The American Journal of Pathology, Vol. 183, No. 2, August 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Regulation of Pathogenic Th17 Cell Differentiation by IL-10 in the Development of Glomerulonephritis

Ruihua Zhang,* Qin Li,*† Peter Y. Chuang,† Geming Lu,* Ruijie Liu,‡ Jianjun Yang,* Liang Peng,* Yan Dai,‡ Zihan Zheng,* Chen-Feng Qi,§ John Cijiang He,‡ and Huabao Xiong*

From the Immunology Institute and the Division of Nephrology,‡ Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York; the Shanghai Blood Center,† Shanghai, China; and the Laboratory of Immunogenetics,§ National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland*

Accepted for publication
May 1, 2013.

Address correspondence to Huabao Xiong, M.D., Ph.D., Immunology Institute, Box 1630, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, New York, NY 10029-6574; or John Cijiang He, M.D., Division of Nephrology, Department of Medicine, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, New York, NY 10029-6574. E-mail: huabao.xiong@mssm.edu or cijiang.he@mssm.edu.

Although it is clear that T helper (Th)17 cells play a pathologic role in the pathogenesis of several inflammatory diseases, the contribution and regulation of pathogenic Th17 cells in the development of glomerulonephritis are still not fully understood. Herein, we show that IL-10–deficient mice exhibit exacerbation of glomerulonephritis after induction with anti–glomerular basement membrane globulin, with enhanced pathogenic Th17 immune responses. We further demonstrate that *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells develop more severe glomerulonephritis after induction of anti–glomerular basement membrane disease, with more infiltration of inflammatory cells into the kidneys. Finally, IL-17 and interferon γ double-positive cells were significantly increased in *IL-10*$^{-/-}$ CD4^{+} T-cell cultures under pathogenic Th17 conditions compared with wild-type cell cultures. These findings suggest that T-cell–derived IL-10 plays a critical suppressive role in the control of pathogenic Th17 cell differentiation and highlights the importance of IL-10 as protection against glomerulonephritis development. *(Am J Pathol 2013, 183: 402–412; http://dx.doi.org/10.1016/j.ajpath.2013.05.001)*

T helper (Th)17 cells, which are clearly distinct from Th1 and Th2 cells, have been defined as an additional Th cell subset that mediates proinflammatory and autoimmune responses through the production of Th17 signature cytokines, including IL-17A, IL-17F, and IL-22.[1–4] Synergy between the cytokines transforming growth factor (TGF)-β and IL-6 induces *in vitro* development of Th17 cells,[5–8] whereas IL-23 promotes the survival and expansion of Th17 cell populations.[2,5,9,10] IL-23 is also believed to play an important role in the development of pathogenic Th17 cells.[11] Several transcription factors are involved in the regulation of Th17 cell differentiation. Among them, RORγt, a member of the orphan nuclear receptor family, has been identified as the master transcription factor for Th17 cell development.[12] Other transcription factors, including RORα, STAT3, IRF4, and IRF8, are also involved in the control of Th17 cell differentiation.[13] In addition, the differentiation of Th17 cells is also regulated by several positive and negative feedback loops involving IL-21, IL-23R, IL-10, and IL-27.[6,7,14–18] There is increasing evidence that Th17 cells are involved in the pathogenesis of various autoimmune/inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, and asthma.[19] Thus, a more complete understanding of the molecular mechanisms involved in the regulation of Th17 immune responses and its roles in different inflammatory diseases should provide insights into the pathogenesis and treatment of inflammatory diseases.

Glomerulonephritis (GN) is a renal disease observed as inflammation in glomeruli and small blood vessels of the kidneys.[20] The presentation of GN may include hematuria, proteinuria, and a variable degree of renal failure.[20] The mechanisms underlying the pathogenesis of GN are incompletely understood. It has been believed that Th1-mediated immune responses are involved in the development of

Supported by NIH grants P01 DK072201 and R56AI091871 and by the Broad Medical Research Program of The Broad Foundation (H.X.).

R.Z. and Q.L. contributed equally to this work.

http://dx.doi.org/10.1016/j.ajpath.2013.05.001

GN,[21,22] but more recent studies suggest that Th17 cells instead of Th1 cells contribute to the pathogenesis of GN.[23–26] However, the functions and regulation of Th17 cells in the development of GN still need to be further explored.

IL-10, identified by Mosmann and colleagues in 1989,[27] is a pluripotent cytokine produced by many activated immune cell types, including Th cells, B cells, macrophages, monocytes, and keratinocytes.[28] IL-10 activates through the IL-10 receptor (IL-10R), which is expressed on a variety of cell types.[28] The IL-10R is composed of two chains, IL-10R1 and IL-10R2.[28] Interaction of IL-10 with the IL-10R results in STAT3-mediated signal transduction.[29] IL-10 inhibits Th1 cell differentiation and IL-12 production in macrophages. We recently demonstrated that IL-10 plays a negative role in the regulation of Th17 cell development.[18] IL-10–deficient mice spontaneously develop colitis; a condition once attributed to an enhanced Th1 immune response. Recent studies suggest that Th17 cells may contribute to the development of colitis in IL-10–deficient mice.[30] Kitching et al[31] reported that endogenous IL-10 regulates Th1 immune responses that induce crescentic GN. However, the importance of IL-10 in the regulation of pathogenic Th17 cell differentiation during the development of GN is still not fully understood.

In the present study, we show that mice deficient in IL-10 exhibit more severe GN after induction with anti–glomerular basement membrane (aGBM) globulin, with enhanced Th17 immune responses. We further demonstrate that *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4$^+$ T cells develop more severe GN after induction of aGBM disease, with more infiltration of inflammatory cells into the kidneys. Finally, the IL-17 and interferon (IFN)-γ double-positive cell populations were significantly higher in *IL-10*$^{-/-}$ CD4$^+$ T-cell cultures under pathogenic Th17 conditions compared with wild-type (WT) cell cultures, and the double-positive cells were significantly increased in *IL-10*$^{-/-}$ mice with GN. These findings suggest that IL-10 plays a critical suppressive role in the control of pathogenic Th17 cell differentiation and highlights the importance of IL-10 as protection against GN development.

Materials and Methods

Mice

C57BL/6J and IL-10–deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the barrier facility at the Icahn School of Medicine at Mount Sinai (New York, NY). The animal study protocols were approved by the Institutional Animal Care and Use Committees of the Icahn School of Medicine at Mount Sinai.

Antibodies

The following antibodies were purchased from BD Biosciences (San Diego, CA), as conjugated to fluorescein isothiocyanate, phosphatidylethanolamine, phosphatidylethanolamine-Cy5, perCP-Cy5.5, or APC: CD4 (L3T4), CD8 (53-6.7), CD3e (145-2C11), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD45RB (C363-16A), IL-17 (TC11-18H10), IFN-γ (XMG1.2), and isotype controls. Antibodies for IL-2 (JES6-1A12), RORγ (B2D), IL-10 (JES5-16E3), and Foxp3 (FJK-16S) were purchased from eBioscience Inc. (San Diego, CA).

Induction of Accelerated aGMB GN

WT and *IL-10*$^{-/-}$ mice ($n = 8$ per group) were sensitized by s.c. injection of 0.5 mg of sheep globulin (SG) in Freund complete adjuvant. Five days later, SG-sensitized mice were injected i.p. with sheep anti-mouse GMB antibody at 30 mg per mouse. Renal injury and systemic immune responses were assessed 14 days later. For assessment of antigen-specific immune responses, a separate group of mice was injected s.c. with SG in Freund complete adjuvant, and the mice were sacrificed 1 week later.

CD4$^+$ T-Cell Preparation and Differentiation *in Vitro*

Naive CD4$^+$ T cells (CD62L$^+$CD44^{lo}) were prepared by fluorescence-activated cell sorting (FACS) from the spleens and lymph nodes of *IL-10*$^{-/-}$ mice and their WT littermates. The sorted cells were primed for 96 hours with 1 μg/mL of anti-CD3 (145-2C11; BD Biosciences) and 2 μg/mL of soluble anti-CD28 (37.51; BD Biosciences). The cells were rested for 48 hours and were then restimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA) plus ionomycin in the presence of brefeldin A, and intracellular cytokines were measured by flow cytometry. Cells stimulated under neutral conditions were defined as Th0 cells. Cells were stimulated to differentiate into Th1 cells by supplementation with IL-12 plus anti–IL-4 or into Th2 cells by supplementation with IL-4 and anti–IFN-γ. For Th17 cell differentiation, cells were stimulated with 5 ng/mL of TGF-β1, 20 ng/mL of IL-6 added with or without 10 ng/mL of IL-23 (all from R&D Systems, Minneapolis, MN) in the presence of 10 μg/mL of anti–IL-4 antibody (11B11; BD Bioscences).

Assessment of Renal Injuries

Glomerular abnormalities were analyzed on PAS-stained, Bouin-fixed, paraffin-embedded sections (3 μm thick) using coded slides. Abnormalities included crescent formation and severe necrosis. A minimum of 50 glomeruli were analyzed per animal to determine the percentage of crescentic glomeruli. Semiquantitative analysis of tubulointerstitial damage was performed in each mouse as previously published[32,33] using 10 randomly selected cortical areas (×200). Injury was defined as tubular dilation, tubular atrophy, sloughing of tubular epithelial cells, or thickening of the basement membrane.[32,33] Each cortical field was scored (0 to 4) according to the amount of injury: 0 indicates no interstitial damage; 1, 25% of the tubulointerstitium damaged;

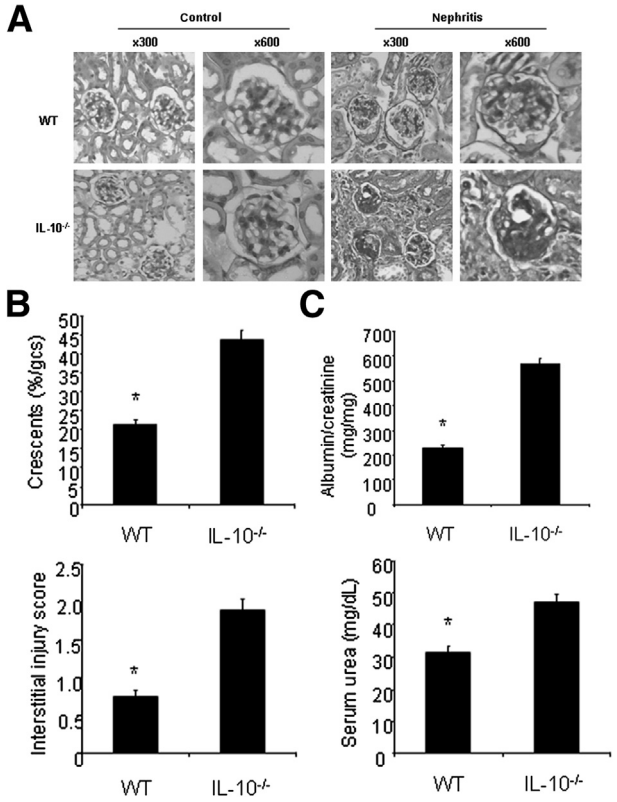


Figure 1 IL-10—deficient mice develop more severe GN. WT and *IL-10*$^{-/-}$ mice were sensitized with SG for 5 days, and the mice were then were injected i.p. with sheep anti-mouse GBM globulin at a dose of 30 mg per mouse. Renal injuries were assessed 14 days later. **A**: PAS staining of glomeruli in WT and *IL-10*$^{-/-}$ mice. **B**: Quantification of histologic injury showed aggravation of GN in *IL-10*$^{-/-}$ mice ($n = 6$ per group). gcs, glomerular cross section. **C**: Functional injury indicated more albuminuria and higher serum urea levels in *IL-10*$^{-/-}$ mice ($n = 6$ per group). Data are given as means ± SD. *$P < 0.05$ versus *IL-10*$^{-/-}$ mice.

2, 25% to 50% damaged; 3, 50% to 75% damaged; and 4, >75% of the tubulointerstitium damaged. For urine collection, mice were housed for 24 hours in metabolic cages with free access to tap water, whereas serum was collected after the mice were sacrificed. Albuminuria was analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience).

Renal Leukocyte Infiltration

Kidney sections were fixed in periodate-lysine-paraformaldehyde for 4 hours, washed with 20% sucrose solution, and then frozen. Tissue sections were cut, and immunofluorescence staining was used to stain for T cells, macrophages, and neutrophils. The antibodies of anti-mouse CD3 (145-2C11), anti—MAC-2, and anti—Gr-1(RB6-8C5) were used as primary antibodies to detect T cells, macrophages, and neutrophils, respectively (BD Biosciences). At least 25 consecutively viewed glomeruli were assessed per animal, and the results were expressed as cells per glomerular cross section.

Intracellular Staining and Flow Cytometry

Cells were stimulated with PMA and ionomycin for 5 hours in the presence of brefeldin A before intracellular staining. Cells were fixed with intracellular fixation buffer (BD Biosciences), incubated with permeabilization buffer, and stained with phosphatidylethanolamine—anti-mouse IL-17, APC—anti—IFN-γ, and phosphatidylethanolamine-Cy5.5 anti-mouse CD4 antibodies. Flow cytometry was performed using a FACSCalibur system (BD Biosciences).

RNA Isolation and Real-Time RT-qPCR

Total RNA was extracted using an RNeasy Plus kit (Qiagen Inc., Valencia, CA), and cDNA was generated using an oligo (dT) primer and the SuperScript II system (Invitrogen, Carlsbad, CA), followed by analysis using iCycler PCR with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Results were normalized based on the expression of ubiquitin. The following primer sets (sense and antisense) were used: IL-17A, 5′-CTCCAGAAGGCCCTCAGACT-AC-3′ and 5′AGCTTTCCCTCCGCATTGACACAG-3′; IL-21, 5′-CGCCTCCTGATTAGACTTCG-3′ and 5′-GCCCC-TTTACATCTTGTGGA-3′; RORγt, 5′-CCGCTGAGAGG-GCTTCAC-3′ and 5′-TGCAGGAGTAGGCCACATTAC-A-3′; and ubiquitin, 5′-TGGCTATTAATTATTCGGTCT-GCA-3′ and 5′-GCAAGTGGCTAGAGTGCAGAGTAA-3′.

Cell Transfer Studies

Splenocytes or CD4$^+$ T cells from WT and *IL-10*$^{-/-}$ mice were injected i.p. into *Rag1*$^{-/-}$ recipients (1×10^7 splenocytes or 4×10^6 CD4$^+$ T cells per mouse in 200 μL of sterile PBS per injection). Mice were then immunized by s.c. injection of 0.5 mg in Freund complete adjuvant, and 5 days later the SG-sensitized mice were i.p. injected with sheep anti-mouse GBM globulin at a dose of 30 mg per mouse. Fourteen days later, renal injury and systemic immune responses were assessed. For evaluation of antigen-specific immune responses, a separate group of mice was weighed every week throughout the course of the experiments. After 5 weeks, the mice were sacrificed and their kidney tissues were excised. The tissues were fixed in 10% buffered formalin and were paraffin embedded. Tissue sections (5 μm thick) were stained with H&E. All the slides were read and scored by an experienced pathologist without previous knowledge of the type of treatment. The degree of inflammation in the epithelium, submucosa, and submuscularis propria was scored separately as described previously.[34]

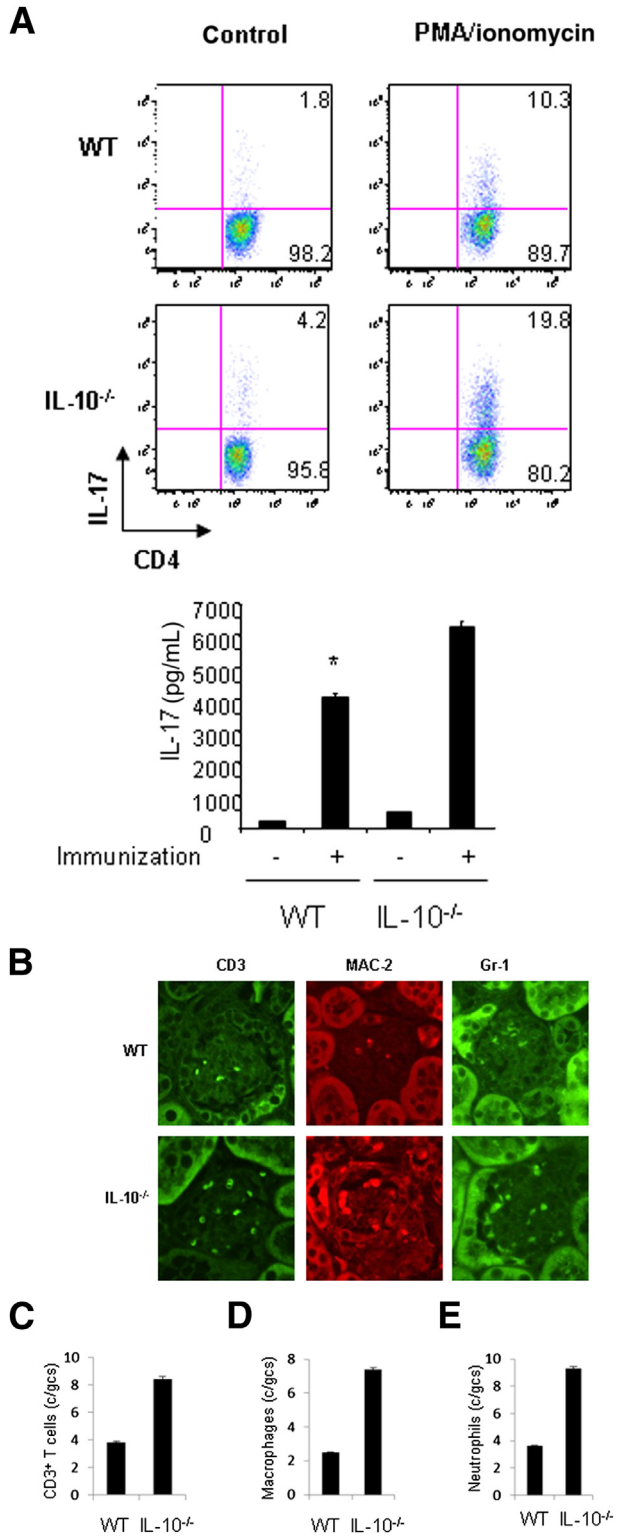
Cytokine ELISA

Supernatants from cell cultures were collected after activation under various conditions, and secreted cytokines in the supernatants were measured by ELISA kits with purified

coating and biotinylated detection antibodies: anti—IL-17 (R&D Systems).

Statistical Analysis

Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.



Results

IL-10 Deficiency Exacerbates GN

To investigate the function of IL-10 in the development of GN, we induced GN by using the aGBM disease model. IL-10—deficient mice developed more severe nephritis after injection of aGBM globulin into sensitized mice compared with WT mice (Figure 1A). *IL-10*$^{-/-}$ mice displayed more severe histologic and functional renal injury and exhibited more glomerular crescents and severe interstitial injury (Figure 1B). In addition, *IL-10*$^{-/-}$ mice also had more albuminuria and higher serum urea nitrogen levels compared with WT mice (Figure 1C). Taken together, the results suggest that anti-inflammatory cytokine IL-10 plays a protective role in the development of GN.

Enhanced Immune Responses in *IL-10*$^{-/-}$ Mice with aGBM Disease

Th17 cells are thought to be pathogenic in several inflammatory diseases. As such, a study of Th17 cell development seems critical to understanding disease progression. We began to investigate the development of Th17 cells in WT and *IL-10*$^{-/-}$ mice with GN. We prepared splenocytes from WT and *IL-10*$^{-/-}$ mice with GN induced by aGBM and restimulated the cells with SG for 24 hours. After stimulation, IL-17—producing CD4$^+$ T cells were analyzed by FACS, and IL-17 protein production was determined by ELISA. The percentage of IL-17—producing CD4$^+$ T cells was significantly higher in *IL-10*$^{-/-}$ cell cultures (19.8%) than in WT cell cultures (10.3%) (Figure 2A). Similarly, IL-17 protein production was significantly enhanced in *IL-10*$^{-/-}$ cell cultures compared with WT cell cultures (Figure 2A). Furthermore, we assessed infiltration of inflammatory cells in the kidneys of WT and *IL-10*$^{-/-}$ mice with GN. Histologic analysis showed that *IL-10*$^{-/-}$ mice have increased numbers of glomerular CD3$^+$ T cells, MAC-2$^+$ macrophages, and Gr-1$^+$ neutrophils compared with WT mice (Figure 2, B—E), suggesting that more inflammatory cells were present in the kidneys of *IL-10*$^{-/-}$ mice with GN. In addition, we also analyzed Th1, regulatory T (Treg), and Th2 cells in WT and *IL-10*$^{-/-}$ mice with GN. Splenocytes from WT and *IL-10*$^{-/-}$ mice with GN were restimulated with SG for 24 hours. IFN-γ—producing, IL-4—producing, and Treg cells were analyzed by FACS. The percentage of IFN-γ—producing CD4$^+$

Figure 2 Enhanced immune responses in *IL-10*$^{-/-}$ mice with GN. **A:** Splenocytes were prepared from WT or *IL-10*$^{-/-}$ mice with GN induced by aGBM, and the cells were activated with SG for 24 hours. IL-17—producing CD4$^+$ T cells were analyzed by FACS, and IL-17 protein levels in the supernatants were determined by ELISA. *$P < 0.05$, versus immunized *IL-10*$^{-/-}$ mice. **B:** WT or *IL-10*$^{-/-}$ mice were sensitized with SG for 5 days, and the mice were then injected i.p. with sheep anti-mouse GBM antibody at a dose of 30 mg per mouse. Renal injuries were assessed 14 days later. The infiltration of immune cells was analyzed by immunostaining in the kidneys. Quantification of T cells (**C**), macrophages (**D**), and neutrophils (**E**) in the kidneys of WT and *IL-10*$^{-/-}$ mice. gcs, glomerular cross section. Data are given as means ± SD.

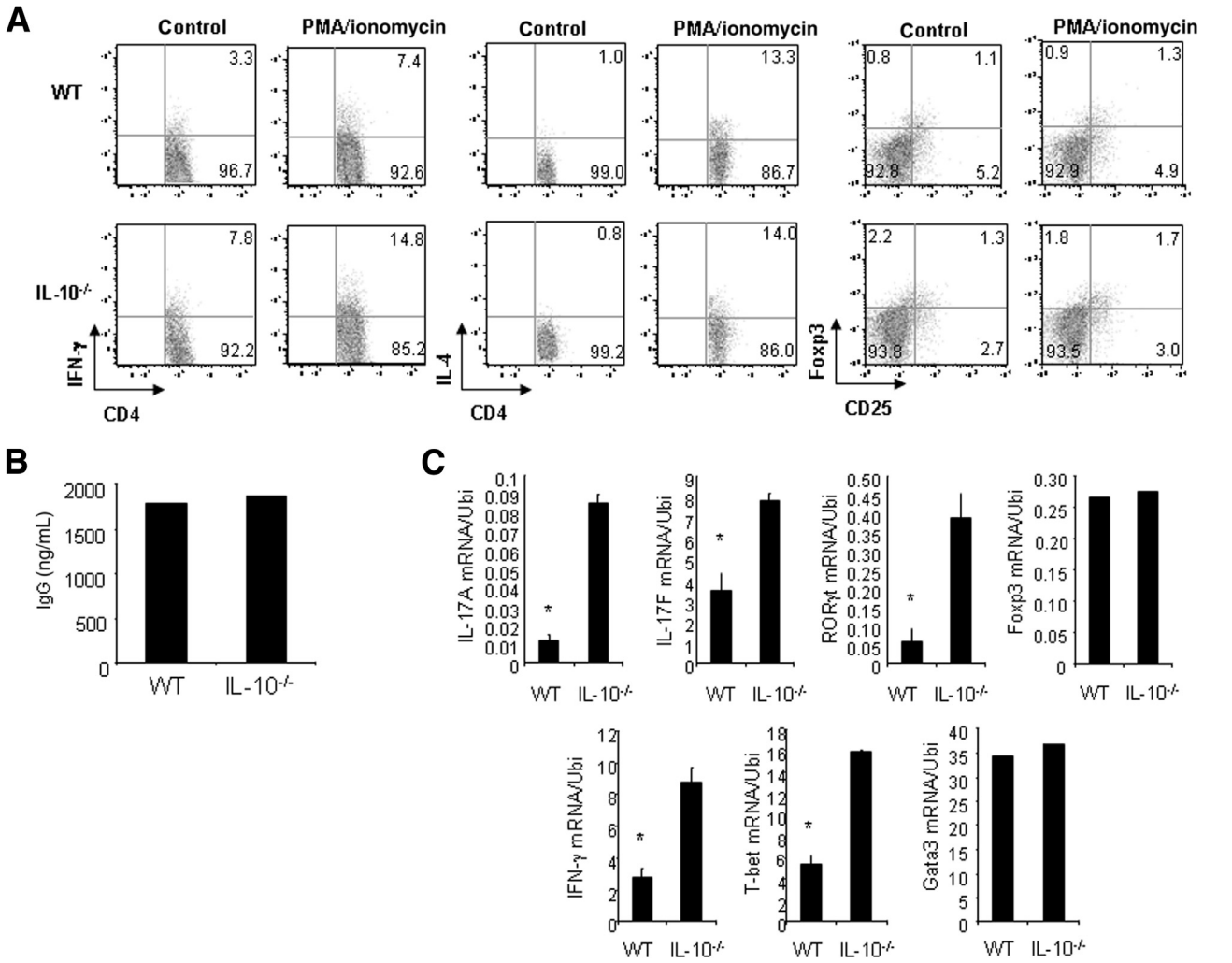


Figure 3 Th17 and Th1 immune responses are enhanced in *IL-10*$^{-/-}$ mice with GN. **A:** Cells from spleens and mesenteric lymph nodes were prepared from WT and *IL-10*$^{-/-}$ mice with GN induced by aGBM, and the cells were activated with SG for 24 hours. IFN-γ—producing, IL-4—producing, and Treg CD4$^+$ T cells were analyzed by FACS. **B:** Serum was collected from WT and *IL-10*$^{-/-}$ mice with GN induced by aGBM, and the serum IgG level was determined by ELISA. **C:** Total RNA was extracted from the kidneys of WT and *IL-10*$^{-/-}$ mice with GN induced by aGBM, and real-time PCR was performed for the analysis of mRNA expression of the indicated genes. *$P < 0.05$ versus immunized *IL-10*$^{-/-}$ mice. Data are given as means ± SD. Ubi, ubiquitin.

T cells was significantly increased in *IL-10*$^{-/-}$ cell cultures (14.8%) compared with WT cell cultures (7.4%) (Figure 3A). The percentages of IL-4—producing and Treg cells were comparable in WT and *IL-10*$^{-/-}$ cell cultures (Figure 3A). Furthermore, IgG levels in WT and *IL-10*$^{-/-}$ mice with GN were similar, ruling out B-cell involvement as a cause of *IL-10*$^{-/-}$ mice developing worse GN (Figure 3B). Real-time PCR analysis indicated that the mRNA expression of signature molecules for Th17 and Th1 cells was significantly enhanced in the kidneys of *IL-10*$^{-/-}$ compared with WT mice with GN (Figure 3C). Transcript levels of transcription factors (Gata3 and FOXP3) for Th2 and Treg cells, however, were not altered (Figure 3C). In addition, IL-10 mRNA expression was slightly increased in WT mice with GN (Figure 4A). To investigate IL-10 expression from different effector T cells, we activated naive CD4$^+$ T cells from C57Bl/6 mice under Th1, Th2, Th17, or Treg cell conditions and found that all these effector T cells express IL-10 (Figure 4B). Taken together, these results suggest that IL-10 negatively regulates Th17 and Th1 immune responses in the development of GN.

Transfer of CD4$^+$ T Cells Induces More Severe GN in *RAG1*$^{-/-}$ Mice

These data suggest that Th17 cells may be involved in the development of GN in *IL-10*$^{-/-}$ mice. Next we wanted to confirm that the disease development is really due to CD4$^+$ T cells. We first prepared splenocytes from WT and *IL-10*$^{-/-}$ mice and transferred the cells into *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ splenocytes developed more severe GN, with more severe histologic injury and functional impairment of the kidney compared with mice reconstituted with WT splenocytes after induction of aGBM disease (Figure 5, A and B). In addition, mice reconstituted with *IL-10*$^{-/-}$ exhibited significantly larger spleens

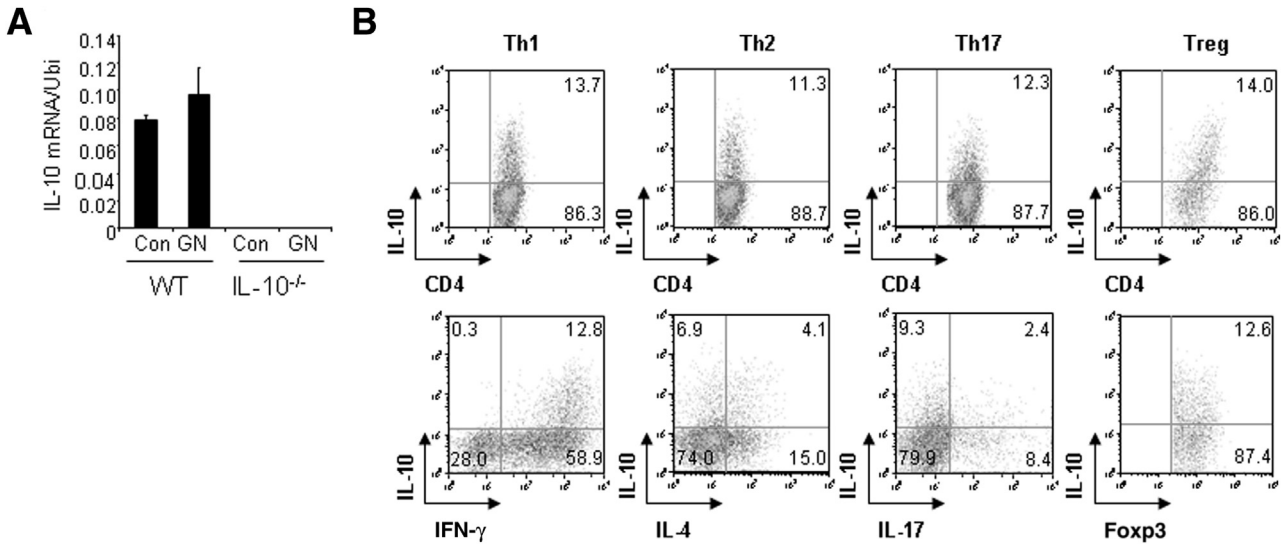


Figure 4 IL-10 is expressed in different effector T cells. **A:** WT and *IL-10*$^{-/-}$ mice were sensitized with SG for 5 , and the mice were then injected i.p. with sheep anti-mouse GBM globulin at a dose of 30 mg per mouse. Total RNA was extracted from the kidneys of WT and *IL-10*$^{-/-}$ mice with GN induced by aGBM or control mice. Real-time PCR was performed for the analysis of IL-10 mRNA expression. Data are given as means ± SD. **B:** Naive CD4^{+} T cells from WT and *IL-10*$^{-/-}$ mice were differentiated under nonpathogenic Th17, pathogenic Th17, or Th1 conditions for 3 days. Cells were then restimulated with PMA/ionomycin for 5 hours; stained for intracellular IL-10, IL-17, IFN-γ, IL-4, and Foxp3; and analyzed by flow cytometry. Representative FACS dot plots gated on CD4^{+} cells and the percentages of IL-17—producing or IFN-γ—producing or double-positive CD4^{+} cells are shown.

(Figure 5C), with more cells in the spleens after induction of GN (Figure 5C), suggesting that alterations of the immune responses and immune microenvironment had occurred. Meanwhile, significantly higher Th17 immune responses were observed in *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ splenocytes (Figure 5D).

To further clarify the results, CD4^{+} T cells were extracted from spleens of WT and *IL-10*$^{-/-}$ mice and then were transferred into *Rag1*$^{-/-}$ mice. After adoptive transfer of CD4^{+} T cells into *Rag1*$^{-/-}$ mice, aGBM disease was induced in these mice. Mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells developed more severe renal disease compared with mice

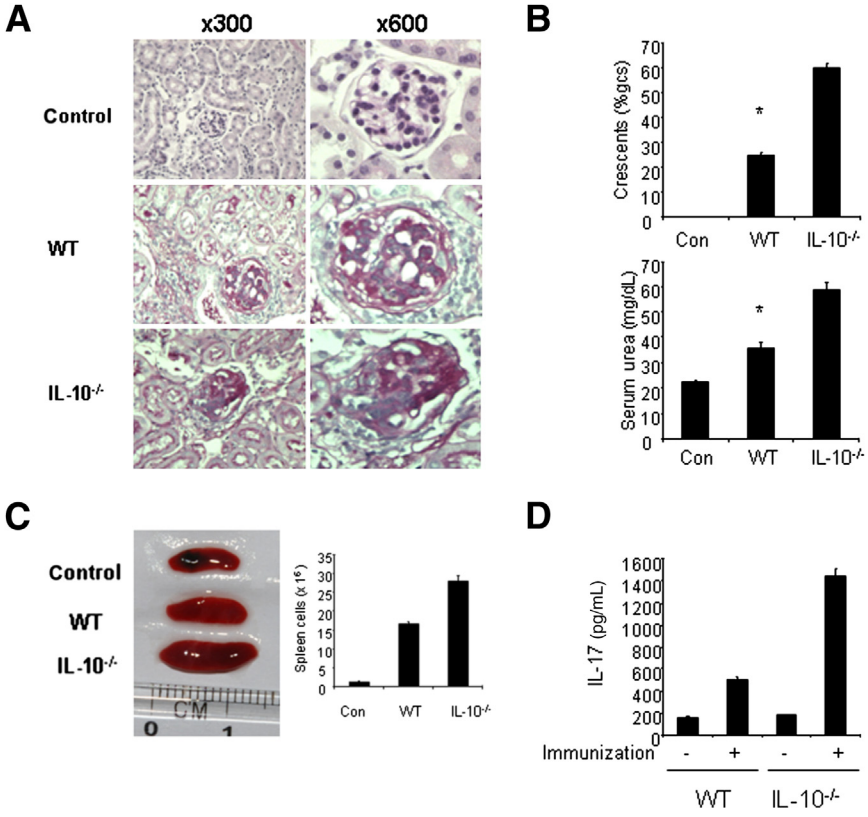


Figure 5 Transfer of *IL-10*$^{-/-}$ spleen cells induces more severe GN in *Rag1*$^{-/-}$ mice. **A:** Spleen cells from WT and *IL-10*$^{-/-}$ mice were prepared and were injected i.p. into *Rag1*$^{-/-}$ mice. Beginning the next day, mice were sensitized with SG for 5 days, and the mice were then injected i.p. with sheep anti-mouse GBM globulin at a dose of 30 mg per mouse. Renal injuries were assessed 20 days later. **A:** PAS staining of glomeruli in mice reconstituted with either WT or *IL-10*$^{-/-}$ spleen cells. **B:** Quantification of histologic injury showed aggravation of GN in mice reconstituted with either WT or *IL-10*$^{-/-}$ spleen cells. gcs, glomerular cross section. *$P < 0.05$ versus mice transferred with *IL-10*$^{-/-}$ spleen cells. **C:** Spleens were enlarged and spleen cells were significantly increased in *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ splenocytes. **D:** Splenocytes were prepared from *Rag1*$^{-/-}$ mice reconstituted with either WT or *IL-10*$^{-/-}$ spleen cells with GN induced by aGBM globulin, and the cells were activated with SG for 24 hours. The supernatants were harvested, and IL-17 protein levels were determined by ELISA. Data are given as means ± SD.

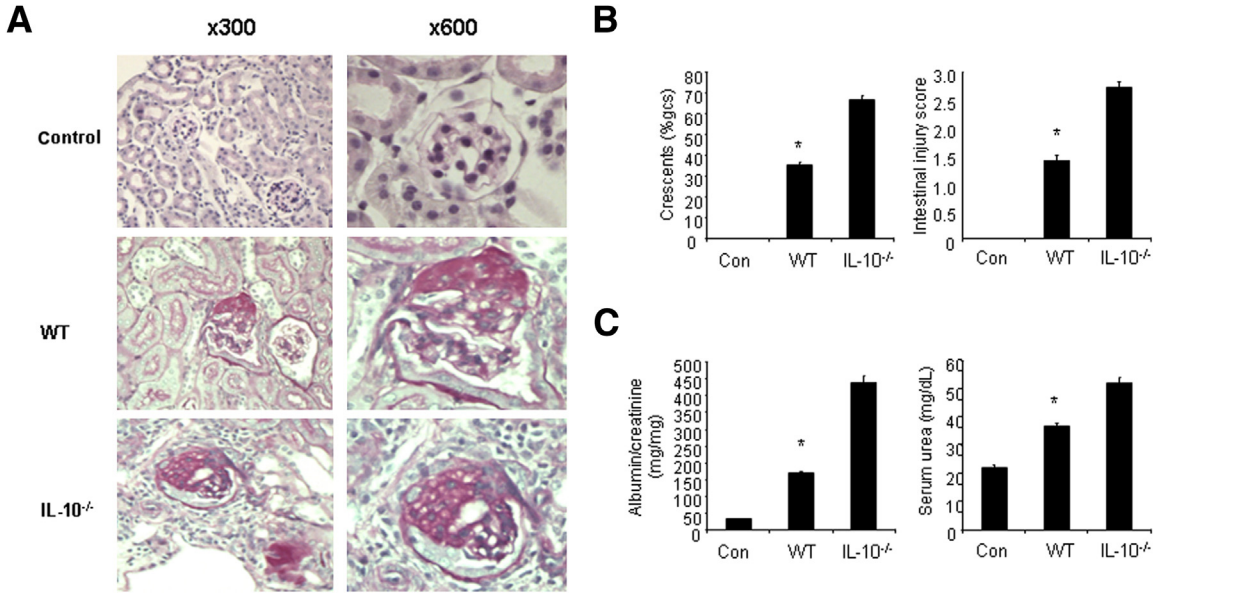


Figure 6 Transfer of *IL-10*$^{-/-}$ CD4^{+} T cells induces more severe GN in *Rag1*$^{-/-}$ mice. **A:** CD4^{+} cells from spleens of WT and *IL-10*$^{-/-}$ mice were prepared and injected i.p. into *Rag1*$^{-/-}$ mice. From next day, mice were sensitized with SG for 5 days, and the mice were then injected i.p. with sheep anti-mouse GBM globulin at a dose of 30 mg per mouse. Renal injuries were assessed 20 days later. **A:** PAS staining of glomeruli in mice reconstituted with either WT or *IL-10*$^{-/-}$ CD4^{+} T cells. **B:** Quantification of histologic injury showed aggravation of GN in mice reconstituted with either WT or *IL-10*$^{-/-}$ CD4^{+} T cells ($n = 6$ per group). gcs, glomerular cross section. **C:** Functional injury indicated more albuminuria and higher serum urea levels in *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells ($n = 6$ per group). Data are given as means ± SD. *$P < 0.05$ versus *IL-10*$^{-/-}$ mice.

reconstituted with WT CD4^{+} T cells (Figure 6A). *IL-10*$^{-/-}$ CD4^{+} T cells transferred to *Rag1*$^{-/-}$ mice developed severe glomerular and interstitial injury (Figure 6B) and higher albuminuria and serum urea (Figure 6C). Taken together, these results demonstrate that the anti-inflammatory cytokine IL-10 plays an important role in the control of GN development, suggesting that IL-10 could be a therapeutic choice for the treatment of immune-related GN.

IL-10 Regulates Pathogenic Th17 Cell Differentiation in GN

To explore how IL-10 regulates pathogenic Th17 cell development during GN, we focused on *Rag1*$^{-/-}$ mice reconstituted with WT and *IL-10*$^{-/-}$ CD4^{+} T cells followed by immunization with SG for 6 days. We found that recipient mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells displayed enlarged spleens and increased splenocyte counts (Figure 7A) compared with mice transferred with WT CD4^{+} T cells. The spleen cells were restimulated with SG for 24 hours, and IL-17–producing CD4^{+} T cells were analyzed by flow cytometry. As expected, the percentage of Th17 cells was significantly higher in mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells (Figure 7B). In addition, significantly more inflammatory cells, including CD3^{+} T cells, MAC-2 macrophages, and Gr-1^{+} neutrophils were present in the kidneys of mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells (Figure 7, C and D). These results demonstrate that *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells develop more severe GN, with enhanced Th17 immune responses.

To further investigate the regulation of pathogenic Th17 cell differentiation by IL-10, we purified naive CD4^{+} T cells from the spleens and lymph nodes of WT and *IL-10*$^{-/-}$ mice and stimulated the cells under nonpathogenic Th17 conditions (TGF-β1 and IL-6), pathogenic Th17 conditions (TGF-β1, IL-6, and IL-23), or Th1 conditions (IL-12) for 3 days. The IL-17–producing CD4^{+} T-cell population was significantly higher in *IL-10*$^{-/-}$ cell cultures compared with WT cell cultures (Figure 8A).[18] Under pathogenic Th17 conditions, the IL-17 and IFN-γ double-positive cell populations also significantly increased, which is similar to the response in IL-17 single-positive cells (Figure 8A). In addition, IFN-γ–producing cells were moderately increased (Figure 8A). IL-17 production and mRNA expression of Th17 signature molecules were also greatly enhanced in *IL-10*$^{-/-}$ mice under pathogenic Th17 conditions (Figure 8, B and C). To confirm that IL-10 regulates the production of Th17 cells *in vivo*, we prepared cells from the spleens and mesenteric lymph nodes of WT and *IL-10*$^{-/-}$ mice with GN and then stimulated those cells with PMA/ionomycin for 6 hours. Stimulated cells were harvested, and the IL-17 and IFN-γ double-positive CD4^{+} T cells were analyzed by FACS. As expected, the percentage of IL-17 and IFN-γ double-positive CD4^{+} T cells was significantly higher in *IL-10*$^{-/-}$ mice than in WT mice (Figure 8D). Taken together, these results suggest that IL-10 derived from T cells regulates pathogenic Th17 cell development in GN.

Discussion

Although Th17 cells have been associated with the pathogenesis of inflammatory diseases, including GN, the regulation of

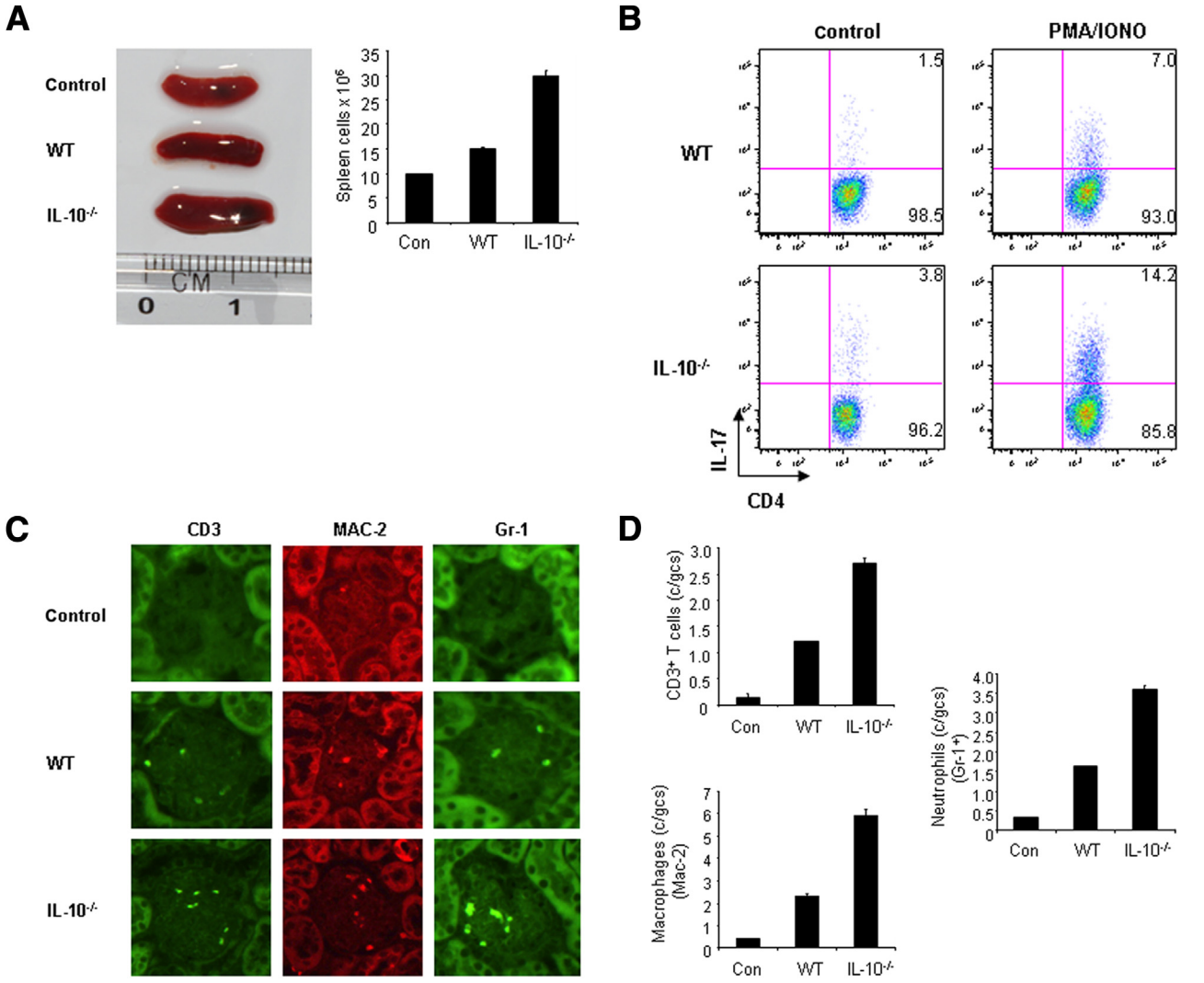


Figure 7 Th17 immune responses are enhanced in *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells. CD4^{+} cells from spleens of WT and *IL-10*$^{-/-}$ mice were prepared and injected i.p. into *Rag1*$^{-/-}$ mice. Beginning the next day, mice were sensitized with SG for 5 days, and the mice were then injected i.p. with sheep anti-mouse GBM globulin at a dose of 30 mg per mouse. The mice were sacrificed 20 days later. **A**: Spleens were enlarged and spleen cells were significantly increased in *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells. **B**: Splenocytes were prepared from *Rag1*$^{-/-}$ mice reconstituted with either WT or *IL-10*$^{-/-}$ spleen cells with GN induced by aGBM globulin, and the cells were activated with SG for 24 hours. The cells were harvested, and IL-17–producing CD4^{+} T cells were analyzed by FACS. **C**: The infiltration of immune cells was analyzed by immunostaining in the kidneys. **D**: Quantification of T cells, macrophages, and neutrophils in the kidneys of mice reconstituted with either WT or *IL-10*$^{-/-}$ CD4^{+} T cells. gcs, glomerular cross section. Data are given as means ± SD.

pathogenic Th17 cell development during the process of inflammatory diseases is incompletely understood. Therefore, it is of great importance to identify the different factors that are responsible for the suppression of pathogenic Th17 cell differentiation in the target organs of inflammatory diseases. In the present study, we demonstrated that IL-10–deficient mice exhibit exacerbation of GN after induction with aGBM globulin, with enhanced Th17 immune responses. We further demonstrated that *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells develop more severe GN with aGBM globulin induction, which is associated with increased infiltration of inflammatory cells into the kidneys. Finally, the IL-17 and IFN-γ double-positive cell populations were significantly greater in *IL-10*$^{-/-}$ CD4^{+} T-cell cultures under pathogenic Th17 conditions compared with WT cell cultures, and double-positive cells were also significantly increased in *IL-10*$^{-/-}$ mice with disease. These results suggest that IL-10 plays a critical role in the control of pathogenic Th17 cell differentiation during GN development.

IL-10 is critical in setting immune response magnitude.[28] Its genetic ablation or inhibition leads to spontaneous colitis and some other autoimmune and inflammatory diseases. Previously, Kitching et al[31] reported that endogenous IL-10 protects against crescentic GN by suppressing Th1 immune responses. In addition, El-Shemi et al[35] showed that IL-10 gene transfer suppressed experimental GN.[35] However, the exact actions performed by IL-10 in preventing the development of GN are not well known. Recently, the new

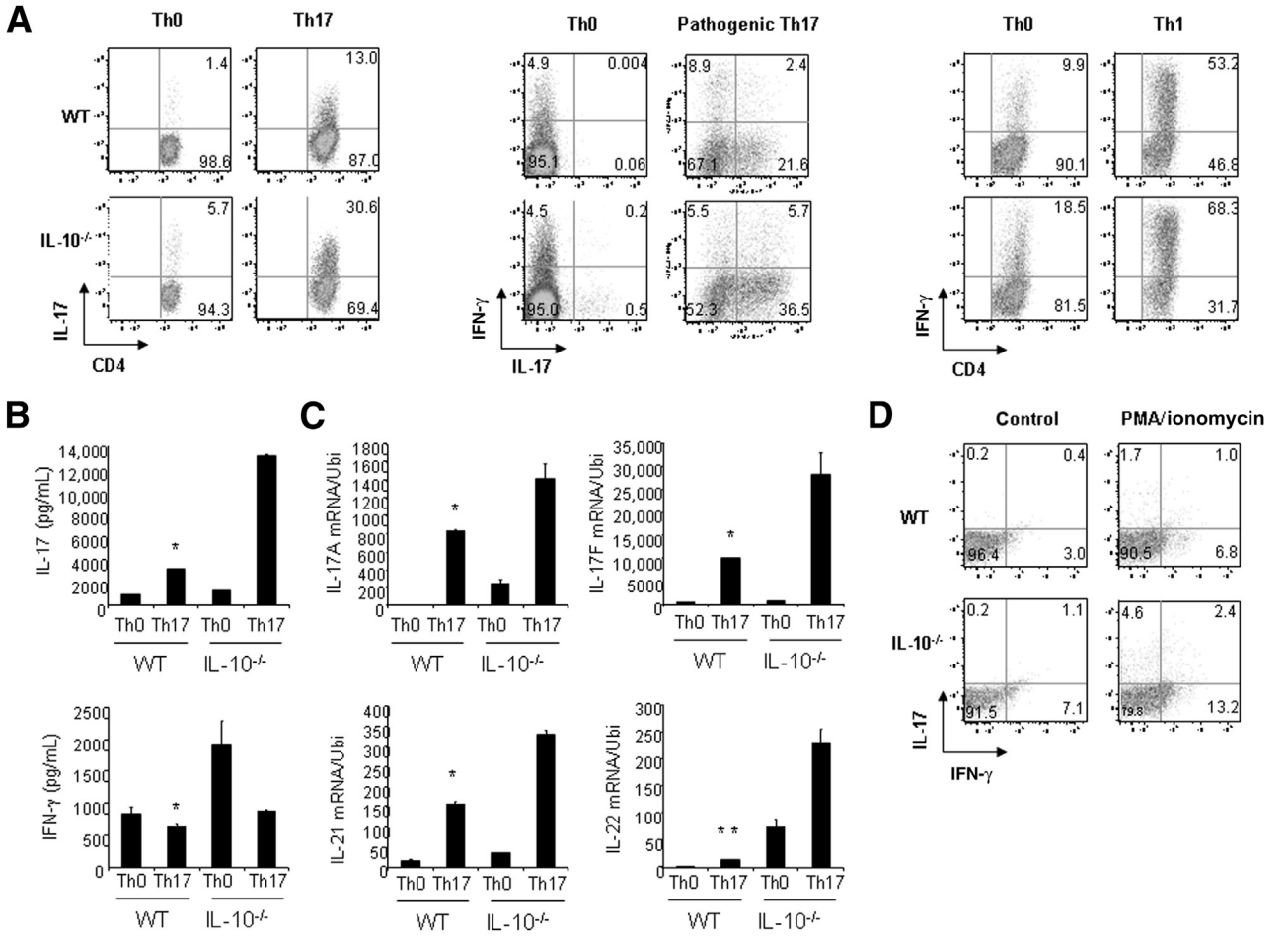


Figure 8 IL-10 suppresses pathogenic Th17 cell differentiation. **A:** Naive CD4$^+$ T cells from WT and *IL-10*$^{-/-}$ mice were differentiated under nonpathogenic Th17, pathogenic Th17, or Th1 conditions for 3 days. Cells were then restimulated with PMA/ionomycin for 5 hours, stained for intracellular IL-17 and IFN-γ, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4$^+$ cells and the percentages of IL-17—producing or IFN-γ—producing or double-positive CD4$^+$ cells are shown. **B:** IL-17 and IFN-γ protein levels in the supernatants of cell culture under the pathogenic Th17 conditions prepared in **A** were determined by ELISA. **C:** Total RNA was extracted from the pathogenic Th17 cells prepared in **A,** and real-time RT-qPCR was performed for the detection of mRNA expression of cytokines. **D:** Cells from spleens and mesenteric lymph nodes were prepared from WT or *IL-10*$^{-/-}$ mice with GN induced by aGBM, and the cells were activated with PMA/ionomycin for 24 hours. IL-17 and IFN-γ double-positive cells were analyzed by FACS. Data are given as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$ versus *IL-10*$^{-/-}$ Th17 cells.

diagram indicates that Th17 cells instead of Th1 cells play the critical role in the development of inflammatory diseases. Therefore, the role of IL-10 in the development of immune-related GN needs to be revisited. In the present study, we demonstrated that IL-10—deficient mice exhibit worse GN in a mouse model induced with aGBM globulin, with enhanced Th17 immune responses. These results suggest that Th17 immune responses may be involved in the exacerbation of GN in IL-10—deficient mice.

IL-10 has been detected in human GN, in which IL-10 mRNA and protein are found in the more severe glomerular lesions.[36] IL-10 mRNA has been detected in the kidneys of rats with accelerated anti-GBM GN.[37] The expression of IL-10 in a disease situation may reflect initiation of protective mechanisms in the context of severe glomerular injury; however, the molecular regulation of IL-10 expression in a disease condition is still not fully understood. IL-10 is produced by different cell types, including macrophages, B cells, mesangial cells, keratinocytes, and T cells.[28] In the present study, we focused on T-cell—expressed IL-10 in the pathogenesis of GN. *Rag1*$^{-/-}$ mice were reconstituted with either WT CD4$^+$ T cells or *IL-10*$^{-/-}$ CD4$^+$ T cells followed by induction with anti-GBM GN. The results show that *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ developed more severe GN and renal injuries compared with mice transferred with WT cells. In addition, more Th17 cells were induced in mice reconstituted with *IL-10*$^{-/-}$ CD4$^+$ T cells as well. These results indicate that IL-10 produced by T cells negatively regulates Th17 cell differentiation, resulting in the development of GN. Although we found that different Th cells produce IL-10 *in vitro*, it is still not clear which effector Th cells are an important cellular source for IL-10 expression in the development of GN.

Because Th1 and Th17 immune responses are significantly strengthened in *IL-10*$^{-/-}$ mice, it has been difficult to resolve

the exact importance of which one (Th1 or Th17) is essential for the immune-related diseases in *IL-10*$^{-/-}$ mice. Yen et al[30] investigated IL-10 and IL-23p19 or IL-10 and IL-12p35 double knockout mice and found that IL-10 and IL-12p35 double-knockout mice develop colitis in a similar way as IL-10 single-knockout mice. Although IL-10 and IL-23p19 double-knockout mice do not develop colitis,[30] their results strongly support the idea that Th17 but not Th1 immune responses contribute to the pathogenesis of inflammatory diseases in *IL-10*$^{-/-}$ mice. In the present study, we observed that *IL-10*$^{-/-}$ or *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells developed worse GN in terms of glomerular crescent formation, serum urea nitrogen level, and inflammatory cell infiltration in the kidneys. Worsening of GN is correlated with enhanced Th17 and Th1 cell development in *IL-10*$^{-/-}$ mice. Taken together, we believe that Th17 and Th1cells regulated by IL-10 may contribute to the worsening of GN in *IL-10*$^{-/-}$ mice. However, the relative importance of Th17 and Th1 cells to the development of GN needs to be further explored in future studies.

Although Th17 cells have been associated with the induction of autoimmune/inflammatory diseases, emerging data suggest that not all Th17 cells are pathogenic and that exposure to IL-23 is crucial for their ability to induce autoimmunity.[11,14,38,39] To investigate the regulation of nonpathogenic and pathogenic Th17 cell development by IL-10, we purified naive CD4^{+} T cells from WT and *IL-10*$^{-/-}$ mice and activated the cells under either nonpathogenic conditions or pathogenic conditions. The IL-17-single-positive cells were significantly increased in *IL-10*$^{-/-}$ cell culture under nonpathogenic or pathogenic conditions compared with WT cell cultures. However, IL-17/IFN-γ double-positive cells were significantly increased in *IL-10*$^{-/-}$ cell cultures under only pathogenic Th17 conditions. In addition, the double-positive cells were also significantly increased in *IL-10*$^{-/-}$ mice with GN. These results imply that IL-10 preferentially regulates pathogenic Th17 cell development. It is known that TGF-β1 is essential for nonpathogenic Th17 cell differentiation, whereas autonomously produced TGF-β3 from Th17 cells is critical in the induction of pathogenic Th17 cells.[11] Although the present study demonstrated that IL-10 is clearly involved in the regulation of pathogenic Th17 cell induction, the molecular mechanisms underlying this phenomenon are incompletely understood. A future study will focus on the regulation of key transcription factors, including RORγt, IRF4, Ahr, and TGF-β3, by IL-10 under pathogenic Th17 conditions.

In summary, these studies clearly demonstrate that *IL-10*$^{-/-}$ mice or *Rag1*$^{-/-}$ mice reconstituted with *IL-10*$^{-/-}$ CD4^{+} T cells develop aggravated GN with enhanced Th17 and Th1 cell phenotypes. In addition, *IL-10*$^{-/-}$ mice display enhanced nonpathogenic and pathogenic Th17 cells. We suggest a novel regulation of pathogenic Th17 cell development by IL-10. These results support the concept that IL-10 expressed by T cells may play an important role in the control of pathogenesis of inflammatory diseases by controlling nonpathogenic and pathogenic Th17 immune responses.

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