Galectin-9 ameliorates anti-GBM glomerulonephritis by inhibiting Th1 and Th17 immune responses in mice

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Zhang Q, Luan H, Wang L, He F, Zhou H, Xu X, Li X, Xu Q, Niki T, Hirashima M, Xu G, Ly Y, Yuan J. Galectin-9 ameliorates anti-GBM glomerulonephritis by inhibiting Th1 and Th17 immune responses in mice. Am J Physiol Renal Physiol 306: F822–F832, 2014. First published January 29, 2014; doi:10.1152/ajprenal.00294.2013. —Antiglomerular basement membrane glomerulonephritis (anti-GBM GN) is a Th1- and Th17-predominant autoimmune disease. Galectin-9 (Gal-9), identified as the ligand of Tim-3, functions in diverse biological processes and leads to the apoptosis of CD4+Tim-3+ T cells. It is still unclear how Gal-9 regulates the functions of Th1 and Th17 cells and prevents renal injury in anti-GBM GN. In this study, Gal-9 was administered to anti-GBM GN mice for 7 days. We found that Gal-9 retarded the increase of Scr, ameliorated renal tubular injury, and reduced the formation of crescents. The infiltration of Th1 and Th17 cells into the spleen and kidneys significantly decreased in Gal-9-treated nephritic mice. The reduced infiltration of Th1 and Th17 cells might be associated with the downregulation of CCL-20, CXCL-9, and CXCL10 mRNAs in the kidney. In parallel, the blood levels of IFN-γ and IL-17A declined in Gal-9-treated nephritic mice at days 21 and 28. In addition, an enhanced Th2 cell-mediated immune response was observed in the kidneys of nephritic mice after a 7-day injection of Gal-9. In conclusion, the protective role of Gal-9 in anti-GBM GN is associated with the inhibition of Th1 and Th17 cell-mediated immune responses and enhanced Th2 immunity in the kidney.

galactin-9; Tim-3; Th17 cells; Th2 cells; anti-GBM glomerulonephritis

RAPIDLY PROGRESSIVE GLOMERULONEPHRITIS (RPGN) is characterized by rapidly declining renal function and the formation of glomerular crescents. Three major categories of crescentic GN have been defined: anti-glomerular basement membrane (anti-GBM) crescentic GN, immune-complex crescentic GN, and pauci-immune crescentic GN (7). Anti-GBM GN is the most aggressive form of GN and has the greatest probability of renal insufficiency and the highest frequency of crescent formation at the time of diagnosis (10). A number of studies indicate that Th1 cell-mediated immunity plays an important role in the pathogenesis of anti-GBM GN (19, 26), and Th17 cells also contribute to the progression of anti-GBM GN (18, 25). Knockout of T-bet, which is a Th1-specific transcription factor, or deletion of IL-17 can attenuate renal injury in experimental anti-GBM GN (20, 28). Therefore, we hypothesized that the blockade of the activation of Th1 and Th17 cells or the elimination of activated Th1 and Th17 cells might prevent the progression of anti-GBM GN.

The T-cell immunoglobulin domain and mucin domain (Tim) family is a group of molecules with a conserved structure and important immunologic functions. Tim molecules, such as Tim-1 and Tim-3, are expressed on many types of immune cells and play important roles in innate and adaptive immunity (21). Tim-1, as known as kidney injury molecule-1 (Kim-1) (20), has been associated with hepatitis A virus infection, kidney injury, and Th2 cell-mediated immunity (20, 21). Tim-3 is involved in Th1- and Th17 cell-mediated immunity and has been identified as the ligand of galectin-9 (Gal-9) (37).

Gal-9 is one of the β-galactoside binding lectins and causes the apoptosis of Th1 cells and the suppressed development of Th17 cells (16, 37). Recent studies also suggest that Gal-9 can suppress the differentiation of Th17 cells in a Tim-3-independent manner and increase the infiltration of IL-4-secreting Th2 cells in experimental myocarditis (13, 16, 37). In a number of studies, Gal-9 has been administered to experimental autoimmune disease models and displayed therapeutic effects, including for experimental allergic encephalomyelitis, experimental autoimmune arthritis, and autoimmune diabetes (23, 35, 37). Our previous studies also revealed that Gal-9 prolongs the survival of allografts through inhibition of the activation of CD8+ T cells and Th17 cells (8, 32).

In experimental anti-GBM GN, Tim-3 has been observed to be upregulated in kidneys and lymph nodes, and the blockade of Tim-3 exaggerated T-cell-mediated immunity and aggravated nephritis (22). Furthermore, the administration of Gal-9 reduced the excretion of urinary protein and the formation of cellular crescents in rat nephrotoxic serum nephritis. These benefits were associated with the apoptosis of activated CD8+ T cells, not CD4+ T cells, in the spleen (29). However, the mechanisms by which Gal-9 attenuates nephritis remain unclear, and it is unknown how Gal-9 changes the profiles of Th1, Th2, and Th17 cells in anti-GBM GN. In the present study, we demonstrated that the administration of Gal-9 decreased the proportion of CD4+Tim-3+ T cells in the spleen and kidneys, and an enhanced Th2 cell-mediated immune response was observed in the spleen and kidneys following Gal-9 administration. Our results suggest that Gal-9 not only suppressed the immune response by elimi-
nating CD4⁺Tim-3⁺ T cells but shifted the immune response from Th1 to Th2 in anti-GBM GN.

MATERIALS AND METHODS

Mice. For this study, 6- to 8-wk-old male C57BL/6 mice were obtained from the animal facilities of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All mice were permitted free access to water and a standard laboratory diet and housed at room temperature (23–26°C) and 50% humidity with a 12-h light-dark cycle. They received humane care in accordance with international guidelines and national law. The protocol in the current study was approved by the Animal Care and Use Committee of Huazhong University of Science and Technology.

Preparation of rabbit anti-mouse GBM antiserum. GBMs were harvested from C57BL/6 mice kidneys by a differential sieve technique, followed by sonication and detergent treatment as previously described (21a). GBMs were briefly washed in PBS and mixed with an equal volume of complete Freund’s adjuvant (Sigma-Aldrich). All mice were immunized subcutaneously with a combination of rabbit IgG (0.02 mg/g; Sigma-Aldrich, St. Louis, MO) and complete Freund’s adjuvant (Sigma-Aldrich) at 50% humidity with a 12-h light-dark cycle. They received humane care in accordance with international guidelines and national law. The protocol in the current study was approved by the Animal Care and Use Committee of Huazhong University of Science and Technology.

Induction of anti-GBM glomerulonephritis in mice. All mice were randomly divided into three groups: control, PBS-treated, or Gal-9-treated nephritis. All mice were preimmunized subcutaneously with a combination of rabbit IgG (0.02 mg/g; Sigma-Aldrich, St. Louis, MO) and complete Freund’s adjuvant (Sigma-Aldrich) at day 0 before the injection of anti-GBM serum. Anti-GBM serum (0.02 ml/g) was administered to induce nephritis at day 0. Then, nephritic mice were treated with PBS or Gal-9 (100 μg; kind gift from Prof. Mitsuomi Hirashima and Dr. Toshiro Niki) from day 1 through day 7. Mice were euthanized at days 7, 14, 21, and 28.

Histology. All mice were euthanized at selected intervals and kidneys were immediately harvested and fixed with 4% paraformaldehyde. Fixed renal tissues were embedded in paraffin, cut into 3-μm sections, and subjected to periodic acid Schiff (PAS) for histological analysis. Glomerular abnormalities include crescent formation, glomerular sclerosis, and tubulointerstitial injury. The histologic evaluation was performed by examining a minimum of 50 glomeruli per section at ×600 magnification. The PAS score is defined as follows: 0 = no deposits of PAS-positive material, 1 = up to one third, 2 = one-third to two-thirds, and 3 = more than two-thirds of the glomerular cross-section stain positive for PAS. Tubulointerstitial injury scores are defined as follows: 0 = no injury, 1 = less than 25%, 2 = 25–50%, 3 = 50–75%, and 4 = more than 75%. To analyze the deposition of rabbit IgG and mouse IgG in kidneys, snap-frozen tissue sections (5 μm) were stained with FITC-sheep anti-rabbit IgG (Sigma-Aldrich; 1:100) and FITC-sheep anti-mouse IgG (Sigma-Aldrich; 1:100) antibodies.

Renal function. Blood samples were collected at days 7, 14, 21, and 28 and tested with serum creatinine (Scr) kit (BioAssay Systems, Hayward, CA). Urine samples were obtained by bladder puncture at day 28 for testing urine albumin (BCA Kit; Beyotime, Wuhan, China) and creatinine (BioAssay Systems).

Single-cell suspension from spleen and kidney. Splenocytes were prepared according to standard laboratory procedures. Spleens were finely minced and sequentially passed through 200-mesh sieves. Erythrocytes were lysed with RBC lysis buffer (Biolegend, San Diego, CA). Previously described protocols for renal cells isolation were used (11a). Briefly, kidneys were finely minced and digested with 1.6 mg/ml collagenase I (Sigma-Aldrich) and 200 μg/ml DNase I (Sigma-Aldrich) in RPMI 1640 (Hyclone, Logan, UT) for 30 min at 37°C. The cell suspensions were sequentially filtered through 70- and 40-μm nylon meshes and washed with HBSS without Ca²⁺ and Mg²⁺ (Hyclone). Erythrocytes were lysed with RBC lysing buffers.

CD4⁺Tim-3⁺ T cells isolation and stimulation. Splenocytes were harvested from C57BL/6 mice according to standard laboratory protocol. CD4⁺ T cells were isolated by negative selection using EasySep Mouse CD4⁺ T cell enrichment kit according to manufacturer’s instructions (STEMCELL Technologies, Vancouver, Canada). The purity of CD4⁺ T cells was over 90% as confirmed by FACS. Purified CD4⁺ T cells were cultured in RPMI 1640 (Hyclone) supplemented with 10% FBS (Hyclone), anti-CD3 (2.5 μg/ml), and anti-CD28 (5 μg/ml) at 37°C in a humidified atmosphere (5% CO₂) incubator for 72 h. Then, activated CD4⁺ T cells were cultured with 7.5 μg/ml Gal-9 or Gal-9 plus α-lactose in 96-well plates. After a 6-h incubation, the profiles of T cell subsets were detected by FACS. Western blot and real-time PCR.

Flow cytometry. Antibodies used for multicolor flow cytometric analysis were as follows: FITC anti-CD4, PE anti-CD69, PE anti-Tim-3, PE anti-IFN-γ, PE anti-IL-4, and PE anti-IL-17A antibodies (eBiosciences, San Diego, CA). For staining of intracellular markers, cells were incubated for 20 min at 4°C in Cytofix/Cytoperm (Biolegend) to permeabilize cell membranes. Intracellular markers were stained according to standard laboratory procedures. Cytometry was performed on BD FACS Calibur System (BD Bioscience).

Real-time PCR. Total RNA was extracted from renal tissues using Trizol (Invitrogen, Carlsbad, CA). Then, 1 μg of RNA was used to synthesize cDNA (Toyobo). Real-time PCR was performed in 96-well plates with SYBR Green PCR Master Mix (Toyobo). β-Actin served as an internal standard for normalization. The sequences of the primers used in this study are listed in Table 1.

Western blotting. Protein extracts were prepared from cultured CD4⁺ T cells. Equal amounts of protein (60 μg) were separated by 10% SDS-PAGE and then blotted onto PVDF membranes. Antibodies were as follows: rabbit anti-T-bet (eBioscience; 1:250), rat anti-GATA-3 (eBioscience; 1:250), rat anti-RORγt (eBioscience; 1:250), rabbit anti-β-actin (CST; 1:4,000), and mouse anti-GAPDH (Epitomics; 1:4,000) antibodies. The immunolabeled proteins were detected by AP solution. The optical density of the scanned blot was quantified using ImageJ.

ELISA. Blood samples were collected at multiple time points. The levels of IFN-γ, IL-4, IL-17A, IL-12, IL-10, and transforming growth factor-β₁ (TGF-β₁) were tested by ELISA in accordance with the manufacturer’s instructions (eBioscience). The absorbance of the final reactant was determined at 450 nm with an ELISA plate reader (BioTek).

Statistical analysis. The results are expressed as the means ± SE. All data were obtained from at least three independent experiments. Data are analyzed by Student’s t-test or one-way ANOVA. A value of P < 0.05 was considered significant.

RESULTS

CD4⁺Tim-3⁺ T cells are increased in anti-GBM GN. To explore the kinetic profiles of CD4⁺ T-cell subsets in anti-GBM GN, we isolated splenocytes from anti-GBM GN mice at days 7, 14, 21, and 28 (Fig. 1). We observed a significant increase of CD4⁺Tim-3⁺ T cells in anti-GBM GN from day 7 onwards with a peak at day 14 (Fig. 1, A and B). The increased CD4⁺Tim-3⁺ T cells existed through the entire period of observation (from days 7 to 28). In addition, the expression of Tim-3 mRNA was upregulated in the kidneys at the later time points (see Fig. 6). Next, we analyzed Th1, Th2, and Th17 subsets in the spleen. After the induction of anti-GBM GN, Th1 and Th17, but not Th2, cells increased significantly in the spleen, and the greatest proportions of Th1 and Th17 cells occurred at day 14 (Fig. 1, D–F). As expected, an increase of CD4⁺CD69⁺ T cells was detected in anti-GBM GN, and the percentage of CD4⁺CD69⁺ T cells at day 28 was even
Table 1. Primers used for real-time PCR

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<th>Genes</th>
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<td>β-Actin Forward</td>
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higher than that in the early phase (day 7 vs. day 28, P < 0.05; Fig. 1C).

Gal-9 ameliorates the renal injury in anti-GBM GN. To investigate whether Gal-9 could prevent renal injury in anti-GBM GN, Gal-9 was applied to nephritic mice from day 1 through day 7. Anti-GBM GN caused the deterioration of renal function (Fig. 2A) and significant deposition of the mouse IgG in glomeruli after day 7 (Fig. 2B). The crescents and protein casts were observed at days 21 and 28 (Fig. 2, D and E). Following the administration of Gal-9, we found that the deterioration of renal function had been significantly slowed in anti-GBM GN (Fig. 2A). At day 28, the urine albumin-to-creatinine ratio in mice treated with Gal-9 was lower than that in anti-GBM GN mice (Fig. 2A). In addition, Gal-9 decreased the deposition of mouse IgG in glomeruli but did not change the distribution of rabbit IgG in glomeruli (Fig. 2, B and C). As well as the benefits to renal function, Gal-9 attenuated the renal structural damages in anti-GBM GN, which included reduced crescent formation, a lower PAS score, and mild interstitial injury (Fig. 2, D and E). In addition, Gal-9 administration decreased the expression of TGF-β1 mRNA in kidneys, which is associated with renal fibrosis (see Fig. 6H). Taken together, these results suggest that Gal-9 could attenuate the renal injury and delay the deterioration of renal function in anti-GBM GN.

Gal-9 inhibits the infiltration of Th1 and Th17 cells in anti-GBM GN. As anti-GBM GN is mediated by Th1 and Th17 cells, we hypothesized that Gal-9 might suppress Th1- and Th17-mediated immune responses in anti-GBM GN. Gal-9 led to a decrease of CD4+Tim-3+ T cells in the spleen after a 7-day continuous administration, and this effect was irreversible (Fig. 3, A and B). In addition to inhibiting CD4+Tim-3+ T cells, Gal-9 reduced the proportions of both Th1 and Th17 cells in the spleen (Fig. 3, C and E). Similarly, quantification of cytokine expression in blood revealed high levels of IFN-γ and IL-17A in anti-GBM GN mice, and the administration of Gal-9 decreased the production of IFN-γ and IL-17A at day 28 (Fig. 4, A and B).

To assess the profiles of Th1 and Th17 cells in kidneys, single cells isolated from kidneys were analyzed using FACS. We observed abundant CD4+IFN-γ+ and CD4+IL-17+ T cells in the kidneys of anti-GBM GN mice. However, the administration of Gal-9 decreased the infiltration of Th1 and Th17 cells in kidneys (Fig. 5). At days 21 and 28, the kidneys of Gal-9-treated nephritic mice displayed an increase of CD4+IFN-γ+ T cells compared with nephritic mice, but it was significantly lower than that in anti-GBM GN mice (Fig. 5, A and B). In addition, Gal-9 markedly inhibited the infiltration of Th17 cells in the kidneys of anti-GBM GN mice (Fig. 5, E and F), and the proportion of CD4+IL-17+ T cells in the kidneys of Gal-9-treated nephritic mice was almost the same as that in the kidneys of nonnephritic mice. These results are consistent with what we observed in the spleen. In addition, we analyzed the expression of cytokine mRNAs in the kidneys and found that the expressions of IFN-γ and IL-17A mRNAs were downregulated in Gal-9-treated nephritic mice at day 28 (Fig. 6, A and B).

Gal-9 administration enhances Th2 cell-mediated immune response in anti-GBM GN. The Th2 cell-mediated immune response has been reported to play a protective role in anti-GBM GN that is associated with the suppression of Th1 cell-mediated immune response (28). Given the suppressive effect of Gal-9 on Th1 and Th17 cells in anti-GBM GN, we were interested in whether Gal-9 treatment could regulate the Th2 immune response as well. FACS revealed increased infiltration of CD4+IL-4+ T cells in the kidneys of anti-GBM GN mice (Fig. 5, C and D), but we did not observe a higher proportion of Th2 cells in the spleen (Fig. 1E). Interestingly, Gal-9 administration led to the further increase of CD4+IL-4+ T cells in both the spleen and kidneys (Figs. 3D and 5D). Although Gal-9-treated anti-GBM GN mice had higher levels of IL-4 and IL-10 in the peripheral blood, compared with C57BL/6 mice, there was no significant difference between PBS- and Gal-9-treated nephritic mice (Fig. 4, C and E). In addition, the increased expression of IL-4 mRNA was disclosed in the kidneys of Gal-9-treated anti-GBM GN mice (Fig. 6C). These suggest that Gal-9 enhances the Th2 cell-mediated immune response in the kidneys of anti-GBM GN mice.

Gal-9 decreases the intrarenal expression of proinflammatory chemokines in anti-GBM GN mice. The infiltration of leukocytes is associated with the expression of chemokines (11), and a number of chemokines have been determined as being involved in glomerulonephritis (1, 4–6, 14, 15). To
determine whether Gal-9 affects the expression of chemokines in anti-GBM GN, we measured the intrarenal mRNA expression of CCL-2, CCL-4, CCL-5, and CXCL-1, which have been reported to be associated with nephritis (1, 4, 5, 14). All of these four chemokine ligands were upregulated in the kidneys of anti-GBM GN mice (Fig. 6). However, after Gal-9 administration, the expressions of CCL-2, CCL-5, and CXCL-1 decreased at day 21 (Fig. 6, D, F, and G). Gal-9 did not suppress the expression of CCL-4 mRNA (Fig. 6E).

We also detected the expressions of CXCL-9, CXCL-10, CXCR-3, and CCL-20, which have been reported to be associated with Th1 and Th17 cell recruitments (9, 12, 30, 33). Gal-9 downregulated the intrarenal mRNA expressions of CXCL-9, CXCR-3, and CCL-20 at days 21 and 28 (Fig. 6), but the reduction of CXCL-10 mRNA in the kidneys only occurred at day 28 following the administration of Gal-9 and not at day 21. These results suggest that the protective function of Gal-9 in anti-GBM GN is associated with the downregulated expression of proinflammatory chemokines.

Gal-9 inhibits the activation of Th1 and Th17 cells, which is dependent on the binding with Tim-3 in vitro. To investigate how Gal-9 regulates the function of CD4+ T cells in vitro, purified CD4+ T cells were isolated from the spleens of C57BL/6 mice and stimulated by anti-CD3 and anti-CD28 antibodies in the presence of Gal-9 or Gal-9 plus α-lactose. α-Lactose binds to the carbohydrate-binding domain of Gal-9 and limits the engagement between Gal-9 and Tim-3. After a 6-h stimulation, CD4+ T cells were collected to evaluate the level of apoptosis. Gal-9 increased the proportion of annexin-V−PI− cells, and α-lactose was able to protect CD4+ T cells from the apoptosis induced by Gal-9 (Fig. 7, A and B). Next, we assayed the profiles of T-cell subsets in an in vitro experiment with FACS. The proportions of Th1 and Th17 cells were decreased in the presence of Gal-9 (Fig. 7C). However, after blocking the binding between Gal-9 and Tim-3 with α-lactose, the proportions of CD4+IFN-γ+ and CD4+IL-17A+ T cells increased (Fig. 7C). These findings suggest that the inhibition of Gal-9 of Th1 and Th17 cells is Gal-9/Tim-3 dependent.

Furthermore, we measured the expression of Tim-3 mRNA in CD4+ T cells by real-time PCR. We found that Tim-3 expression was significantly inhibited by Gal-9 (Fig. 8A). In addition, Gal-9 suppressed the expression of the Th1-related transcription factor T-bet and Th17-related transcription factor RORγt but not the Th2-related transcription factor GATA-3 (Fig. 8, B and C). On the contrary, GATA-3 increased significantly in the presence of Gal-9 (Fig. 8, B and C). The regulation by Gal-9 of T-bet and RORγt was Tim-3 dependent, as blocking the binding of Tim-3 and Gal-9 with α-lactose reversed the downregulation of T-bet and RORγt.

**DISCUSSION**

The present study demonstrated that Gal-9 administration was able to attenuate the development of anti-GBM GN in mice by inhibiting Th1- and Th17-mediated immune response in vitro and in vivo. In addition, an enhanced Th1-mediated immune response was observed in the kidneys following Gal-9 administration. Furthermore, our in vitro experiments indicated that the regulation of Gal-9 in the differentiation of CD4+ T cells was Tim-3 dependent and associated with the expressions of T-cell specific transcription factors.
Fig. 2. Galectin-9 (Gal-9) ameliorates the renal injuries in anti-GBM glomerulonephritis. A: serum creatinine (Scr) was detected at days 7, 14, 21, and 28. The deterioration of renal function in nephritic mice occurred from day 14, but the administration of Gal-9 preserved the renal function and led to a mild rise in Scr at days 21 and 28. In addition, Gal-9 treatment decreased the proteinuria-to-creatinine ratio at day 28, compared with that in nephritic mice. Bars represent the means ± SE; n = 4–8 per group at each time point. B and C: representative photographs of the deposition of mouse IgG (B) and rabbit IgG (C) in the kidneys of nephritic or Gal-9-treated mice (original magnification, ×400). D: representative photographs of periodic acid Schiff (PAS)-stained kidney sections in control, nephritic, and Gal-9-treated mice (original magnification, ×400). E: quantification of crescents, PAS-positive deposits, and interstitial injury in nephritic mice receiving either PBS or Gal-9. *P < 0.05, **P < 0.01.
Anti-GBM GN is an autoimmune disease that is associated with systemic and organ-specific autoimmunity (26). CD4+ T cells play a crucial role in the initiation of the immune response that leads to the crescentic injury (27) in anti-GBM GN. Tim-3 has been revealed to be specifically expressed on IFN-γ-secreting CD4+ Th1 and CD8+ Tc1 cells and participate in the antitumor immune response, autoimmune response, and graft rejection (2, 8, 32). It has been reported that the kidneys of nephrotoxic nephritis rats express higher level of Tim-3 mRNA, and most of the Tim-3+ cells in those kidneys have been reported to be CD4+ T cells (22). However, the time course of the appearance of CD4+ Tim-3+ cells in anti-GBM GN remains unclear. Our data indicate that abundant CD4+ Tim-3+ T cells were in the spleen of anti-GBM GN mice as early as day 7, and the highest proportion of CD4+ Tim-3+ T cells was observed at day 14. Although the number of

Fig. 3. Gal-9 administration inhibits Th1- and Th17-mediated immune responses in the spleens of anti-GBM GN mice. A: flow cytometric analysis of splenocytes isolated from anti-GBM GN mice at days 7, 14, 21, and 28 in nephritic mice receiving either PBS or Gal-9. Data are representative of 3 independent experiments. B–E: quantification of the percentage of Tim-3+, IFN-γ+, IL-4+, and IL-17A+ cells in CD4+ T cell subsets in nephritic mice or Gal-9-treated nephritic mice at days 21 and 28. Bars represent the means ± SE; n = 3–4 each group for days 7 and 14; n = 6–8 each group for days 21 and 28. *P < 0.05, **P < 0.01.
CD4+ Tim-3+ T cells decreased slightly during the late stage of nephritis (days 14–28), it was significantly higher than in normal C57BL/6 mice.

Paust et al. (17) reported the increasing secretion of IFN-γ by splenocytes in nephrotoxic serum nephritis (NTN). This is consistent with our results. They also described the early upregulated production of IL-17 on day 3, which is followed by a peak on day 5, and the decreased secretion of IL-17 after day 7. However, in our study, the increase of CD4+ IL-17A+ T cells occurred from day 7 onwards until day 28. This difference might be due to the different experimental techniques applied in each study. In the previous study, the systemic Th17 immune response was evaluated by detecting cytokine secretion from restimulated splenocytes; however, we detected CD4+ IL-17A+ T cells directly using FACS. Recently, several groups revealed the existence of IL-17-producing macrophages in different models (24, 31), which raises the possibility that Th17 cells might not be the only source of IL-17 secreted by stimulated splenocytes.

Next, we measured the renal and systemic Th2 cell-mediated immune response in anti-GBM GN. The upregulated production of IL-4 was detected in the blood, and there was an increased proportion of Th2 cells in the kidneys rather than in the spleen. The abundant infiltration of Th1 and Th2 cells in the kidneys of anti-GBM GN mice was associated with the upregulated levels of CCL-4, CCL-5, CXCL-9, and CCL-20 mRNAs in the kidneys, which have been reported to be involved in T-cell migration and nephritis (1, 9, 14, 15, 17).

A recent study revealed that blockade of the Tim-3 pathway with anti-Tim-3 antibody aggravates renal injury in nephritis and that was associated with the proliferation and apoptosis of kidney cells, instead of activating T cells (22). In our study, a different strategy has been applied. Gal-9 is a member of the galectin family of carbohydrate-binding proteins and plays a multifaceted role in T-cell development, homeostasis, and apoptosis (35). We hypothesized that the administration of Gal-9 could suppress T-cell-mediated immunity and protect the kidneys in experimental anti-GBM GN. We found that Gal-9 administration decreased the deposition of mouse IgG and attenuated renal injury, which included less formation of crescents and milder interstitial injury. Although Gal-9 was not able to prevent the initiation of renal injury in anti-GBM GN mice, the deterioration of renal function was delayed following Gal-9 administration.

A previous study reported the protective effect of Gal-9 in NTN and disclosed that Gal-9 administration could reduce IgG deposition and induce the apoptosis of CD8+ T cells but not CD4+ T cells in the spleens of NTN rats (29). However, this study did not determine how Gal-9 affected the functions of Th1 and Th17 cells, which have been identified as the major pathogenic cell types in anti-GBM GN (17, 26). To address this question, first, we analyzed the kinetic profile of CD4+ Tim-3+ T cells. As we expected, Gal-9 administration inhibited the
increase of CD4+Tim-3+ T cells in the spleen and downregulated the expression of Tim-3 mRNA in the kidneys. This is associated with the activation of the Gal-9/Tim-3 pathway in which Gal-9 causes the apoptosis of CD4+Tim-3+ T cells by binding to Tim-3 on the surface of T cells (37). In addition, a decline in the number of Th1 and Th17 cells was detected in the spleen following a 7-day continuous injection of Gal-9. Moreover, we detected lower levels of IFN-γ and IL-17A in
Fig. 6. Expression of cytokines and proinflammatory chemokines in the kidneys of nephritic mice. A–M: intrarenal mRNA expressions of IFN-γ, IL-17A, IL-4, CCL-5, CCL-4, CXCL-1, CCL-2, TGF-β1, Tim-3, CXCL-9, CXCR-3, CCL-20, and CXCL-10 were determined at days 21 and 28 by real-time PCR. Relative quantitation (RQ) was calculated using the ddCT method with normalization of mRNA expression to the endogenous control β-actin. Bars represent the means ± SE; n = 3–4 each group. *P < 0.05, **P < 0.01.
the blood of Gal-9-treated nephritis mice. In line with the profiles of Th1 and Th17 cells in the spleen, Gal-9 also inhibited the infiltration of Th1 and Th17 cells in the kidneys. Furthermore, we observed abundant Th2 cells and the increased expression of IL-4 mRNA in the kidneys following Gal-9 administration, but the levels of IL-4 and IL-10 in the blood did not increase correspondingly. We did not observe increased Th2 cells in the spleens of Gal-9-treated nephritis mice as well. Similarly, Xiong and colleagues (13) reported increased production of IL-4 and IL-10 in the heart of myocardiatic mice following Gal-9 administration. Taken together, we provided evidence that Gal-9 administration could inhibit Th1 and Th17 cells in the spleen and kidneys and enhance the renal Th2 immune response in anti-GBM GN. The limitation of our study might be that we are not able to determine if the increased Th2 population in the kidneys is caused by Gal-9 directly or is secondary to the inhibition of the Th1 and Th17 immune responses.

The mechanism of Gal-9 regulating the differentiation of CD4$^+$ T cells is partially dependent on the interaction with Tim-3 (16, 37). Our study revealed that Gal-9 inhibited the expression of Th1- and Th17-specific cytokines (IFN-γ and IL-17A) and transcription factors (T-bet and RORγt) in vitro and this effect could be reversed by α-lactose, which blocks the binding between Gal-9 and Tim-3. In addition, in the presence of Gal-9, the proportion of Th2 cells in activated CD4$^+$ cells increased significantly, and α-lactose could not suppress the increase of Th2 cells. This indicates that the regulation of Gal-9 to Th2 cells was cell-contact independent. Several studies have demonstrated that IL-17 inhibited Th1 cells differentiation (12, 29), so we added anti-IL-17A antibody to neutralize the endogenous IL-17A in the in vitro experiments, but anti-IL-17A did not interfere with Gal-9 suppression of Th1 and Th17 differentiation. Furthermore, we found that the expression of Tim-3 mRNA was downregulated in the presence of Gal-9 both in vitro and in vivo. In regard to the apoptosis of CD4$^+$Tim-3$^+$ T cells induced by Gal-9 (37), the lower expression of Tim-3 mRNA might be attributed to the decreased amount of Tim-3$^+$ T cells. In addition, a recent study indicated that Tim-3 expression is in part regulated by the Th1-specific transcription factor T-bet, which binds to the Tim-3 promoter directly and drives Tim-3 expression (3, 36). This raises the additional possibility that the downregulation of Tim-3 mRNA might result from the decreased expression of T-bet in activated CD4$^+$ T cells.

In summary, our studies revealed that Gal-9 administration ameliorated renal injury in anti-GBM GN. This protective effect is associated with the inhibition of Th1- and Th17-cell-mediated immune responses in the spleen and kidneys. The enhanced Th2-mediated immune response in the kidneys might also play a role in protecting kidneys from anti-GBM GN.

**DISCLOSURES**

Drs. T. Niki and M. Hirashima are board members of GalPharma Co., Ltd. Although there are patents and products in development, this does not alter these authors’ adherence to all of the American Journal of Physiology-Renal Physiology polices on sharing data and materials, as detailed in the guide for authors.
AUTHOR CONTRIBUTIONS

Author contributions: Q.Z., H.L., and H.Z. performed experiments; Q.Z., L.W., F.H., and J.Y. analyzed data; Q.Z., X.X., Q.X., and J.Y. drafted manuscript; L.W. and H.Z. interpreted results of experiments; X.X. and X.L. prepared figures; X.L., T.N., M.H., G.X., Y.L., and J.Y. edited and revised manuscript; Y.L. and J.Y. conception and design of research; Y.L. and J.Y. approved final version of manuscript.

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