflammation. Moreover, PPARα was expressed in both Lin^-CD127^+ cells and $Lin^-T\text{-}bet^+$ cells (Supplementary Figure S4).

DISCUSSION

We report a novel progressive mechanism of anti-GBM GN, in which ILC1s are involved. From the analysis of phenotypic and functional characteristics, we demonstrated the infiltration of ILC1s in the development of anti-GBM GN in rats and humans. Moreover, PPARα agonist treatment attenuated glomerular injury in anti-GBM GN rats through regulation of ILC1s. These data demonstrate that ILC1s are involved in the progression of anti-GBM GN and could be considered a novel therapeutic target. Furthermore, a strategy targeting PPARα activation for the regulation of ILC1s might be a useful treatment for anti-GBM GN.

This study is the first, to our knowledge, to identify infiltrating $CD8^+$Lym in necrotizing and crescentic glomeruli as ILC1s and report the involvement of ILC1s in the development of anti-GBM GN. Our findings are supported by those of Roan *et al.*,[37] who reported that ILC1s contain $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ populations. Although some populations of NK cells are believed to express CD8, the ILC1s that we identified do not express CD161 (an NK cell marker in rats). Moreover, the expression of Eomes in ILC1s is significantly lower than that in conventional NK cells. Therefore, this population of ILC1s differs

Figure 3 | (continued) following the induction of anti-GBM GN. Cell infiltration was quantified by the mean number of these cells per glomerulus in 30 glomerular cross sections (GCSs). (**c**) Quantitative real-time polymerase chain reaction analysis of mRNA for *PPARα* and *enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase* (*Ehhadh*) in isolated glomerular-infiltrating $CD8^+$Lym and Mφ. Results are normalized to *β-actin* and are presented as relative expression levels compared with those of Mφ (set as 1). (**d**) Quantitative real-time polymerase chain reaction analysis of mRNAs for *PPARα* and *Ehhadh* in isolated glomeruli. Results are normalized to *β-actin* and are presented as relative expression levels compared with those of vehicle group (set as 1). (**e–g**) Serum creatinine (**e**) and urinary albumin excretion (**f**) amounts and frequency of necrotizing and crescentic glomeruli (**g**) in vehicle or fenofibrate treatment groups on days 3, 5, and 7 following the induction of anti-GBM GN. (**g**) The frequencies of necrotizing and crescentic glomeruli are expressed as the mean percentage of glomeruli with legions in 100 glomeruli in each kidney sample. (**h**) Representative light microscopic findings (periodic acid–Schiff stain). (Continued)

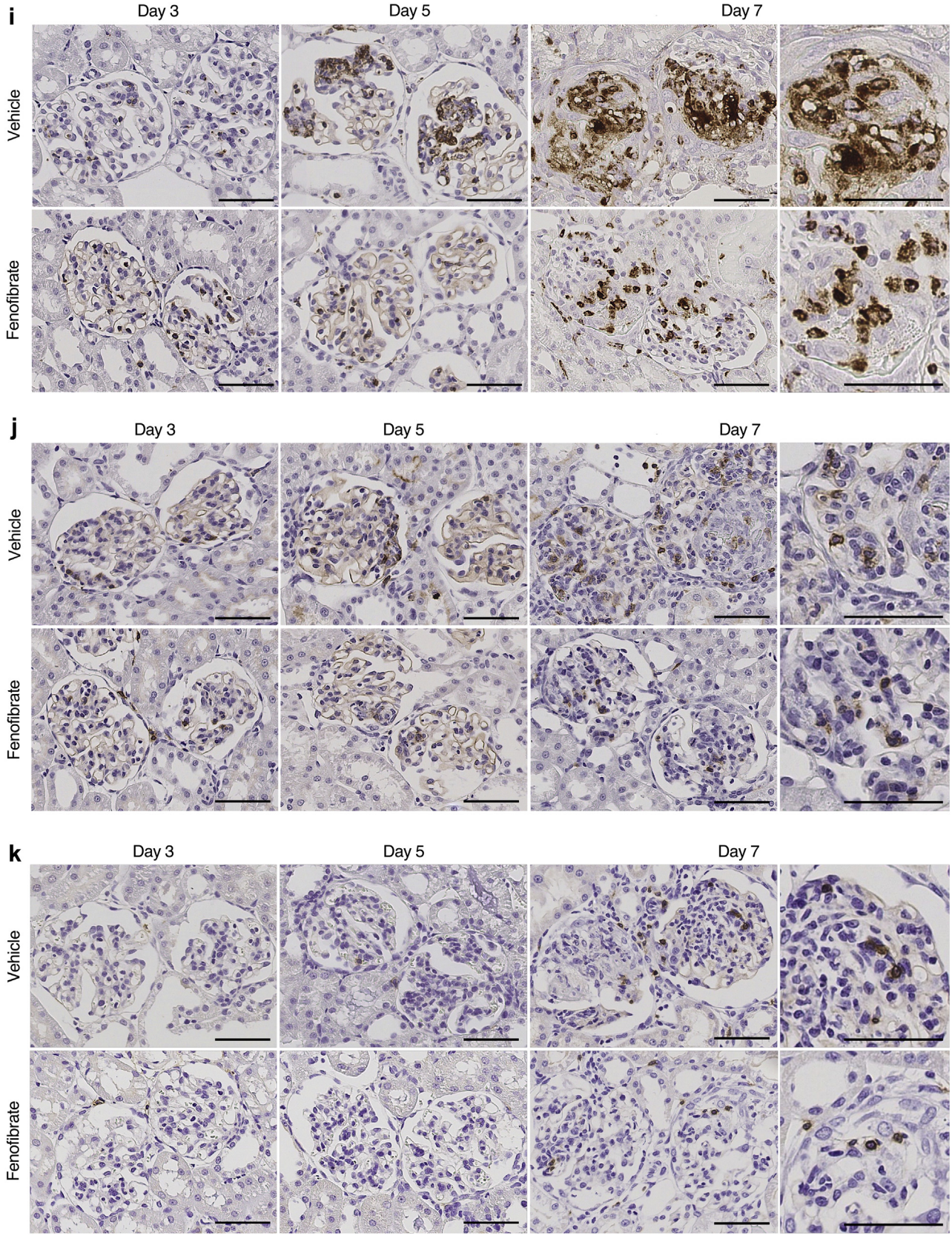

Figure 3 | (Continued) (**i–k**) Representative immunohistochemical staining for CD68 (**i**), CD8 (**j**), and CD3 (**k**) in vehicle or fenofibrate treatment groups on days 3, 5, and 7 following the induction of anti-GBM GN. (Continued)

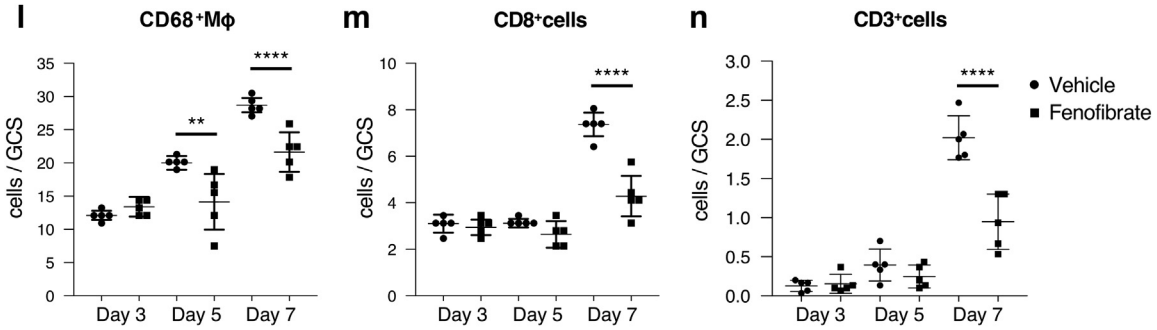

Figure 3 | (Continued) (**l–m**) Numbers of glomerular-infiltrating CD68$^+$Mφ (**l**), CD8$^+$Lym (**m**), and CD3$^+$ (**n**) cells were evaluated in vehicle or fenofibrate treatment groups on days 3, 5, and 7 following the induction of anti-GBM GN. Infiltration of CD68$^+$Mφ, CD8$^+$Lym, and CD3$^+$ cells was quantified by the mean numbers of CD68-positive, CD8-positive, and CD3-positive cells per glomerulus in 30 GCSs, respectively. Values represent the mean ± SD of evaluations from each group (**c**: $n = 6$ per group; **b, d–g, l–n**: $n = 5$ per group). $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$. Original magnification ×400 (**h–k**) and ×600 (**a, i–k [day 7 right]**). Bar = 50 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

from conventional NK cells. Wu *et al.*[38] reported the identification of a CD8αα$^+$ major histocompatibility complex class II$^+$ cell with both antigen-presenting functions and NK-like cytotoxicity. In their anti-GBM GN rat model, these cells infiltrated into glomeruli on days 35 to 40 after disease induction and suppressed inflammation by inducing apoptosis in effector CD4$^+$ T cells. Based on differences in the roles of inflammatory responses and the time phase of infiltration, those cells differ from the ILC1s identified herein. Indeed, in this study, more than 95% of glomerular-infiltrating CD8$^+$Lym did not express RT1B (data not shown). Van Kaer *et al.*[39] reported that innate CD8α$^+$ cells are involved in innate immunity against bacterial infection in both mouse and human intestinal epithelium. Such CD8$^+$ cells do not express either T cell receptor or CD127 in a major subset of ILC populations and produce IFN-γ. However, the same cells express CD103; thus, they differ from the ILC1s described in the current study. In our study, CD127 was not expressed in rat ILC1s (data not shown). However, several investigators have revealed the presence of a subset of ILC1s that do not express CD127 in the liver, salivary grand, or tonsils.[6,40] Further studies are required to clarify the phenotypic differences between rat and human ILCs.

Studies from recent years have shown that ILCs are associated with various inflammatory diseases.[5–8,37] Huang *et al.*[41] and Cao *et al.*[42] recently reported that ILC2s induced by IL-25 or IL-33 treatment attenuated renal ischemic/reperfusion injury by producing large amounts of Th2-related cytokines, such as IL-4 and IL-13, and by inducing alternatively activated Mφ. Furthermore, Riedel *et al.*[43] demonstrated that ILC populations reside in the healthy human kidney and ILC2s are a major ILC subtype in the kidneys of humans and mice. However, the association between ILC1s and kidney diseases has not been clarified. ILC1s play an essential role in the initiation and regulation of inflammation in the innate immune response. In our study, ILC1s infiltrated into glomeruli during early stages of disease in anti-GBM GN rats and appeared within glomeruli in patients with anti-GBM GN. Interestingly, the larger number of ILC1s that infiltrated into glomeruli without crescents supports a role for ILC1s in the early progression of anti-GBM GM. We also found that ILC1s are the main IFN-γ-producing cells in the early phase of anti-GBM GN. IFN-γ, a Th1 cytokine, plays an important role in Mφ activation.[44] Following the induction of anti-GBM GN, IFN-γ knockout mice developed less severe glomerular injury than wild-type mice by regulating Mφ activation.[45] Phoon *et al.*[46] reported that T-bet knockout anti-GBM GN mice showed attenuated renal injury and a reduction in the number of glomerular-infiltrating Mφ. Because T-bet is an essential transcription factor not only in Th1 cells, but also in ILC1s, the lack of ILC1s may result in the suppression of Mφ activation and attenuation of anti-GBM GN. Previous studies reported that Th1 and Th17 cells were involved in the progression of experimental anti-GBM GN.[16–19] However, in our study, few CD3$^+$ T cells were detected in the glomeruli in anti-GBM GN, despite the presence of many glomerular-infiltrating CD8$^+$ILC1s. This may have occurred because our experimental model shows only the early phase of the immune response (i.e., the innate immune response) in anti-GBM GN. Moreover, CD8$^+$Lym play an important role in the development of anti-GBM GN in rats.[11,12] Therefore, these results lead us to believe that ILC1s could serve as a novel therapeutic target in the early phase of anti-GBM GN.

PPARα agonists reduce proinflammatory cytokines, including TNF-α and IFN-γ, by regulating the transcription of their genes by repressing nuclear factor-κB and activator protein-1 signaling in inflammatory-induced disorders.[30,47–49] In this study, the PPARα agonist directly inhibited the activation of ILC1s induced by IL-12 and IL-18 (Figure 5a–d). Zhang *et al.*[29] demonstrated that PPARα signaling suppresses IFN-γ production from CD4$^+$, CD8$^+$ T, and NK T cells. However, such suppressive effects were not observed in NK cells. Thus, the CD8$^+$ILC1s described in the current study differ from NK cells, and we reveal a novel anti-inflammatory mechanism of PPARα via the regulation of ILC1s. Previous studies have shown the anti-inflammatory effects of PPARα on Mφ.[50,51] In this study, we have similarly shown that the administration of a PPARα agonist directly inhibited

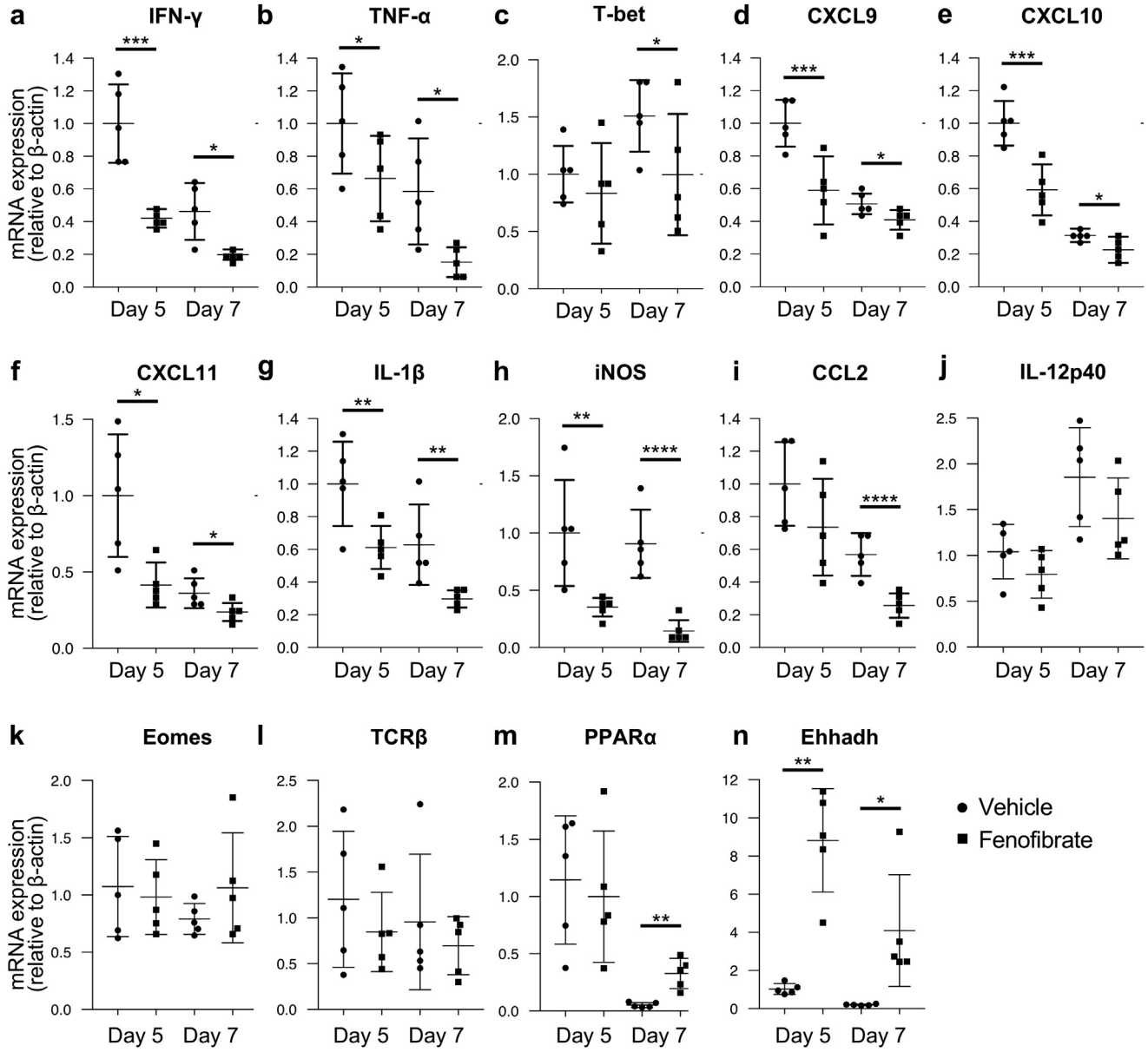

Figure 4 | Treatment with a peroxisome proliferator-activated receptor-α (PPARα) agonist suppressed the glomerular expression of group 1 innate lymphoid cell–related cytokines and chemokines *in vivo*. Quantitative real-time polymerase chain reaction analysis of mRNA for *interferon-γ* (*IFN-γ*) (**a**), *tumor necrosis factor-α* (*TNF-α*) (**b**), *T-box factor expressed in T cells* (*T-bet*) (**c**), *CXC chemokine ligand 9* (*CXCL9*) (**d**), *CXCL10* (**e**), *CXCL11* (**f**), *interleukin-1 β* (*IL-1β*) (**g**), *inducible nitric oxide synthase* (*iNOS*) (**h**), *C-C motif chemokine ligand 2* (*CCL2*) (**i**), *IL-12p40* (**j**), *eomesodermin* (*Eomes*) (**k**), *T-cell receptor β* (*TCRβ*) (**l**), *PPARα* (**m**), and *enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase* (*Ehhadh*) (**n**) in isolated glomeruli of vehicle- or fenofibrate-treated rats on days 5 and 7 following the induction of anti-glomerular basement membrane glomerulonephritis. Results are normalized to *β-actin* and are presented as the relative expression compared with that of the vehicle group on day 5 (set as 1). Values represent the mean ± SD of evaluations from each group ($n = 5$ per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, and ****$P < 0.001$.

Mφ activation by IFN-γ stimulation. These findings suggested that PPARα serves to inhibit the activation of innate immune cells, such as Mφ and ILCs, in inflammatory diseases.

Although treatment with fenofibrate significantly downregulated glomerular expression of inflammatory cytokine and chemokine mRNAs in this study, there was a delay between downregulation of these mRNAs and downregulation of *T-bet* mRNA. We demonstrated that $CD8^+$ILC1s composed the majority of IFN-γ-expressing cells in the early phase of anti-GBM GN. Fenofibrate directly inhibited cytokine and chemokine production from $CD8^+$ILC1s, Mφ, podocytes, and GEnCs. Thereafter, recruitment of $CD8^+$ILC1s was indirectly inhibited through the fenofibrate-induced suppression of ILC1-related chemokines. Although there were no significant statistical differences between the vehicle and fenofibrate groups in the number of glomerular-infiltrating $CD8^+$Lym or in the expression of *T-bet* mRNA on day 5, the mean values of these variables showed a slight

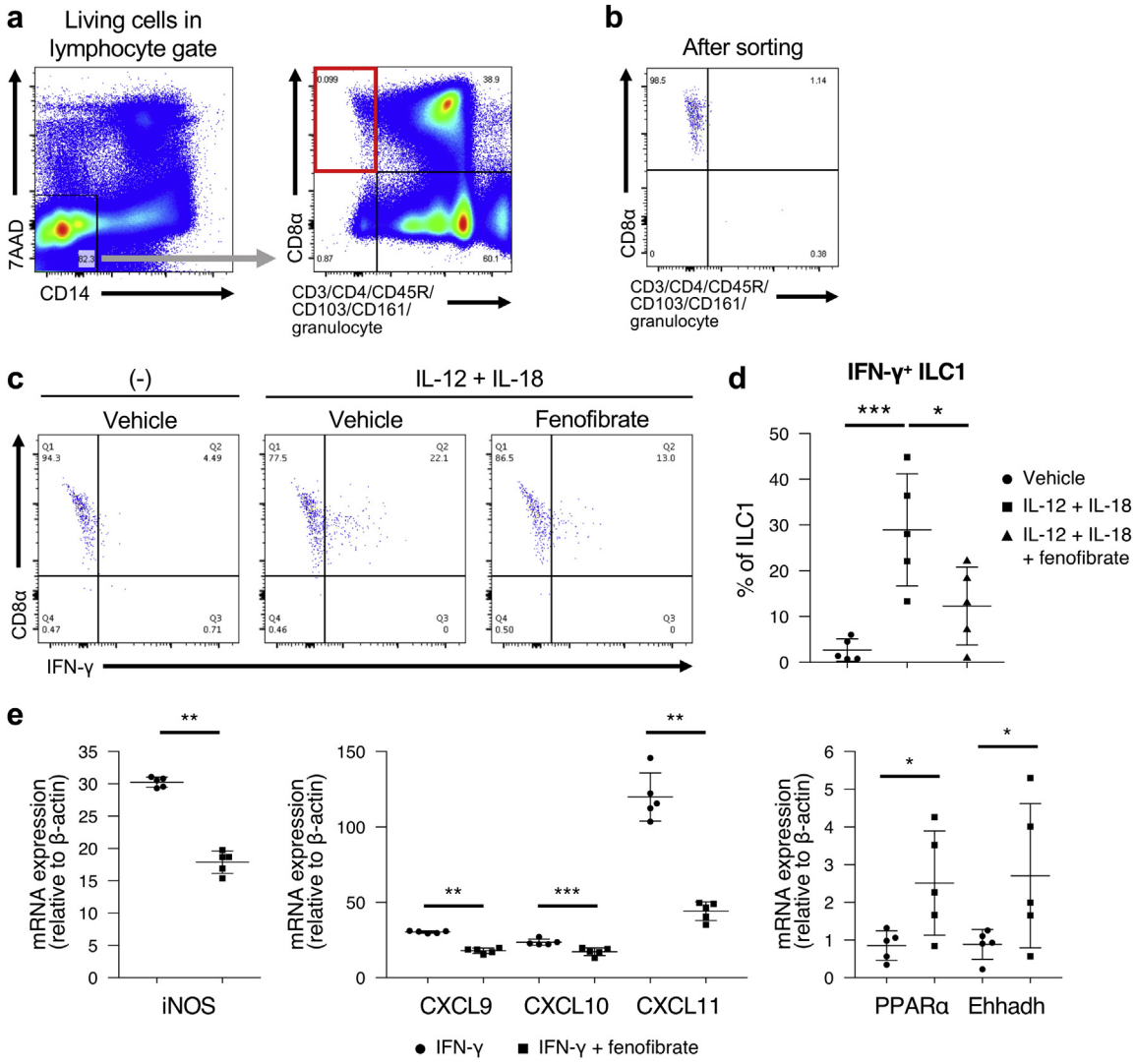

Figure 5 | Peroxisome proliferator-activated receptor-α (PPARα) directly inhibited the activation of group 1 innate lymphoid cells (ILC1s) and macrophages *in vitro*. (**a**) Representative flow cytometric analyses showing the gating strategy to sort living CD8α$^+$ lineage marker-negative (CD3$^-$CD4$^-$CD14$^-$CD45R$^-$CD103$^-$CD161$^-$granulocyte$^-$) ILC1s (red outlined area, right panel) from rat splenocytes. (**b**) Representative flow cytometric analyses of sorted CD8α$^+$ ILC1s. CD8α$^+$ ILC1 purity was more than 95%. (**c**) Interferon-γ (IFN-γ) production of highly purified spleen CD8α$^+$ ILC1s after stimulation for 16 hours with or without the combination of interleukin-12 (IL-12) and IL-18, as assessed by flow cytometry. Numbers in gates (outlined areas) and quadrants indicate the percentages of cells in each. (**d**) Frequencies of IFN-γ–positive ILC1s in the vehicle group and IL-12 and IL-18 stimulation groups with or without fenofibrate treatment. (**e**) Quantitative real-time polymerase chain reaction analysis of mRNAs for *inducible nitric oxide synthase* (*iNOS*), ILC1-related chemokines, *PPARα*, and *enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase* (*Ehhadh*) in cultured macrophages. Results are normalized to *β-actin* and are presented as relative expression levels compared with those of unstimulated macrophages (set as 1). Values represent the mean ± SD of evaluations from each group ($n = 5$ per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$. CXCL9, CXC chemokine ligand 9.

tendency toward reduction in the fenofibrate group. These results suggest that fenofibrate predominantly affects the expression of ILC1-related cytokines and chemokines rather than the recruitment of T-bet$^+$ cells (e.g., CD8$^+$ILC1s).

Several studies have reported a protective role for PPARα in podocytes through preservation of nephrin expression in glomerular injury models.[52,53] However, few reports are available regarding the anti-inflammatory roles of PPARα in GEnCs and podocytes. The production of chemokines from endothelial cells and podocytes leads to local recruitment of inflammatory cells, while ILC1s reportedly express CXC chemokine receptor 3, in a manner similar to that of T cells.[4,34,37,54–56] In this study, PPARα treatment inhibited ILC and T-cell migration into glomeruli via downregulation of ILC1-related chemokines in glomeruli, including GEnCs and podocytes. Further studies are needed to reveal the therapeutic effect of PPARα on lymphocytes, including ILC1s, in various inflammation-induced disorders.

Our study had several important limitations. First, although we evaluated the effects of PPARα on ILC1 and Mφ, no other leukocytes were evaluated. ILC1-related chemokines are indeed produced by Mφ, podocytes, and GEnCs; however,

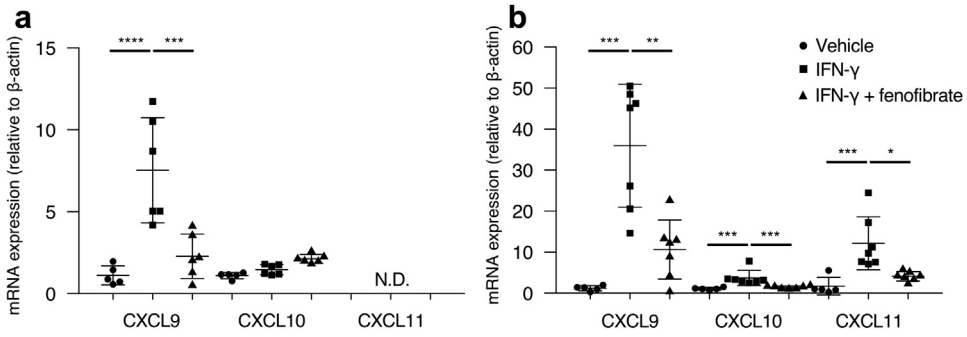

Figure 6 | Treatment with peroxisome proliferator-activated receptor-α agonist inhibited the expression of group 1 innate lymphoid cell–related chemokines in glomerular endothelial cells and podocytes. Quantitative real-time polymerase chain reaction analysis of mRNAs for group 1 innate lymphoid cell–related chemokines, including *CXC chemokine ligand 9* (*CXCL9*), *CXCL10*, and *CXCL11*, in rat glomerular endothelial cells (**a**) and mouse podocytes (**b**). Results are normalized to *β-actin* and are presented as relative expression compared with that of the vehicle group (set as 1). Values represent the mean ± SD of evaluations from each group (glomerular endothelial cells, $n = 5$ per vehicle group; $n = 6$ per group for other groups. Podocytes, $n = 5$ per vehicle group; $n = 7$ per group for other groups). *$P <$ 0.05, **$P < 0.01$, ***$P < 0.005$, and ****$P < 0.001$. IFN-γ, interferon-γ; N.D., not detected.

they are also produced by dendritic cells and neutrophils.[57,58] Several studies have reported that PPARα regulates the activation of T cells and dendritic cells.[59–61] Moreover, the activation of neutrophils is reportedly regulated by NF-κB signaling, which can be inhibited by PPARα.[30,47–49,62] Although ILC1s, Mφ, podocytes, and GEnCs were the focus of this study because of their relevance to the early progression of anti-GBM GN,[9–14,32,33] other leukocytes may be regulated by PPARα. Second, we could not evaluate IL-18 expression in anti-GBM GN. IL-18 is a member of the highly proinflammatory IL-1 family of cytokines; it is synthesized as a biologically inactive precursor protein, which is cleaved prior to secretion as a bioactive cytokine.[63] Therefore, the level of *IL-18* mRNA does not accurately reflect inflammatory status. Previous studies demonstrated that IL-12 and IL-18 were needed to activate ILC1s;[1–5] thus, IL-18 was administered to cultured ILC1s with IL-12 in this study. Finally, we examined only the preventive administration of a PPARα agonist. Further studies are therefore needed to elucidate the therapeutic effect of PPARα in advanced anti-GBM GN.

In conclusion, we determined the functional significance of ILC1s in the progression of anti-GBM GN and the therapeutic effects of PPARα on ILC1s. ILC1s are novel immune cells in the field of renal disease and thus the clarification of ILC1s and the progression of renal disease, including GN, may lead to the development of a novel specific therapy for these diseases.

METHODS

Full methods are available in the Supplementary Methods.

Rat models

We used a Wistar Kyoto rat model of anti-GBM GN, as previously published.[9,10] To investigate the $CD8^+$Lym profile, 5 rats were killed on day 7 following injection of the anti-GBM antibody. To evaluate the effects of PPARα, rats were treated with oral fenofibrate (300 mg/kg per body weight per day) (Wako, Osaka, Japan) or vehicle once daily beginning 1 day before injection of the anti-GBM antibody for killing.

Histopathology and immunohistochemistry

Kidney tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin for light microscopy examination. Tissue sections were stained with hematoxylin and eosin and periodic acid–Schiff for histopathological examination. For immunohistochemistry, paraffin-embedded tissue sections were stained using Histofine Simple Stain Rat MAX PO (MULTI; Nichirei, Tokyo, Japan) following the protocol of the standard avidin-biotin-peroxidase complex technique. For immunofluorescence staining in rats, 4-μm frozen sections were stained by the standard indirect and direct technique. In human kidney biopsy specimens, immunofluorescence multiplex staining was performed with the PerkinElmer Opal Kit (Perkin-Elmer, Waltham, MA) as previously described.[64] Antibody details can be found in the Supplementary Methods.

Quantification of serum creatinine and albuminuria

Serum creatinine was measured using The DetectX Serum Creatinine kits from Arbor Assays (Ann Arbor, MI). Albuminuria was determined by a Rat Albumin ELISA Kit from Bethyl Laboratories (Montgomery, TX).

Isolation of rat glomeruli

Glomeruli were isolated by a differential sieving method from harvested kidneys as described previously.[65] Under light microscopy, tubular contamination was <5%.

Cell preparation

Disrupted spleens and isolated glomeruli were digested for 30 minutes at 37 °C in RPMI 1640 medium (Wako) supplemented with collagenase D (1 mg/ml; Roche Life Science, Mannheim, Germany) and DNase I (100 μg/ml; Sigma-Aldrich, St. Louis, MO). Next, the digested cell suspension was passed through a 40-μm cell strainer. The panning method was performed to enrich $CD8^+$Lym from splenocytes according to Wysocki and Sato.[66]

Flow cytometry and cell sorting

For cell surface labeling, cell suspensions were stained with fluorochrome-bound antibodies for 30 minutes at 4 °C. For

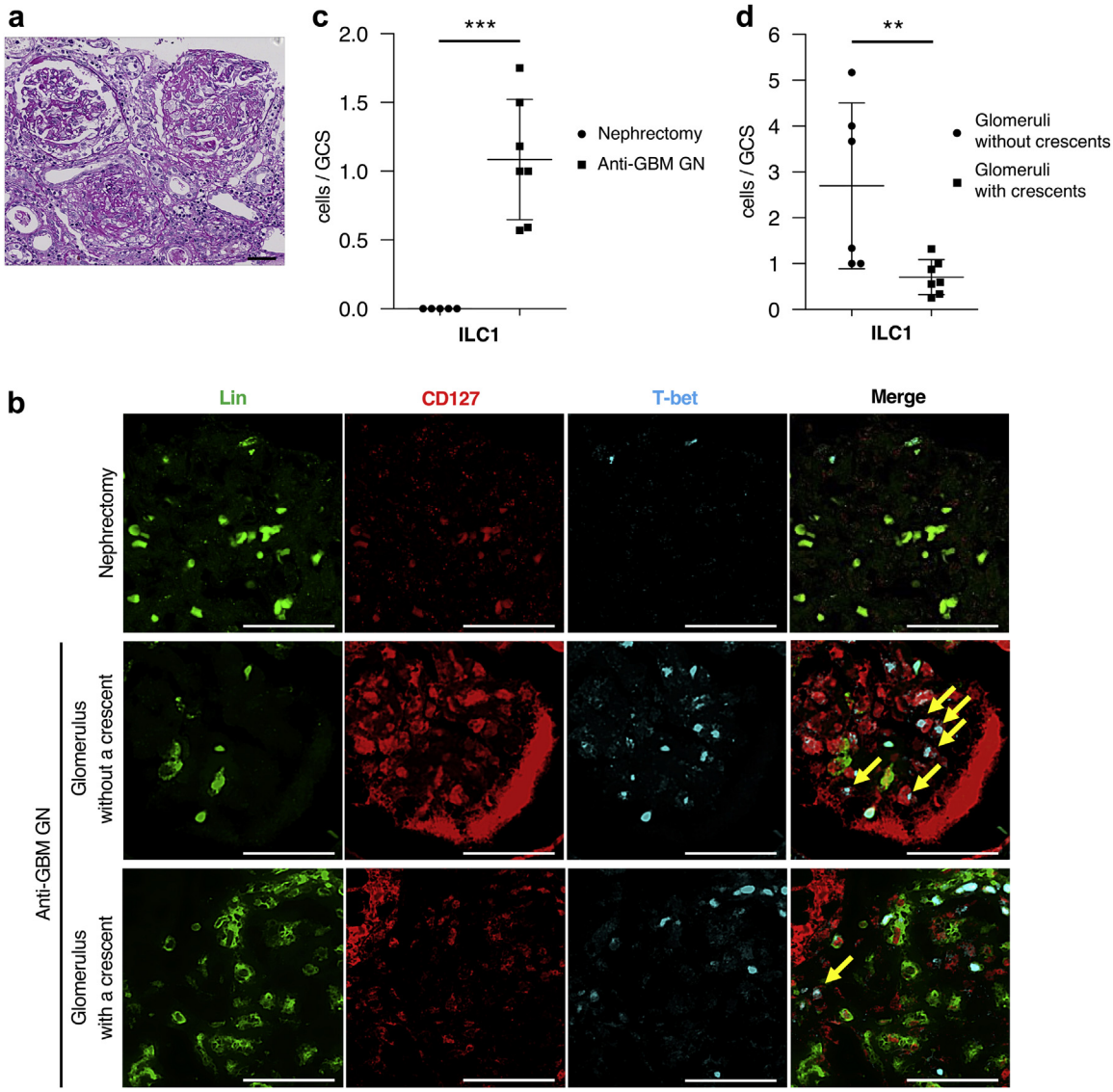

Figure 7 | Group 1 innate lymphoid cells (ILC1s) infiltrated into glomeruli in human anti-glomerular basement membrane (anti-GBM) glomerulonephritis (GN). (**a**) Representative light microscopic findings in kidney biopsy specimens of patients with anti-GBM GN (periodic acid–Schiff stain). (**b**) Representative immunofluorescence staining for lineage marker (Lin) (CD3, CD16, CD19, CD20, CD56, and CD68; green), CD127 (red), and T-box factor expressed in T cells (T-bet) (cyan) in unaffected renal tissue from patients undergoing total nephrectomy due to renal cell carcinoma (top), glomeruli without a crescent (middle), and with (bottom) a crescent in human anti-GBM GN. Arrows indicate ILC1s ($Lin^{-}CD127^{+}T\text{-}bet^{+}$ cells). Original magnification ×100 (**a**) and ×400 (**b**). Bar = 50 μm. (**c**) Number of glomerular-infiltrating ILC1s in unaffected renal tissue and human anti-GBM GN. (**d**) Number of glomerular-infiltrating ILC1s in glomeruli with or without crescents in human anti-GBM GN. One case had no glomeruli without crescents. Values represent the mean ± SD of evaluations from each group ($n = 5$ per nephrectomy group, $n = 7$ per anti-GBM group, $n = 6$ per glomeruli-without-crescents group, and $n = 7$ per glomeruli-with-crescents group). $^{**}P < 0.01$ and $^{***}P < 0.005$. GCS, glomerular cross section. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

intracellular cytokine analysis, monensin (35 mg/kg per body weight), obtained from Sigma-Aldrich, was administered i.p. 6 hours before killing. Intracellular staining was performed using an Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Data were collected by FACSCanto II (BD Biosciences, San Jose, CA), and cells were sorted by FACSAria II (BD Biosciences). Data were analyzed by FlowJo software 10.4.2 (BD Biosciences). Antibody details can be found in the Supplementary Methods.

qRT-PCR analyses

Total RNA was extracted by ISOGEN II (Nippon Gene, Tokyo, Japan) and transcribed into cDNA by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A cDNA copy of sorted cells was created with the CellAmp Whole Transcriptome Amplification Kit (Takara Bio, Siga, Japan) according to the manufacturer's instructions. qRT-PCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) using a QuantStudio 5 (Applied Biosystems). The primer sequences used for qRT-PCR are shown in Supplementary Table S2.

Cell culture experiments

Cyclin-dependent kinase 4-transformed mouse podocytes were provided by Dr. Jeffrey B. Kopp (University College London, UK) and generated according to the protocol of Sakairi *et al.*[67] Podocytes were incubated with recombinant mouse IFN-γ (10 ng/ml) from Peprotech (Rocky Hill, NJ). Rat primary kidney GEnCs were purchased from Cell Biologics (Chicago, IL). GEnCs were incubated with recombinant rat IFN-γ (10 ng/ml) or TNF-α (20 ng/ml) from BioLegend (San Diego, CA). Purified $CD8^+$Lym were stimulated with combinations of recombinant rat IL-12 (50 ng/ml) and IL-18 (50 ng/ml) from Genscript (Piscataway, NJ). Rat Mφ were induced from bone marrow cells collected from femur and tibia of normal Wistar Kyoto rats in accordance with the protocol of Avni *et al.*[68] Mφ were incubated with recombinant rat IFN-γ (10 ng/ml). Cells were incubated for 24 hours in the absence or presence of 10 μmol/l fenofibrate.

Human material

Human kidney biopsy specimens were obtained from 7 patients who underwent a kidney biopsy and were diagnosed with anti-GBM GN by a pathologist in our hospital. The unaffected tissues of kidneys from 5 patients who underwent total nephrectomy due to renal cell carcinoma were used as control renal tissues. The study was performed in accordance with the Declaration of Helsinki and was approved by the ethical committee of our hospital (30-07965).

Statistical analyses

Continuous variables are expressed as the mean ± SD. The normality of the quantitative data was checked by the Kolmogorov-Smirnov test. For normally distributed variables, Student's *t*-test was used to compare variables between 2 groups; 1-way analysis of variance with Dunnett's *post hoc* test was used to compare variables among 3 or more groups. For nonparametric variables, the Mann-Whitney *U* test was used to compare variables between 2 groups; the Kruskal-Wallis test with Dunn's *post hoc* test was used to compare variables among 3 or more groups. All statistical analyses were performed using the SPSS version 25.0 statistical software package (IBM Corp., Armonk, NY).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods.

Figure S1. (**A**) Representative flow cytometric analyses of glomeruli isolated from Wistar Kyoto rats on day 7 following the induction of anti-glomerular basement membrane glomerulonephritis. Right panel shows the expression of CD8α on $CD45^+$ lineage marker $(Lin)^-$ ($CD3^-CD4^-CD14^-CD45R^-CD103^-CD161^-$granulocyte$^-$) glomerular-infiltrating leukocytes (red outlined area, left panel). Macrophages (Mφ) were defined as $CD14^+$ leukocytes (black outline area, left panel). (**B**) Frequency of $CD8^+Lin^-$ cells and $CD8^-Lin^-$ cells in glomerular-infiltrating Lin^- cells. Lym, non-T lymphocytes.

Figure S2. The phenotype of $CD8^+$ non-T lymphocytes ($CD8^+$Lym) differs distinctly from that of natural killer (NK) cells. (**A**) Representative flow cytometric analyses showing the gating strategy to sort out living $CD161^+$ lineage marker $(Lin)^-$ ($CD3^-CD4^-CD14^-CD45R^-CD103^-$granulocyte$^-$) NK cells (black lined area, right panel) from rat splenocytes. (**B**) Quantitative real-time polymerase chain reaction analysis of mRNAs for *eomesodermin* (*Eomes*) and *T-box factor expressed in T cells* (*T-bet*) in sorted glomerular-infiltrating $CD8^+$Lym and spleen NK cells. Results are normalized to *β-actin* and are presented as relative expression compared with that of $CD8^+$Lym (set as 1). Values represent the mean ± SD of evaluations from $CD8^+$Lym and NK cells ($n = 6$ per group). $^*P < 0.05$.

Figure S3. Peroxisome proliferator-activated receptor-α was unable to suppress the activation of glomerular endothelial cells (GEnCs) both *in vitro* and *in vivo*. Quantitative real-time polymerase chain reaction analysis of mRNAs for *intercellular adhesion molecule-1* (*ICAM-1*) and *vascular cell adhesion molecule-1* (*VCAM-1*) in (**A**) isolated glomeruli and (**B**) cultured rat GEnCs. Results are normalized to *β-actin* and are presented as relative expression compared with that of the vehicle group on day 5 and unstimulated GEnCs (set as 1), respectively. Values represent the mean ± SD of evaluations from each group (isolated glomeruli, $n = 5$ per group; GEnCs, $n = 5$ per group). TNF-α, tumor necrosis factor-α.

Figure S4. Representative immunofluorescence staining for lineage marker (Lin) (CD3, CD16, CD19, CD20, CD56, and CD68; green), peroxisome proliferator-activated receptor-α (PPARα) (cyan), and CD127 (**A**) or T-box factor expressed in T cells (T-bet) (**B**) (red) in human anti-glomerular basement membrane glomerulonephritis. Original magnification ×400. Arrows indicate $Lin^-CD127^+PPARα^+$ cells (**A**) and Lin^-T-$bet^+PPARα^+$ cells (**B**). Bar = 50 μm.

Table S1. The numerical values corresponding to each figure.

Table S2. Quantitative real-time polymerase chain reaction primer sequences.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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