Voltage-gated potassium channel Kv1.3 blocker as a potential treatment for rat anti-glomerular basement membrane glomerulonephritis

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The potassium (K⁺) channel is encoded by an extended superfamily of 76 genes and exhibits the largest diversity among all known ion channels in humans (25). The K⁺ channel hyperpolarizes the membrane potential and modulates excitability appropriate for the cell’s function. Multiple K⁺ channel subtypes are usually expressed together on the cell surface membrane, and the expression pattern of K⁺ channels gives each cell its unique function. Therefore, a particular cell’s function could be controlled if the cell-type-specific K⁺ channel were to be selectively activated or blocked (8, 22). K⁺ channels are expected to be key drug targets for the treatment of a variety of diseases (60). Examples include J) a Kv1.5 blocker for atrial fibrillation (14), 2) a KCNQ2/3 activator for epilepsy and use as an analgesic agent (24), and 3) a KCNQ1 (Kv2.1) blocker for diabetes (62). Recently, researchers (8, 23) have observed distinct patterns in the expression of the voltage-gated K⁺ channel Kv1.3 and the calcium-activated K⁺ channel KCa3.1 that depend on the state of T-cell activation and differentiation.

Naïve T cells (TN) are mature T cells that have not yet encountered an antigen. Following an encounter with an antigen, the T cells divide and differentiate. Most of their progeny become short-lived effector cells, while some become long-lived memory cells. Effector memory T cells (TEM) are one type of memory cell. This cell type can move directly to the sites of inflammation and exert effector functions. Central memory T cells (TCM) are another type of memory cell that migrates to the lymph node before moving to the site of inflammation, requires longer to differentiate into effector cells, and does not secrete many cytokines (41, 48). In rats, the subsets of memory T cells are divided by the expression pattern of the lymph node homing receptors CD62L (L-selectin) and CCR7 and the leucocyte common antigen CD45RC as follows: TN, CD45RC⁻CCR7⁻CD62Llow; TEM, CD45RC⁻CCR7⁻CD62Llow; and TCM, CD45RC⁻CCR7⁻CD62Lhigh. TEM express significantly higher levels of Kv1.3 channels and lower levels of KCa3.1 channels than TN and TCM (22, 59). Therefore, Kv1.3 blockers affect TEM selectively by depolarizing their membrane potential, thereby attenuating the Ca²⁺ signaling pathway necessary for T-cell activation (8).

Many studies have reported an association between Kv1.3-expressing TEM and autoimmune disease. Disease-associated autoreactive T cells from the blood of patients with multiple sclerosis, rheumatoid arthritis, or type 1 diabetes display a TEM phenotype characterized by Kv1.3high in the blood, whereas T cells specific for disease-irrelevant antigens from the same patient populations or T cells specific for auto-antigens in...
control populations are CCR7<sup>−/−</sup>Kv1.3<sup>low</sup> T<sub>N</sub> or T<sub>CM</sub> (10, 43, 59). In rat models of experimental autoimmune encephalomyelitis and allergic contact dermatitis, the phenotype of T cells at the sites of inflammation is CCR7<sup>−</sup>CD45RC<sup>−</sup>Kv1.3<sup>high</sup> TEM (7, 55). Furthermore, the administration of a Kv1.3 blocker selectively suppresses the proliferation of TEM without persistently suppressing T<sub>N</sub> and T<sub>CM</sub> (8, 9, 55).

Although the expression of Kv1.3 channels has not yet been investigated in progressive renal diseases, several authors have speculated that TEM could be related to the progression of investigated in progressive renal diseases, several authors have association of Kv1.3-expressing TEM with anti-GBM in rats and functions (30). In the work reported here, we investigated the injury through macrophage recruitment and activation akin to glomeruli and by a high rate of crescent formation in the accumulation of T cells and monocytes/macrophages in the glomeruli (51). T cells have been found to induce glomerular injury through macrophage recruitment and activation akin to delayed-type hypersensitivity as well as through their effector functions (30). In the work reported here, we investigated the association of Kv1.3-expressing TEM with anti-GBM in rats and subsequently investigated whether a Kv1.3 blocker, Psora-4, could prevent renal damage.

MATERIALS AND METHODS

Study design. Five- to six-week-old male Wistar-Kyoto rats obtained from Charles River (Atsugi, Japan) were used. Animal care was in accordance with the National Defense Medical College Guidelines for the Care and Use of Laboratory Animals in Research. The study protocols were approved by the Animal Ethical Committee of the National Defense Medical College. Anti-GBM GN was induced by the injection of rabbit anti-rat GBM antibody (19, 20) at a dose of 25.0 μl/100 g body wt on day 0. Psora-4 (5-[4-phenylbutoxy] psoralen) is a potent small-molecule Kv1.3 blocker that blocks Kv1.3 channels in a use-dependent manner; its Hill coefficient is 2 and its EC<sub>50</sub> is 3 nM, a potent small-molecule Kv1.3 blocker that blocks Kv1.3 channels in all closely related Kv1-family channels (Kv1.1, Kv1.2, Kv1.4, and Kv1.7) other than Kv1.5 (EC<sub>50</sub> = 7.7 nM; Ref. 55). Psora-4 was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in a mixture of 17% CremophorEL and 75% PBS to prepare a concentration of 9 mg/ml.

To investigate differences between the results of early and delayed treatments with Psora-4 (n = 6 at each time point), rats were injected with a 9 mg/ml dose of Psora-4 from day 0 to day 21 in the early treatment group and from day 7 to day 21 in the delayed treatment group. The rats received four injections during the first 24 h, three injections during the second 24 h, and two injections from then onward (0.3 ml per dose of vehicle or Psora-4 at a concentration of 9 mg/ml ip). In the vehicle group, the intraperitoneal injection of only the vehicle (without Psora-4) was started on day 0 or day 7 after the injection of the anti-GBM serum. The animals were housed in metabolic cages to collect 24-h urine samples on days 0, 3, 5, 7, 10, 14, 17, and 21; during these 24-h periods, the rats had free access to standard chow and water. Body weight was measured at the end of each 24-h urine collection. Rats were killed on days 0, 3, 7, 14, and 21. Rats injected with normal rabbit IgG were used as an untreated normal control group. Blood was collected from the abdominal aorta, and the serum levels of creatinine were measured using an enzymatic method (SRL, Tokyo, Japan). The kidneys were removed for histological examination. Urinary creatinine was also measured using an enzymatic method (SRL), and the creatinine clearance was calculated.

Histological examination and immunostaining. The kidneys were fixed in 10% formalin and embedded in paraffin. All formalin-fixed kidney sections (3 μm) were stained with periodic acid-methenamine-silver. The percentage of glomeruli with crescent formation was counted by examining 50 consecutive glomeruli stained with periodic acid-methenamine-silver. Sections of the kidney were stained with primary antibodies (see Table 1) using standard indirect immunoperoxidase staining techniques. More than 50 glomeruli from each section were examined under a high-power field (×400), and the number of stained cells was counted. The number of interstitial infiltrating cells in 10 high-power fields was also counted.

Mononuclear cell preparation for flow cytometry analysis and immunohistochemistry. The rats (each group; n = 5) in the normal kidney group and the vehicle group were killed on day 7, and the kidneys were removed. The kidney mononuclear cells were prepared as described previously (3, 37). Peripheral blood mononuclear cells were obtained using the standard method with PANCOLL (PAN Biotech). For three-color flow cytometry, cells from each sample were stained for 30 min at 4°C with the optimal dilution of the antibodies (see Table 1). The labeled cells were analyzed with a flow cytometry analyzer (EPICS XL; Beckman Coulter), and the obtained data were analyzed with Expo. 32 software (Beckman Coulter). Mononuclear cells isolated from the kidney (vehicle group killed on day 7) were also used for immunohistochemistry. The isolated cells were deposited onto microscope slides by centrifugal force (50 g for 5 min) using a Cytospin 4 (Thermo Fisher Scientific), fixed for 10 min with acetone at −20°C before staining, and stained with anti-Kv1.3 mAb as a primary antibody (see Table 1). After being washed in PBS, the cells were incubated with Alexa-594-conjugated anti-mouse IgG antibody (Invitrogen) as the secondary antibody. After blocking with 10% normal mouse serum, the sections were stained with Alexa-488-

Table 1. Antibodies used in this study

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Labeling</th>
<th>Origin</th>
<th>Pretreatment for HIC</th>
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<td>PE-Cy5</td>
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<td>PE-Cy5, PE</td>
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HIC, immunohistochemistry; proteinase K, incubated with proteinase K (Dako) for 8 min; autoclave, boiled in citrated buffer (pH 6) via autoclave (121°C) for 15 min; PE, phycoerythrin. *BD bioscience; *Biolegend; *Serotec; *Alomone Lab; *EPTMICS; *NeuroMab.

Downloaded from http://ajprenal.physiology.org/ by 10.220.33.2 on October 21, 2017
 conjugated anti-rat CD3 mAb or Alexa-488-conjugated anti-ED-1 mAb, followed by incubation with Hoechst 33342 (Sigma) for nuclear counterstaining. The slides were analyzed using confocal microscopy (Zeiss LSM 510).

Magnetic cell sorting. Kidney and peripheral blood cell suspensions were prepared using the same procedure as that used for the flow cytometry analysis. To obtain the CD8+ αβγδTCR+ cell fraction (corresponding to the CD4+ T cells), the cell suspensions were first labeled with CD8 mAb and then depleted using anti-mouse IgG magnetic beads (Dynal Biotech). The depleted fractions were finally isolated αβγδ TCR+ T cells by positive selection using pan-T-cell MACS beads (Miltenyi Biotec). The CD8+ αβγδTCR+ cell fraction (corresponded to CD8+ T cells) was obtained using the same procedure as that used for the CD8+ αβγδTCR+ cells. For the ED-1+ cell fractions, the cell suspensions were first labeled using anti-ED-1 mAb and then positively selected using anti-mouse IgG magnetic beads.

Quantitative reverse transcriptase-PCR. Total RNA was extracted from the renal cortex and magnetically isolated cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). A 5-μg aliquot of total RNA was reverse transcribed with SuperScript reverse transcriptase (Invitrogen). The resulting complementary DNA (cDNA) was then used as a template for real-time quantitative PCR with the TaqMan Gene Expression Assays primer/probe sets for rat IL1-β (Rn00580432_m1), IL-17A (Rn01757168_m1), INF-γ (Rn00594078_m1), TNF-α (Rn09999017_m1), and GAPDH (Rn099999916_s1); TaqMan Mastermix (Applied Biosystems, Foster City, CA) was also used. Real-time PCR was performed using an ABI Prism 7900 sequence detection system (Applied Biosystems). The relative amount of mRNA was calculated using the comparative Ct (ΔΔCt) method. All specific amplification products were normalized against GAPDH mRNA, which was amplified in the same reaction as an internal control.

Statistical analysis. The results are expressed as means ± SD. The data were statistically analyzed using an ANOVA followed by the Fishers correlation test. A P value <0.05 was considered significant.

RESULTS

T cells infiltrating the kidney have an effector memory T-cell phenotype. To identify the phenotype of T cells that had infiltrated the kidney, we performed a flow cytometric analysis of mononuclear cell suspensions from normal and anti-GBM GN kidneys obtained on day 7 (Fig. 1). By the analysis with CD45RC and CD4/CD8α, we found five major distinct populations in the isolated kidney cell suspensions: R1 to R5. In the anti-GBM kidney, the proportion of CD4bright cells (gate R1) and CD4dimcells (R2), and CD8αbrightCD45RC−cells (R3) was increased compared with that in the normal rat kidney. The majority of CD4bright cells (R1) was positive for the T-cell markers CD3 and αβTCR and negative for CD45RC and CD62L, and thus corresponded to TEM. The CD8αbright cells, on the other hand, were divided into CD45RC+ and CD45RC−populations. The CD8αbrightCD45RC−cells (R3) were positive for CD3 and negative for CD62L and thus corresponded to TEM. The CD8αbrightCD45RC+ cells (R4) were positive for CD3 and negative for CD62L and did not correspond to any known phenotype of memory T cells. Interestingly, the majority of the CD8αbrightCD45RC+ (R4) cells was comprised of not γδTCR+, but rather γδTCR+ T cells, unlike the proportions in other T-cell subsets. Most cells in the R2 and R5 subsets were not T cells, since they lacked CD3, αβTCR, or γδTCR.

We found differences in minor T-cell distribution population (CD4brightCD45RC+ cells; Fig. 1, dotted-line circle) between normal and anti-GBM GN kidneys. CD4brightCD45RC+ cells were rarely found in anti-GBM GN rat kidney. A larger proportion of CD4brightCD45RC+ cells was observed in the normal rat kidney (2.9%) compared with anti-GBM GN kidney (0.3%). These CD4brightCD45RC+ cells were positive for the T-cell markers CD3 and αβTCR and negative for CD62L (data not shown). We also found the following differences in T-cell distribution between normal and anti-GBM GN kidneys. The
Expression of the voltage-gated potassium channel Kv1.3 on the proximal tubules of untreated normal rat kidney. In normal rat kidney (without anti-GBM antibody), Kv1.3 was stained in most of the tubular epithelial cells, which contained varying amounts of weakly stained granular material in the cytoplasm. Kv1.3 staining was prominent in the cortex, rather than in the medulla, and was especially prominent on tubules with a brush border, which were suspected of being proximal tubules (Fig. 2A). Kv1.3+ staining was not found in the glomeruli (Fig. 2B), interstitium, or around the vessel walls in untreated normal kidneys.

Expression of the Kv1.3 channel on the glomeruli and interstitium in rats with anti-GBM GN. In the anti-GBM kidney on days 3 and 7 (anti-GBM antibody was injected on day 0), many Kv1.3+ cells appeared in the glomeruli, interstitium, and in and around the vessel walls. Two patterns of heterogeneous staining for Kv1.3 were observed in the glomeruli. One pattern formed a large, round shape in relatively intact glomeruli (Fig. 2C), while the other type was comprised of small irregular deposits, the peripheries of which were sometimes faintly stained, that had accumulated in the injured glomeruli (Fig. 2D). On day 3, a few large round Kv1.3+ cells were observed in the glomeruli. On day 7, large round Kv1.3+ cells were more frequently observed around the peritubular capillaries (Fig. 2E), rather than in the glomeruli. In contrast, small Kv1.3+ deposits were less obvious on day 3, but many of these deposits had accumulated in the injured glomeruli on day 7. Kv1.3+ cells were abundantly observed in blood pooling in the vessels of the spleen (Fig. 2F) and had the same shape and size as infiltrates in the glomeruli and interstitium.

Localization of various cell phenotypes in the kidney of anti-GBM GN. To identify the localization of immune cells, we performed an immunohistochemical study in anti-GBM GN kidney. CD3+ T cells were frequently seen in and around the glomeruli and in the interstitium. Two types of CD3+ staining patterns were distinguished: 1) a large, round type in relatively intact glomeruli (Fig. 3A), and 2) a smaller type in injured glomeruli (Fig. 3B). CCR7+ cells, which were thought to be T\textsubscript{N} or T\textsubscript{CM}, were not detected in the glomeruli, but a small number of these cells were seen in the interstitium around the vessels (Fig. 3C). More CD45RC\textsuperscript{+} cells were detected than CCR7\textsuperscript{+} cells, and most of them were observed around the vessels and rarely in the glomeruli (Fig. 3D). In contrast, numerous CCR7\textsuperscript{+} cells and CD45RC\textsuperscript{+} cells were observed on the parenchyma of the spleen (Fig. 3, C and D, top right insets). Numerous ED-1\textsuperscript{+} macrophages were diffusely accumulated in the glomeruli and interstitium. ED-1\textsuperscript{+} macrophages, which sometimes had a huge foamy appearance, were more variable.
in size and shape and had a higher tendency to aggregate than CD3+ T cells (Fig. 3E).

Kv1.3 was expressed on CD3+ T cells and some ED-1+ macrophages. To better examine the localization of Kv1.3 on immune cell subsets, mononuclear cell suspensions that had been isolated from anti-GBM GN kidney on day 7 were observed after staining with anti-Kv1.3 mAb and anti-CD3 mAb or anti-ED-1 mAb. There were two types of CD3+ T cells that differed in size (large and small, suggesting they were CD8+ T cells and CD4+ T cells, respectively). Kv1.3 channels were expressed on the cell membrane as well as in the cytoplasm of both large and small CD3+ T cells isolated from anti-GBM GN kidney (Fig. 4A). Kv1.3 channels were also expressed on some ED-1+ macrophages, which have a larger cell size with an irregular cell border, compared with ED-1+ macrophages not expressing Kv1.3 (Fig. 4B).

Difference in Kv1.3 expression between CD62L− T cells and CD62L+ T cells. We analyzed the difference in the Kv1.3 expression level between the CD62L− fraction (including TEM) and the CD62L+ fraction (including TN and TCM) of the T cells (Fig. 5). αβ/γδTCR+ T cells, which were isolated by using magnetic cell sorting from the kidney and peripheral blood in the vehicle group on day 7, were used to minimize the contamination of the tubules as much as possible. In the kidney, most of the T cells were CD62L− T cells. Numerous CD62L+ T cells in the peripheral blood, which differed from those in the kidney, were observed. As expected, a higher intensity of Kv1.3 staining was observed in the CD62L+ T cells than in the CD62L− T cells.

Both early and delayed treatments with Psora-4 reduced renal damage. From the above results, we found that Kv1.3-expressing TEM had infiltrated the anti-GBM GN kidney tissue, indicating that the Kv1.3 channel is likely involved in the pathogenesis of anti-GBM GN. We therefore examined whether the blockade of the Kv1.3 channel could prevent renal damage in anti-GBM GN. As shown in Fig. 6A, a sharp rise in urinary protein excretion in the vehicle group (anti-GBM GN control without Psora-4 treatment) appeared on day 5, and marked urinary protein excretion (105.1 ± 19.3 mg/day) developed on day 7 and continued to increase after day 7. Both early treatment (from day 0 to day 21) and delayed treatment (from day 7 to day 21) with Psora-4 significantly reduced urinary protein excretion. No body weight differences between the groups were observed, except on day 21 (Fig. 6B). The increase in kidney weight in the Psora-4 group was significantly smaller than that in the vehicle group (Fig. 6C). Both early and delayed treatments restored creatinine clearances.
which were significantly higher than the creatinine clearance in the vehicle group (Fig. 6D).

Psora-4, a Kv1.3 blocker, reduced crescent formation, and the number of ED-1⁺ macrophages and CD3⁺ T cells but not the number of CCR7⁺ cells. In the vehicle group, crescent formation and severe necrotizing lesions of the glomeruli were observed from day 7 onwards (Fig. 7A). In parallel with the urinary findings, early Psora-4 treatment reduced the proportion of crescentic glomeruli (Fig. 7B) on day 7 (81 ± 6.1 vs. 43 ± 12.1%; P < 0.05). Delayed Psora-4 treatment also significantly reduced the proportion of crescentic glomeruli significantly (Fig. 8).

In the vehicle group, the immunohistochemical staining of renal tissue revealed increased numbers of glomerular ED-1⁺ macrophages (Fig. 7C) and CD3⁺ T cells (Fig. 7E). Numerous ED-1⁺ macrophages appeared in the glomeruli and interstitium in the vehicle group on day 3. In the glomeruli, the number of infiltrating ED-1⁺ macrophages peaked on day 7. The number of ED-1⁺ macrophages in the interstitium continued to increase throughout the experiment. The increased number of ED-1⁺ macrophages in the glomeruli was significantly reduced by Psora-4 treatment in both the early and the delayed treatment groups (Figs. 7D and 8). The increased number of ED-1⁺ macrophages in the interstitium was not reduced by delayed Psora-4 treatment but was significantly reduced by early Psora-4 treatment. A few CD3⁺ T cells were seen in the glomeruli and interstitium during the early stage (day 3). The number of CD3⁺ T cells had increased in the glomeruli and interstitium on day 7 and continued to increase after day 7. Psora-4 treatment caused significant reductions in these T cells in both the early and the delayed treatment groups (Figs. 7F and 8). A small number of CCR7⁺ cells were noted around the peritubular capillaries (Fig. 7G), but these cells were never seen in the glomeruli of either the vehicle or the Psora-4-treated group. Interestingly, Psora-4 treatment did not reduce the number of CCR7⁺ cells, compared with the vehicle group (Figs. 7H and 8).

Psora-4 prevented the increased mRNA expressions of inflammatory cytokines in anti-GBM GN. Figure 9 shows the mRNA expression levels of inflammatory cytokines. In the vehicle group, the expression of IFN-γ mRNA was elevated, peaking during the early stage on day 3 (at a level 52.5-fold greater than the level in untreated normal rat kidney without the induction of anti-GBM GN) but promptly decreasing after day 7. The expression of IL-17A mRNAs peaked on day 7 (at level that was 119.1-fold greater than their levels in normal rat kidney, respectively). The expressions of IL-1β and TNF-α mRNA showed similar trends, with elevated expression levels observed on both days 3 and 7 and reduced expression levels...
observed on day 14. Early Psora-4 treatment significantly reduced these increases in the expression of cytokine mRNAs. On the other hand, delayed Psora-4 treatment did not reduce these increases with the exception of IL-17A, which was significantly reduced on day 14.

Cytokine mRNA profiles in isolated CD8- T cells, CD4+ T cells, and ED-1+ macrophages from anti-GBM GN. As shown in Fig. 10, IL-17A mRNA was predominantly expressed in the CD8- T-cell fraction (corresponding to CD4+ T cells) at levels 50.1-fold greater than those in CD4+ T cells and 140.0-fold greater than those in ED-1+ macrophages. IFN-γ mRNA was predominantly expressed in the CD4+ T-cell fraction (corresponding to CD8- T cells) at levels 8.1-fold greater than those in CD8- T cells and 18.8-fold greater than ED-1+ macrophages. IL1-β and TNF-α were predominantly produced by the ED-1+ macrophages fraction, rather than the T-cell fraction.

DISCUSSION

The results of this study demonstrated that numerous Kv1.3+ cells had infiltrated the kidneys of rats with anti-GBM GN. A flow cytometry analysis using a mononuclear cell suspension obtained from anti-GBM GN kidney revealed that most CD4+ T cells and some CD8+ T cells had a TEM phenotype (CD45RC-CD62L-). Double-immunofluorescent staining showed that Kv1.3 channels were highly expressed on CD3+ T cells and some ED-1+ macrophages (Fig. 4). Subsequently, we found that early treatment with a Kv1.3 blocker, Psora-4, reduced the numbers of CD3+ T cells and ED-1+ macrophages, glomerular crescent formation, and inflammatory cytokines and eventually restored renal function and reduced urinary protein excretion in anti-GBM GN. In addition, delayed Psora-4 treatment, which was started after crescent formation had been established, also reduced renal damage and restored renal function. Interestingly, while the total number of CD3+ T cells was reduced by Psora-4 treatment, the number of CCR7+ cells, which are expressed on TCM or TN, did not change. These results suggest that the Kv1.3 blocker Psora-4 may exert its immunosuppressive effect by selectively suppressing CCR7+ TEM without affecting CCR7+ TCM/T EM, as reported previously (10, 36). These findings indicated that Kv1.3-expressing TEM as well as some macrophages were responsible for the pathogenesis of anti-GBM GN and that Kv1.3 blocker may be useful for the treatment of patients with RPGN.

We showed that most of the T cells that had infiltrated the anti-GBM GN kidney tissues had a TEM phenotype and that these TEM expressed high mRNAs level of inflammatory cytokines. These TEM induced glomerular injury through a cytokine-secreting effector function, such as the secretion of IFN-γ during the early stage and of IL-17 during the late stage. TEM are essential mediators of numerous chronic inflammatory autoimmune diseases (10, 17, 54, 55). TEM exert an immediate effector function, which is achieved through the prompt secretion of large amounts of cytokines, such as IFN-γ or IL-4, at the site of antigen deposition and the initiation of a localized inflammatory immune response. A flow cytometry analysis of T cells in the urine of patients with IgA nephropathy, Henoch-Schönlein purpura nephritis, or ANCA-associated vasculitis showed that the urinary T cells mainly exhibited a TEM phenotype (CD45RC-CD62L-). These TEM were also observed in and around the glomeruli, and the number of TEM was correlated with the degree of glomerular cell infiltration and crescent formation (45). In an experimental model, Totsuka and colleagues (18, 53) succeeded in inducing colitis by the adoptive transfer of only colitogenic CD4+ TEM into SCID mice (which do not contain any naïve T cells) and demonstrated a significant role of TEM in the development of colitis. In the field of kidney disease models, the infiltration of TEM-secreting IL-17 or IFN-γ was observed in the kidneys of unilateral ureteral obstruction (16) and ischemic reperfusion models (5).

Beside T cells, glomerular accumulation of macrophages is the striking feature in crescentic glomerulonephritis. Depletion

Fig. 6: A: effect of early and delayed Psora-4 treatments on urinary protein excretion. *P < 0.05 vs. vehicle group. B: body weight. C: kidney weight. D: creatinine clearance (Ccr). Each plot shows the means ± SD for 6 rats. Closed circles, vehicle group. Open circles, early Psora-4 group (from day 0 to day 21). Double circles, delayed Psora-4 group (from day 7 to day 21). *P < 0.05 vs. vehicle group.
studies (29, 47) have shown that macrophages can induce glomerular injury in experimental crescentic glomerulonephritis, suggesting that modulation of macrophage activation may be an important therapeutic strategy for the treatment of crescentic glomerulonephritis. Macrophages can produce many molecules that may cause renal damage. Macrophage-derived mediators, such as IL-1, TNF-α, macrophage migration inhibitory factor, and procoagulant activity, have been implicated in the development of crescent formation (6, 38). Administration of IL-1β and TNF-α exacerbates glomerular injury in anti-GBM GN, whereas blocking these cytokines suppresses the induction of glomerular injury (32, 50, 52).

Our results using whole kidney samples showed that the expressions of IL-1β and TNF-α mRNA, which were predominantly expressed on ED-1+ macrophages, were upregulated in the vehicle group and that Psora-4 significantly suppressed these increases. The time trend of IL-1β and TNF-α was paralleled with the number of ED-1+ macrophages in the anti-GBM kidney, suggesting that Psora-4 could have some effects on macrophages either directly or indirectly by modu-
coexpressed potassium channels, as exemplified by the upregulation can be rapidly escaped by the upregulation of other role in maintaining the resting membrane potential on TEM natural killer cells, the Kv1.3 channel only plays a dominant present on T cells as well as on macrophages, B cells, and been reported in a study examining the brain tissue of subjects group.

Figs. 8 and 9. Quantification analysis of crescent formation, ED-1 + macrophages, CD3 + T cells, and CCR7 + cells. Results are means ± SD for each group. Closed bar, the vehicle group. Dotted bar, the early Psora-4 group. Hatched bar, the delayed Psora-4 group. Mφ, macrophages; glo, glomerulus; IS, interstitium. *P < 0.05 vs. vehicle.

Fig. 10. Cytokine profiles in cells isolated from the vehicle group on day 7. To determine which cell populations expressed these cytokines, we isolated and fractionated the cells that had infiltrated the kidney using magnetic cell sorting. Open bar, CD8 + T cells (corresponding to CD4 + T cells). Closed bar, CD4 + T cells (corresponding to CD8 + T cells). Hatched bar, ED-1 + macrophages. Isolated cells were analyzed for mRNA expression using real-time RT-PCR. Whole mononuclear cells isolated from untreated normal rat kidney (without the induction of anti-GBM GN) using the density gradient centrifugation method were used for calibration. Results are means ± SD for each group (n = 3).

On the other hand, monocytes and macrophages express Kv1.5, the calcium-activated channels KCa1.1 and KCa3.1, and the inward-rectifier Kir2.1 in addition to Kv1.3 channels (11, 56). Especially, the association of Kv1.5 and Kv1.3 comprise the major voltage-dependent K + channel in activated macrophages (11, 56). Macrophages express different Kv1.3-to-Kv1.5 ratios in cell type specification, leading to biophysically and pharmacologically distinct channels (58). High Kv1.3-to-Kv1.5 ratios, observed in bone marrow derived macrophages and TNF-α-activated Raw 264.7 macrophages, are sensitive to Kv1.3 blocker, whereas low Kv1.3-to-Kv1.5 ratios as observed in control Raw 264.7 macrophages have a low sensitivity to Kv1.3 blocker (57). The presence of Kv1.5 in macrophage lineage cells should be taken into account when designing Kv1.3-based therapies. Psora-4, a potent small-molecule Kv1.3 blocker (EC50 = 3 nM), is only 2.5-fold selective over the Kv1.5 channel (EC50 = 7.7 nM; Ref. 55), suggesting that Psora-4 may exert a direct inhibitory effect on some of the activated macrophages through the inhibition of the Kv1.3 and Kv1.5 channels, as well as an indirect effect on macrophages, through the suppression of T cells.

Several types of Kv channels, such as KCNQ1, KCNA10, and Kv1.3, are highly expressed at the apical membrane of renal tubules. Kv1.3 channels are reportedly expressed on the cortical collecting duct of the human kidney and may be involved in K + secretion (61). Since serum and glucocorticoid-induced kinase stimulates the activity of Kv1.3 channels (28), increases in the K + channel activity induced by serum and glucocorticoid-induced kinase may also contribute to aldosterone-mediated increases in K + secretion. However, adverse effects, such as hyper/hypokalemia, increases in serum creatinine and blood urea nitrogen levels, or morphological changes in the kidneys, have not been reported with the use of Kv1.3 blockers in rodent models (55). In a previous report (40), the acute and chronic administration of Kv1.3 blocker did not induce any apparent hematologic toxicity, and no marked fluctuations in the frequencies of the major T-cell lineages were noted, except for a temporary decrease in circulating...
lymphocytes with a coincident increase in neutrophils immediately after the administration of Kv1.3 blocker.

Kv1.3 blockers may have potential advantages over current immunomodulatory therapies with regard to several points. First, Kv1.3 blockers may not increase susceptibility to infections, because naïve and long-lived T<sub>EM</sub> (the main memory pool) would escape from its inhibition (10, 40). In addition, the Kv1.3 channel regulates glucose transporter type 4 trafficking in skeletal muscle and adipose tissue; therefore, Kv1.3 blockers are expected to be useful for the management of insulin resistance and diabetes (34).

The induction of anti-GBM GN increased the expression of the mRNAs of various inflammatory cytokines, and these increases were suppressed by Psora-4 treatment. IFN-γ is the most potent activator of mononuclear phagocytes and is predominantly secreted by natural killer and natural killer T cells as part of the innate immune response and by CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells once antigen-specific immunity develops (46). However, the role of IFN-γ in anti-GBM GN remains controversial. Conflicting reports exist as to whether anti-GBM is ameliorated or exacerbated in IFN-γ gene knockout mice (33, 42). In anti-GBM GN, adoptive transfer of IFN-γ-activated macrophages substantially augmented macrophage-mediated renal injury (31). However, a consensus that IFN-γ is required for the induction of autoantibody formation and nephritis in lupus-prone mice has been established (26, 27). In our study, the IFN-γ mRNA level promptly increased on day 3 after the induction of anti-GBM GN, and IFN-γ was predominantly expressed in the CD4<sup>+</sup> T-cell fractions (corresponding to CD8<sup>+</sup> T cells), indicating that IFN-γ-secreted CD8<sup>+</sup> T cells would be the major target of Psora-4 during the early stage of anti-GBM GN.

IL-17 has been defined as a proinflammatory cytokine and is produced by activated T cells (Th17). In humans, CD4<sup>+</sup>CD45RO<sup>+</sup>CCR7<sup>−</sup>CCR6<sup>+</sup>T<sub>EM</sub> have been identified as the principal IL-17-secreting T cells under Th17-polarizing conditions (35). IL-17 is involved in the development of autoimmune disease models, such as experimental allergic encephalomyelitis, allergic contact dermatitis, and type II collagen-induced arthritis (4, 7, 49). Recently, both IL-23 p19<sup>−/-</sup> and IL-17<sup>−/-</sup> mice were reported to develop less severe nephritis as measured by renal function, proteinuria, and frequency of glomerular crescent formations (39). IL-17- and IFN-γ-producing CD3<sup>+</sup> T cells were identified by intracellular cytokine staining after stimulation with PMA/ionomycin in the flow cytometric analysis of isolated renal T cells from nephritic wild-type mice, and IL-17 enhanced the production of the proinflammatory chemokines CCL2/MCP-1, CCL3/MIP-1α, and CCL20/LARC, which are implicated in the recruitment of T cells and monocytes, in mouse mesangial cells in vitro (39). In our real-time RT-PCR results, IL-17 mRNA was highly expressed on day 7 and was predominantly produced by the CD8<sup>+</sup> T-cell fraction (corresponding to CD4<sup>+</sup> T cells). These increases were suppressed by Psora-4 treatment. Therefore, Psora-4 was suspected to exert its effect by suppressing IL-17-secreting CD4<sup>+</sup> T<sub>EM</sub>. In conclusion, the results of this study have demonstrated for the first time that the Kv1.3 blocker Psora-4 has effects in a rat model of anti-GBM GN that are likely mediated by inhibition of T<sub>EM</sub> and macrophages. If shown to be nontoxic in more detailed and long-term clinical toxicity tests, Psora-4 may become a very effective means of treating patients with rapidly progressive glomerulonephritis.

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REFERENCES

F1268  EFFECT OF Kv1.3 BLOCKER ON ANTI-GBM GN


