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의학박사 학위논문

# 항기저막사구체신염에서 Clin three-requiring 9과 Th17 경로 유발 염증과의 관계

Clin three-requiring 9 and Th17 pathway driven inflammation in anti-glomerular basement membrane glomerulonephritis

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의학과 면역학

이 하 정

# Abstract

## Clin three-requiring 9 and Th17 pathway-driven inflammation in anti-glomerular basement membrane glomerulonephritis

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T helper 17 (Th17) lymphocytes promote renal inflammation in anti-glomerular basement membrane glomerulonephritis (anti-GBM GN), and signal transducer and activator of transcription 3 (STAT3) mediates activation of Th17 lymphocytes by interleukin 6 (IL-6) and transforming growth factor beta (TGFβ). Clin three requiring 9 (Ctr9), a subunit of RNA polymerase-associated factor complex (PAFc), regulates the transcription of IL-6/STAT3-dependent genes. Here, the role of Ctr9 in regulating Th17-driven inflammation was investigated in anti-GBM GN.

In mice, STAT3β or IL-17 knockout ameliorated anti-GBM autoantibody-induced renal injury. This phenomenon was associated with decreases in retinoic acid receptor-related orphan receptor γt (RORγt), IL-17, phosphorylated STAT3, and pro-inflammatory cytokines. Compared with wild-type mice, Ctr9 increased in

both STAT3β$^{-/-}$ and IL-17$^{-/-}$ mice injected with anti-GBM IgG, showing a negative correlation with Th17-related transcripts. Small interfering RNA (siRNA)-mediated knockdown of Ctr9 in intrarenal lymphocytes further upregulated Th17-related transcripts, consistent with repression of Th17 differentiation by Ctr9. Interestingly, Ctr9 was also expressed in human and mouse mesangial cells and downregulated in response to anti-GBM IgG or to TGFβ plus IL-17. Ctr9 in mesangial cells was even more repressed in the presence of both anti-GBM IgG and Th17-activating cytokines. Consistent with these findings, renal biopsies obtained from patients with anti-GBM GN showed consistent downregulation of Ctr9 and upregulation of phosphorylated STAT3 and IL-17 in the glomerulus.

In this study, Ctr9 is associated with suppression of Th17 differentiation in anti-GBM GN and repressed by anti-GBM IgG and IL-17 in mesangial cells. This is the first study to examine the association between Ctr9 and Th17 driven renal inflammation in anti-GBM GN. Further study should be considered to clarify whether Ctr9 has an impact on Th17 pathway as a transcriptional regulator in anti-GBM GN.

**Key words:** Anti-glomerular basement membrane glomerulonephritis, Ctr9, STAT3, Th17 pathway

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# INTRODUCTION

## Clinical manifestations of anti-glomerular basement membrane glomerulonephritis (anti-GBM GN)

Anti-glomerular basement membrane (GBM) glomerulonephritis (GN) is an autoimmune GN characterized pathologically by proliferation of mesangial and parietal epithelial cells and clinically by subacute, progressive nephritis that eventually leads to the loss of renal function within several weeks to months. Anti-GBM GN is caused by circulating auto-antibodies that react with α3- and/or α5-chain of type IV collagen in the GBM. These autoantibodies disrupt the glomerular filtration barrier and induce proliferation of mesangial and parietal epithelial cells leading to the collapse of glomerular capillary loops and loss of functioning glomeruli. Despite immunosuppressive therapy and plasmapheresis, renal survival for anti-GBM GN remains poor (14).

## Th17 pathway in autoimmune diseases

Since 1986, the Th1/Th2 hypothesis has explained how hosts induce different adaptive immune responses to eliminate various pathogens (1). This two major different immune responses are cell-mediated immune response driven by type 1

CD4 T-helper cells (Th1) and humoral immune response mediated by type 2 CD4 T-helper cells (Th2). Th1 cells enhance the cellular immunity against virus or intracellular pathogens or drive antibody mediated responses in certain subclasses of the G isotype of immunoglobulin (Ig) antibody, specifically termed IgG2a. On the contrary, Th2 cells play a key role in host defense against extracellular pathogens and in helping B-cells to produce antibodies. Although the initial differentiation is stimulated similarly by innate immune system responding to microbial antigens, parasitic antigens or allergens, the effector cytokines that are subsequently produced are different. Th1 cells secrete IFNγ and IL-12, activate macrophages, and consequently mediate organ specific autoimmune diseases including psoriasis, Crohn's disease, sarcoidosis, and acute allograft rejection in kidney. Th2 cells produce various cytokines including IL-4, IL-5, IL-10 and IL-13, which are responsible for strong antibody production, eosinophil activation and inhibition of several macrophage functions. Abnormal Th2 responses drive atopic disorders in genetically susceptible individuals and systemic sclerosis. Over the past decades, this Th1/Th2 paradigm explained many phenomena in adaptive immunity.

Recently, the Th1/Th2 paradigm have been contested by a third subset of effector Th cells that produce IL-17 (Th17 cells) (12). Th17 played distinguished effector role from Th1 and Th2 cells. Primary function of Th17 cells appear to clear pathogens that are not adequately handled by Th1 or Th2 cells. However, Th17 cells induce tissue inflammation and are associated with the main pathogenesis of

various autoimmune diseases. Experimental autoimmune encephalitis (EAE) contributed to identify the pathogenic role of Th17 cells for the first time. The IFNγ and IFNγ receptor deficient mice, as well as other Th1 deficient mice that lack IL-12p35, IL-12 receptor, and IL-18 were not saved from EAE, rather developed more severe injury. Th17 cells, different from Th1 cells, induced EAE by producing IL-17. This experimental findings were also reproduced in the human autoimmune encephalitis. Beside autoimmune encephalitis, Th17 cells are proved to play an important role in autoimmune diseases including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and autoimmune crescentic GN. Table 1 displayed Th17 pathway associated autoimmune diseases. Moreover, a few therapeutic challenges that inhibit Th17 response were performed in patients with psoriasis and Crohn's disease, although these biologic agents have failed to demonstrate significant efficacy and posed long-term safety concerns. New therapeutic targets that regulate Th17 immune response in autoimmune disease should be warranted.

Table 1. Th17 response associated autoimmune diseases

| Cytokine | Animal models | Human diseases |
|---|---|---|
| IL-17A | Collagen induced arthritis | Multiple sclerosis |
| IL-17F | Kerato-conjunctivitis sicca | Rheumatoid arthritis, Inflammatory bowel disease, Psoriasis, Lupus nephritis, Sjogren's syndrome |
| IL-22 | | Rheumatoid arthritis, Inflammatory bowel disease, Psoriasis, Lupus nephritis, Sjogrens's syndrome |
| IL-21 | Experimental autoimmune encephalomyelitis, Collagen induced arthritis, Murine lupus, Keratoconjunctivitis sicca | Inflammatory bowel disease, Psoriasis, Lupus nephritis |
| IL-26 | | Crohn's disease |
| IL-9 | Experimental autoimmune encephalomyelitis | |
| CCL20 | Experimental autoimmune encephalomyelitis, Collagen induced arthritis | Multiple sclerosis, Rheumatoid arthritis, Inflammatory bowel disease, Psoriasis |

## Th17 pathway in autoimmune crescentic GN

In most cases of crescentic GN, cellular immune effectors, particularly T cells, are suggested to play a key role. Among them, $CD4^+$ T cells have a central role in renal injury by direct accumulation in the glomeruli as effectors. Several studies suggest a pathogenic role for the Th17 pathway in nephrotoxic autoimmune GN, lupus nephritis, anti-myeloperoxidase induced GN, and anti-GBM GN models. Table 2 summarized the recent evidences about the role of Th17 pathway in autoimmune crescentic GN. In 2009, Paust et al demonstrated that IL-23/IL-17 signaling promoted renal injury without association of Th1 pathway. At the same time, Holdsworth and Kitching probed an independent role of IL-23 in anti-GBM GN animal model. They also differentiated the role of Th17 from Th1 cells in proliferative GN models as follows. Both Th1 and Th17 cells can induce proliferative GN. Th17 cells induce albuminuria early, with persistent accumulation of leukocytes. Th1 cells lead to a slower rise in albuminuria, but more macrophage activation and delayed type hypersensitivity reactions, including crescent formation and fibrinoid necrosis. Th17 pathway was also demonstrated its independent role in lupus nephritis. However, most previous studies focused on downstream effector molecules, including those involved in IL-17, IL-23, and TNF signaling, and not on the regulatory mechanism.

Table 2. Th17 response associated autoimmune glomerular diseases

| Disease model | Manipulation | Outcome |
|---|---|---|
| Nephrotoxic autoimmune GN | IL-23 p19 KO<br>IL-17 KO | IL-23/IL-17 pathway contributed renal injury |
| | CCR6KO | CCR6 contributes to kidney directed migration of Th17 cells |
| Planted antigen induced autoimmune crescentic GN | Th1 vs Th17 polarized ovalbumin | Th1 and Th17 effector cells can induce glomerular injury |
| Anti-MPO induced GN | IL-17 KO | Il-17A contributes to the pathophysiology of anti-MPO GN |
| Anti-GBM GN | IL-23p19 KO<br>IL-12p40 KO<br>IL-12p35 KO | Auto-reactivity to anti-GBM antigens directed primarily by IL-23 |
| | ROR-γt KO | ROR-γt promotes the development of crescentic GN by directing nephritogenic Th17 pathway |
| Lupus nephritis | MRL-Faslpr lupus nephritis | IL-23/IL-17 axis contributes to the development of lupus nephritis |
| | NewZealand Black* | TNF signaling regulates Th17 pathway related autoimmune LN |

## Role of Th17 pathway in anti-GBM GN induced renal injury

In anti-GBM GN, T cells play an important role in promoting or limiting renal inflammation initiated by anti-GBM autoantibodies. Th1 and Th17 cells promote inflammation (29), whereas $CD4^+CD25^+$ regulatory T cells limit the extent of inflammation (32). In particular, Th17 cells have been established as a crucial player in the pathogenesis of renal inflammation in anti-GBM GN (22-25, 28, 29). Th17 cells differentiate from naïve T cells upon stimulation by IL-6 and TGFβ (1, 16, 31). IL-6 activates signal transducer and activator of transcription 3 (STAT3) via phosphorylation by Janus kinase 2 (JAK2) (18, 33). STAT3 activated by IL-6 induces a Th17-defining transcription factor retinoic acid receptor-related orphan receptor γt (RORγt), which further induces IL-17, IL-21, IL-22, and IL-23 receptor (9, 34). In anti-GBM GN, knockout of IL-17, IL-23p19, or RORγt significantly reduces renal inflammation (25, 28), suggesting that modulation of Th17 pathway might help reduce renal injury in anti-GBM GN.

## Ctr9 regulates Th17-driven inflammation

STAT3 phosphorylated by JAK2 forms homo- or heterodimers with STAT3 or STAT1 and migrates into the nucleus and binds to its target promoters. STAT3 dimers interact with DNA or histone proteins at the promoter of their target genes, and this interaction is mediated by RNA polymerase-associated factor complex

(PAFc) (35, 36, 38). PAFc is an evolutionarily conserved mediator of general transcriptional processes including transcription initiation, elongation, RNA processing, and histone modification (10). Among other subunits of PAFc, Ctr9 provides a structural scaffold in PAFc assembly (4), and depletion of Ctr9 essentially prevents the formation of PAFc by downregulating other subunits (20, 40). Interestingly, Ctr9 selectively regulates the transcription of IL-6-responsive genes. In mice injected with lipopolysaccharide, Ctr9 in hepatocytes regulates the transcription of IL-6-responsive genes by modulating STAT3-DNA interaction (38). In T cells, Ctr9 inhibits Th17 differentiation by repressing IL-17 transcription: without IL-6, Ctr9 proteins are normally bound to the gene body of Il17a and thereby repress its transcription; with IL-6, Ctr9 is released from the gene body of Il17a, allowing transcription to proceed (35). Another study on Ctr9 in the regulation of IL-6-responsive genes has shown that depletion of Ctr9 decreased the association of negative elongation factor and increased the association of cyclin-dependent kinase 9, a kinase component of the positive transcription elongation factor at the elongation checkpoint (36). These findings imply that Ctr9 could act as a negative regulator of Th17-driven inflammation in anti-GBM GN.

Here, I investigated the role of Ctr9 in modulating Th17-driven renal inflammation in anti-GBM GN. In the first part of this study, I sought to define the role of Ctr9 in Th17 cell-mediated inflammation in anti-GBM GN using STAT3β and IL-17 knockout mice. STAT3β-deficient mice were used because indiscriminate knockout of STAT3 is embryonically lethal (30). STAT3β is an

isoform of STAT3 that lacks 55 C-terminal amino acids containing a transactivation domain and instead has unique 7C-terminal amino acids (2, 27). Although originally reported to exert dominant-negative action on STAT3α (2), STAT3β was later found to have distinct intracellular kinetics and biological functions (17, 21). STAT3β can enhance phosphorylation and prolong nuclear retention of STAT3α (21). Independently of STAT3α, STAT3β alone can rescue STAT3-null cells from embryonic lethality and induce acute phase response genes (17). STAT3β also seems to have an anti-inflammatory property, as STAT3β-deficient mice injected with lipopolysaccharide demonstrated more severe organ damage and slower recovery from endotoxin-induced shock (17, 37). In case of anti-GBM GN, It was observed that STAT3β knockout mitigates autoantibody-induced inflammation through downregulation of IL-17. This finding was largely reproduced in IL-17 knockout mice, which displayed significantly less renal injury compared to their wild-type counterparts. In both models, Ctr9 negatively correlated with Th17 activity and renal inflammation. Consistent with its role as a repressor of Th17 differentiation, RNA interference-mediated disruption of Ctr9 in intrarenal lymphocytes enhanced Th17 differentiation and IL-17 production.

In the second part of this study, the expression of Ctr9 in mesangial cells and its correlation with Th17 conditions were investigated. Mesangial cells are important in glomerular diseases as they proliferate and produce a wide range of cytokines and chemokines in response to immune complexes, autoantibodies, and cytokines (6). It was shown that anti-GBM autoantibodies or IL-17 downregulate Ctr9 and

induce IL-6 in mesangial cells. These findings were reproduced in a human T lymphocyte-mesangial cell co-culture system, in which anti-GBM IgG isolated from a patient with anti-GBM GN augments IL-17 production and represses Ctr9 expression in mesangial cells grown under the Th17-differentiating condition (i.e. IL-6, TGFβ, and IL-23). Thus, IL-6, IL-17 and Ctr9 seem to participate in a reciprocal regulatory loop involving Th17 lymphocytes and mesangial cells. Finally, I examined the expression of Ctr9 and IL-17 in human biopsies obtained from patients with anti-GBM GN and normal subjects.

# MATERIALS AND METHODS

## Induction of anti-GBM GN in STAT3β$^{-/-}$ and IL-17$^{-/-}$ mice

The Institutional Animal Care and Use Committee of the Biomedical Research Institute of Seoul National University Hospital reviewed and approved the animal experiment protocol. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Research Council and the NIH guide for the Care and Use of Laboratory Animals. Generation of C57BL/6.STAT3β$^{-/-}$ mice was described previously (37). To induce anti-GBM GN, I intravenously injected 4.5 mg of rabbit anti-GBM IgG into C57BL/6 and C57BL/6.STAT3β$^{-/-}$ mice, and BALB/c and BALB/c.IL-17$^{-/-}$ mice (donated by Dr. Yoon-Keun Kim, Pohang University of Science and Technology, Pohang, Korea). The rabbit anti-GBM IgG was prepared from a rabbit immunized with homogenized murine renal cortex in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA), as reported previously (13). As negative controls, age-matched (8~10-week-old) wild-type male C57BL/6 and BALB/c mice (Orient Company, Seoul, Korea) received phosphate-buffered saline (300 μL) instead of anti-GBM IgG. All animals were bred under specific pathogen-free conditions. The genotype of each animal was confirmed by PCR analysis. At day 0, 3, 7, 14, and 21, blood urea nitrogen (BUN) was determined using an auto-analyzer (Wako Chemical Industries, Ltd., Osaka, Japan), and 24-hour urine protein excretion rate was determined using Bradford method with normalization against urine creatinine

concentrations. The extent of renal injury and changes in protein and mRNA abundances of Th17-related genes were assessed by the expression of intrarenal cytokines, immunohistochemistry, Western blotting, and quantitative real-time PCR.

In the STAT3$\beta^{-/-}$ experiment, CD3- or F4/80-positive cells were counted in at least 30 glomeruli per mouse kidney (n = 8). The mean values are expressed as cell per glomerular cross-section (cells/gcs). Sections were evaluated in a blinded fashion. To analyze STAT3 binding to the promoters of Il17a, Stat3, and Ctr9, I examined previously published STAT3 chromatin immunoprecipitation with deep sequencing (ChIP-seq) datasets [GSM540722 from (5); GSM1601721, GSM1601724, and GSM1601726 from (8)] on the UCSC genome browser (11). The UCSC genome browser snapshots for these datasets are available in Figure 2E.

## Silencing of Ctr9 in splenocyte-derived Th17 cells

Naïve $CD4^+$ T cells ($CD4^+$CD62Lhi) were obtained from spleens of C57BL/6 wild-type mice using a MACS $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated T cell populations was 99%. For Th17 differentiation, naïve $CD4^+$ T cells were incubated with murine (m) TGFβ1 (5 ng/mL) and mIL-6 (20 ng/mL) in the presence of anti-CD3 (5 μg/mL) and anti-CD28 (10 μg/mL) activating antibodies and anti-mIFN-γ (10 μg/mL) and anti-mIL-

4 (10 μg/mL) neutralizing antibodies. Concentrations of IL-17 in the lysed tissue or supernatant were determined using a sandwich ELISA kit (R&D Systems, Wiesbaden, Germany). For Ctr9 silencing, 1 μmol Accell siRNA targeting murine Ctr9 (Dharmacon, Ettenleur, the Netherlands) or 1 μmol Accell scrambled non-targeting siRNA (Dharmacon) were added. Twenty-four hours before transfection of cells, cells were washed, detached with trypsin, diluted 1:5 with fresh medium without antibiotics ($1 \sim 3 \times 10^5$ cells/mL), and transferred to 24-well plates (500 μL/well). Specific silencing of Ctr9 was confirmed by at least three independent experiments. Because IL-17 production from Th17 cells was highest on day 3, we treated Th17 cells with siRNAs for 3 days.

## Silencing of Ctr9 in intrarenal lymphocytes isolated from mice with anti-GBM GN

Kidneys were harvested from C57BL/6 mice 3 days after injecting anti-GBM IgG. For quantitative flow cytometry analysis, mononuclear cells were isolated from kidney homogenates using a Stomacher 80 Biomaster laboratory paddle blender (Seward Ltd., Worthing, Sussex, UK). Single-cell suspensions were prepared by passing the homogenate through a 40-μm cell strainer. Kidney cells were re-suspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and overlaid onto 72% Percoll. After centrifugation at 1000×g at 25℃ for 30 min, mononuclear cells were isolated from the interface and were incubated with

fluorescence-conjugated mouse monoclonal antibodies against CD4 and IL-17 (BD Biosciences, Franklin Lakes, NJ, USA). For intracellular cytokine staining, $5 \times 10^5$ renal mononuclear cells were plated in RPMI 1640 supplemented with 10% fetal calf serum, 100 U penicillin/mL, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Ctr9 knockdown using siRNAs was performed in the same manner as the spleen-derived Th17 cells. Then, cells were incubated with phorbol 12-myristate 13-acetate (PMA, 100 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) in the presence of Brefeldin A (10 μg/mL, BD Pharmingen, San Diego, CA, USA) at 37℃ for 4 h. After activation, T cells were permeabilized with BD Cytofix/Cytoperm™ solution according to the manufacturer's instructions (BD Pharmingen), stained with antibodies, and fixed with 1% paraformaldehyde. Fluorescence signals were analyzed using a FACSCalibur instrument and the CellQuest software (BD Biosciences).

## Primary mouse mesangial cells treated with anti-GBM IgG

Primary cultures of mouse mesangial cells were established as reported previously (3). In brief, mesangial cells were isolated from glomeruli using a differential sieving technique. The glomerular fraction was enriched to more than 95% by centrifugation. Cells were incubated at 37℃ with 5% CO2 and propagated in Dulbecco's modified essential medium (DMEM) containing 10 mM D-glucose, 15 % FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM supplemental

glutamine. Mesangial cells were passaged every 72 h. Primary mouse mesangial cells isolated from normal C57BL/6 mice were cultured with or without rabbit anti-GBM IgG (2 and 20 μg/mL) for 48 hours. Cell proliferation was assessed using a colorimetric MTS cell proliferation assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. IL-6 concentrations were determined using ELISA (R&D Systems). IL-6 receptor, STAT3, and Ctr9 mRNAs were quantified using real-time PCR. Protein expressions of phosphorylated STAT3 and Ctr9 were determined using semi-quantitative immunoblotting.

## Mouse mesangial cells in Th17 environment

Mouse mesangial cells (SV40 MES 13, ATCC® CRL-1927™, $5 \times 10^4$/well) were cultured in DMEM containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ with 5% CO2. After incubation in serum-free media for 24h, mouse mesangial cells were stimulated with recombinant TGFβ1 (5 ng/mL), recombinant IL-17 (10 ng/mL) or both for 24 hours. After stimulation, mesangial cell proliferation was quantified using MTS assays (Promega Corporation). IL-6 concentrations in the culture media were determined using ELISA (R&D Systems). Protein expression of Ctr9, phosphorylated STAT3 (pSTAT3), and IL-17 receptors were visualized using immunofluorescence staining. Images were captured using a Leica TCS SP8 STED CW (Leica, Mannheim, Germany) and MetaMorph version 7.8.10 software (Universal Imaging

Downingtown, PA, USA). The proportions of pSTAT3-, IL-17 receptor- or Ctr9-positive cells were determined for quantitative analysis. The mRNA levels of STAT3, RORγt, IL-17, IL-23R and Ctr9 in mesangial cells were determined using quantitative real-time PCR.

## Co-culture of human mesangial cells and PBMCs

Peripheral-blood mononuclear cells (PBMCs) were collected from a healthy blood donor and centrifuged over Ficoll-Hypaque density gradient (Amersham Biosciences, Uppsala, Sweden). After two washes, the cells were re-suspended in a DMEM culture medium. Primary cultures of human mesangial cells were established in the same manner as mouse primary mesangial cells. To obtain human anti-GBM antibody-containing IgG and determine the concentrations of pro-inflammatory cytokines in the circulation, I collected serum samples from a 54-year-old female patient with anti-GBM GN at the time of diagnosis (day 0), day 15, 30, and 90. The Institutional Review Board of Seoul National University Hospital approved the protocols for obtaining serum samples from patients with anti-GBM GN and normal controls (IRB No. 1404-117-515). Informed consent was obtained before starting serum collection. The serum samples were passed through a IgG affinity column (Promega Corporation) to obtain total IgG. Anti-GBM antibody titers were determined using enzyme immunoassay (Bio-Rad Laboratories, Hercules, CA, USA). The concentrations of TGFβ1, IL-6, IL-12, IL-

17, TNFα and IFNγ in the serum samples were determined using the Bio-Plex Pro® system (Bio-Rad Laboratories), an immunoassay system formatted on magnetic beads. The serum samples were diluted 1:4 before measurement. Each sample was analyzed twice, and the mean cytokine concentration was calculated.

To study the response of mesangial cells to anti-GBM IgG and/or Th17 conditions in a co-culture system, mesangial cells were grown in the lower chamber of Transwell culture dishes and then subjected them to different experimental conditions. Human mesangial cells were first plated at a density of 1 x $10^5$ /well, and at 70% confluence the culture media were changed to serum-free DMEM containing 1% FBS. Twenty-four hours later, the culture medium was changed to various experimental conditions: (1) 1% FBS; (2) 1% FBS + anti-GBM Ab-containing IgG (1000 ng/mL) ; (3) 1% FBS + anti-GBM Ab-containing IgG (1000 ng/mL) + PBMCs (in the upper chamber of Transwell dishes, 1 × $10^6$/well), and 1% FBS + anti-GBM Ab-containing IgG (1000 ng/mL) + PBMCs (1 × $10^6$/well) + stimulation (10 ng/mL TGFβ1, 10 ng/mL IL-6, 30 ng/mL IL-23). Total IgG purified from a healthy subject was used as a negative control for anti-GBM Ab-containing IgG. Forty-eight hours later, the concentrations of IL-17 and IL-6 in the supernatant were determined using ELISA (R&D Systems), and the expression of Ctr9 in the mesangial cells was quantified using real-time PCR.

## Determination of Th17 activity in human anti-GBM GN

The Institutional Review Board of Seoul National University Hospital reviewed and approved the protocols for obtaining kidney biopsy tissues from patients with anti-GBM GN and normal controls (IRB No. 1002-045-309). Patients with anti-GBM GN (n = 4) and normal subjects (n = 3) underwent ultrasonography-guided percutaneous needle biopsy. The diagnosis of anti-GBM GN was made by typical pathologic findings on renal biopsy and by elevated anti-GBM antibody titers. Normal controls showed minimal nonspecific change of glomeruli or tubules. The expression of pSTAT3, IL-17, and Ctr9 was visualized by immunohistochemistry, and the expression of IL-17 by immunofluorescence staining. α-smooth muscle actin was used as a cell-proliferation marker and Thy1.1 as a marker for mesangial cells. To quantify Th17-associated mRNA transcripts in human anti-GBM GN, total RNAs were isolated from the biopsy specimen using either an SV total RNA isolation system (Promega Corporation) or an RNeasy-Mini kit (Qiagen GmBH, Hilden, Germany). One microgram of total RNAs were reverse-transcribed using oligo-dT primers and avian myoblastosis virus reverse transcriptase (Promega Corporation). The mRNA abundances of IL-17, STAT3 isoforms (STAT3α and STAT3β), and Ctr9 were determined using quantitative real-time PCR.

## Assessment of renal injury

Light microscopy and immunohistochemistry were performed on 4-μm thick, paraffin-embedded sections. Crescent formation was assessed in periodic acid-

Schiff-stained sections. Glomeruli with cellular crescents were counted in a blinded fashion. A minimum of 50 glomeruli per mouse kidney were evaluated, and the mean value was used as representative for the mouse. Renal tubulo-interstitial injury was assessed in sections stained with Masson's trichrome stain.

## Immunohistochemistry

Kidney sections were deparaffinized and rehydrated with xylene and ethanol. Endogenous streptavidin activity was blocked by 0.3% hydrogen peroxide. Antigens were retrieved by heating paraffin-embedded sections in 10% citrate buffer in a microwave oven, 5 times for 5 min each. The sections were probed with primary antibodies, followed by counterstaining with Mayer's hematoxylin (Sigma-Aldrich). The degree of nonspecific staining was assessed using secondary antibodies and isotype control IgGs. The stained slides were photographed using an Olympus inverted microscope (Olympus Imaging America, Center Valley, CA).

## Immunofluorescence staining

For immunofluorescence staining of kidney tissues, deparaffinized sections of mouse or human kidney specimen were probed with primary antibodies in a blocking agent overnight at 4 ℃, followed by AlexaFluor-conjugated secondary

antibodies (Molecular Probe, Eugene, OR, USA). 4',6-diamidino-2-phenylindole (DAPI, Molecular Probe) was used for counterstaining. For negative controls, the primary antibodies were omitted. Confocal microscopy was performed with a Leica TCS SP8 STED CW (Leica).

## Western blot analysis

Tissues or cells were lysed in RIPA buffer [50 mM Tris/HCl, pH 7.3; 150 mM NaCl; 0.1 mM EDTA; 1% (v/v) sodium deoxycholate; 1% (v/v) Triton X-100; 0.2% NaF; and 100 μM $Na_3VO_4$] supplemented with CompleteTM protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). The kidney homogenate was centrifuged at 12,000 × g for 30 min at 4 ℃, and the protein concentration of the supernatant was determined by Bradford method. Different amounts of extracted protein were separated by SDS-PAGE and transferred onto Immobilon-FL 0.4-μm polyvinylidene difluoride membranes (Millipore, Massachusetts, USA). Tris-buffered saline containing 0.1% Tween-20 was used as the washing buffer. After probing with primary antibodies, anti-rabbit (1:5000; Cell Signaling Technology, Danvers, MA, USA) and anti-mouse (1:6000, for β-actin; Cell Signaling Technology) antibodies were used as secondary antibodies. Detection of labeled proteins was performed by the enhanced chemiluminescence system (ECLTM PRN 2106; Amersham Pharmacia Biotech, Buckinghamshire, UK). The band intensities were analyzed using a Gel documentation system (Bio-Rad Gel Doc

1000 and Multi-Analyst version 1.1). The list of antibodies used in immunohistochemistry, immunofluorescence staining, and Western blotting is available in Table 1.

## Quantitative real-time PCR

Quantitative real-time PCR was carried out using an Applied Biosystem 7500 thermocycler (Applied Biosystems, Foster City, CA, USA). I ran PCR using either SYBR Green method [for human Ctr9, STAT3, STAT3α, STAT3β, IL-17, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); and for mouse STAT3α, STAT3β, and GAPDH; primer sequences are available in Table 2] or custom-designed Assay-on-Demand TaqMan probes and primers (for all the other genes). For SYBR Green method, each reaction (20 μL) contained 20 μL reaction volume containing 1 SYBR Green Master Mix (Applied Biosystems) and 0.2 μM forward and reverse primers. Thermocycler conditions for both methods were as follows: 95 ℃ for 10 minutes followed by 40 cycles of dissociation (95 ℃ for 10s), annealing (55 ℃ for 30s) and elongation (72 ℃ for 30s). Relative quantification was done using ΔCT method (15). GAPDH was used as a reference gene. Two-tailed Student's t-test was used to compare fold changes.

## Statistical analysis

The results were expressed as the mean ± standard deviation or mean ± standard error of mean where indicated. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). When comparing more than two groups, I used one-way ANOVA with Tukey's post-hoc test. Statistical significance was set at $P < 0.05$.

# RESULTS

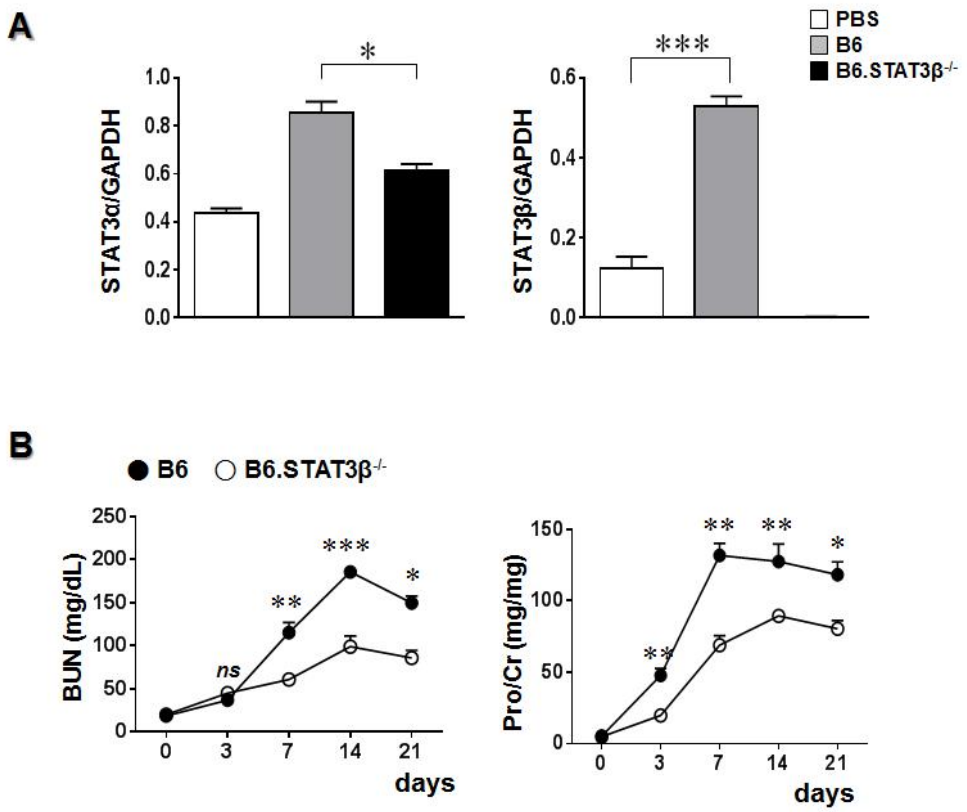
## Ctr9 expression in murine anti-GBM GN negatively correlates with Th17 activation

To correlate renal Ctr9 with Th17 activity in anti-GBM GN, anti-GBM GN was induced in two independent mouse models deficient in Th17 differentiation and observed the renal expression of Ctr9 and Th17-related proteins. In the first animal model, anti-GBM GN was induced in wild-type C57BL/6 and C57BL/6.STAT3$\beta^{-/-}$ (STAT3$\beta^{-/-}$) mice by injecting rabbit anti-GBM IgG. Figure 1A shows relative mRNA abundances of renal STAT3α and STAT3β in each animal group. Compared with vehicle (phosphate-buffered saline), anti-GBM IgG caused a significant upregulation of both STAT3α and STAT3β mRNAs in wild-type C57BL/6 mice. In STAT3$\beta^{-/-}$ mice injected with anti-GBM IgG, STAT3β was almost absent and the expression of STAT3α was not as high as in their wild-type counterparts. These results show that STAT3β knockout decreases the overall STAT3 expression in response to anti-GBM IgG and that STAT3β does not exert a dominant-negative effect on STAT3α.

STAT3β knockout protected against anti-GBM IgG-induced renal injury via downregulation of Th17 activity. STAT3$\beta^{-/-}$ mice had significantly lower blood urea nitrogen and urinary protein excretion up to 21d (Figure 1B); developed fewer cellular crescents (Figure 1C); and displayed significantly less deposition of anti-

GBM IgG along the GBM (Figure 1D) and less infiltration of T cells and macrophages in the glomerulus (Figure 1E). Consistent with Th17 activation, both RORγt and IL-17 increased in wild-type mice injected with anti-GBM IgG. STAT3β knockout significantly attenuated RORγt and IL-17 expression. (Figure 1F).

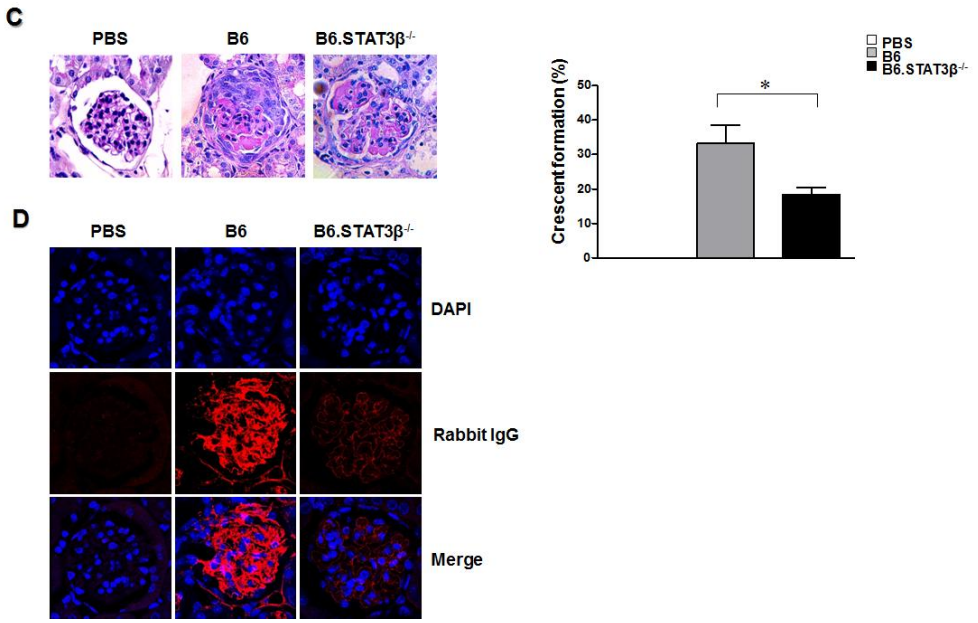
Figure 1. STAT3β knockout ameliorated renal injury in anti-GBM GN.




The extent of renal injury and changes in Th17-related transcripts was compared among PBS-injected wild-type C57BL/6 (PBS), anti-GBM antibody-injected wild-type C57BL/6 (B6), and anti-GBM antibody-injected C57BL/6.STAT3β$^{-/-}$ mice (B6.STAT3β$^{-/-}$). Kidneys were obtained at 7d of anti-GBM GN induction. (A) Relative mRNA abundances of STAT3α and STAT3β in PBS, B6, and B6.STAT3β$^{-/-}$ mice. Despite selective disruption of STAT3β, STAT3α mRNA abundances also moderately decreased in B6.STAT3β$^{-/-}$ mice. (B) Compared with

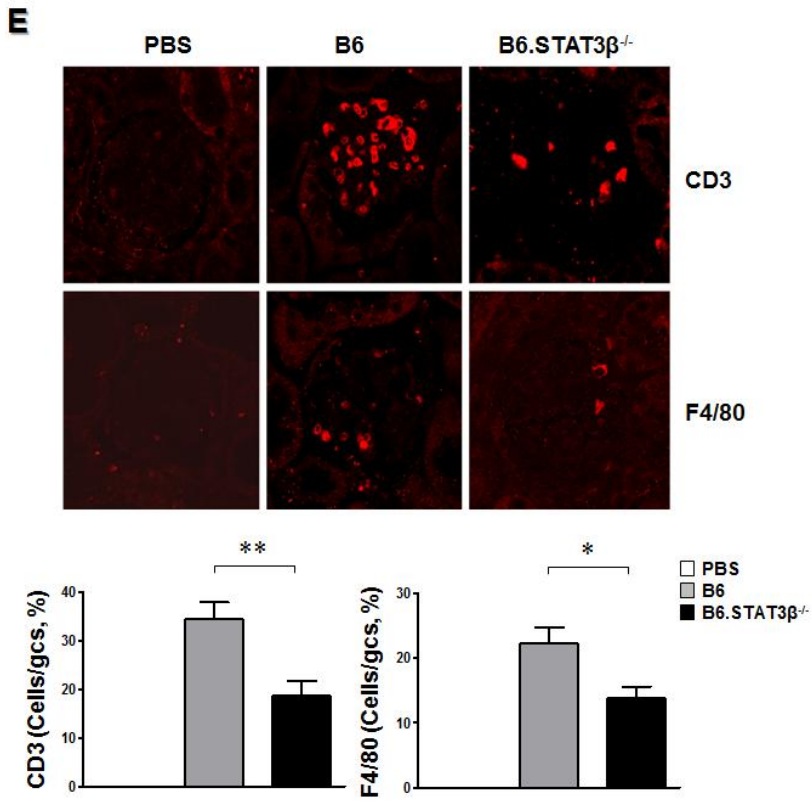
B6 mice, renal injury was significantly attenuated in B6.STAT3β$^{-/-}$ mice (n = 8 per group, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Abbreviations: PBS, phosphate-buffered saline; BUN, blood urea nitrogen; Pro/Cr, random urine protein-to-creatinine ratio;



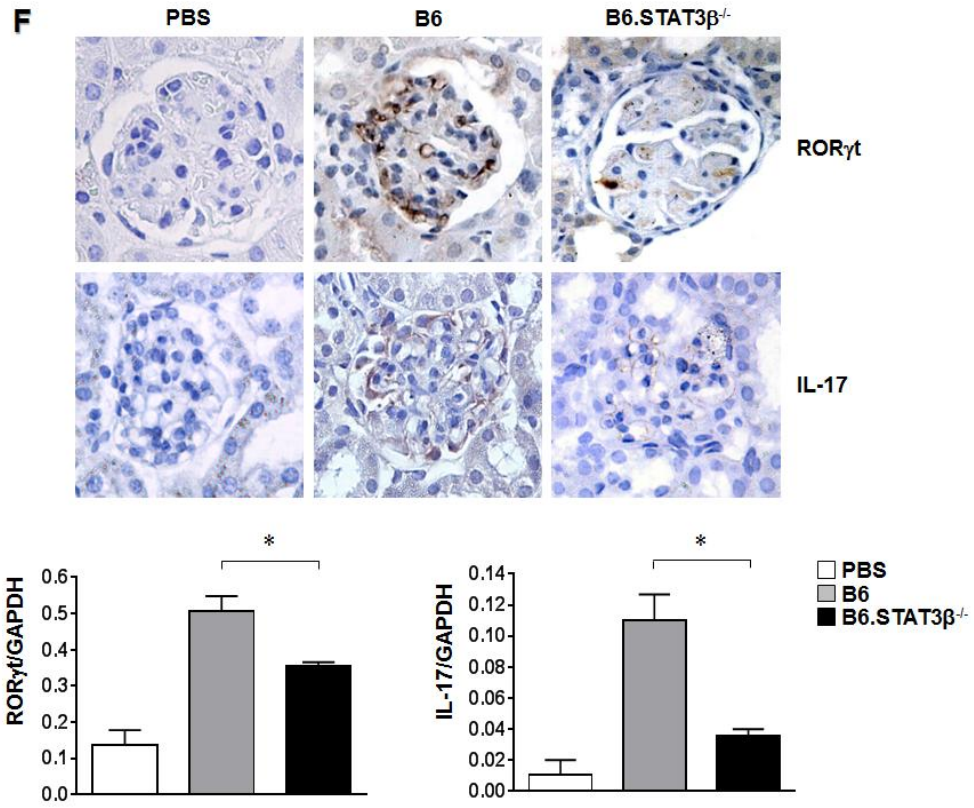

(C) Periodic acid-Schiff staining revealed that B6.STAT3β$^{-/-}$ mice had significantly fewer cellular crescents than B6 mice (n = 8 per group, *$P$ < 0.05, original magnification 400x). (D) On immunofluorescence microscopy, B6.STAT3β$^{-/-}$ mice had much less deposition of anti-GBM antibodies along the GBM.

Abbreviations: PBS, phosphate-buffered saline; DAPI, 4',6'-diamidino-2-phenylindole.




(E) B6.STAT3β$^{-/-}$ mice had fewer T cells (CD3+) and macrophages (F4/80+) infiltrating the kidney than B6 mice (n = 8 per each group, *$P < 0.05$, **$P < 0.01$, original magnification 400x).

Abbreviations: PBS, phosphate-buffered saline; DAPI, 4',6'-diamidino-2-phenylindole.



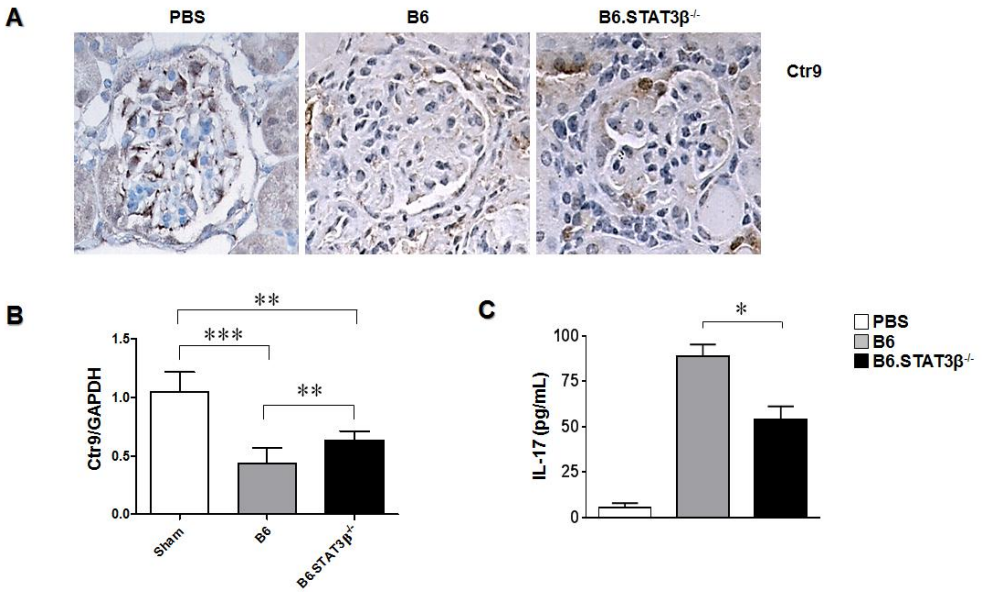

F) B6.STAT3β$^{-/-}$ mice also had lower mRNA and protein expression for RORγt and IL-17 than did B6 mice (means ± standard errors, *$P < 0.05$).

Abbreviations: PBS, phosphate-buffered saline; DAPI, 4',6'-diamidino-2-phenylindole.

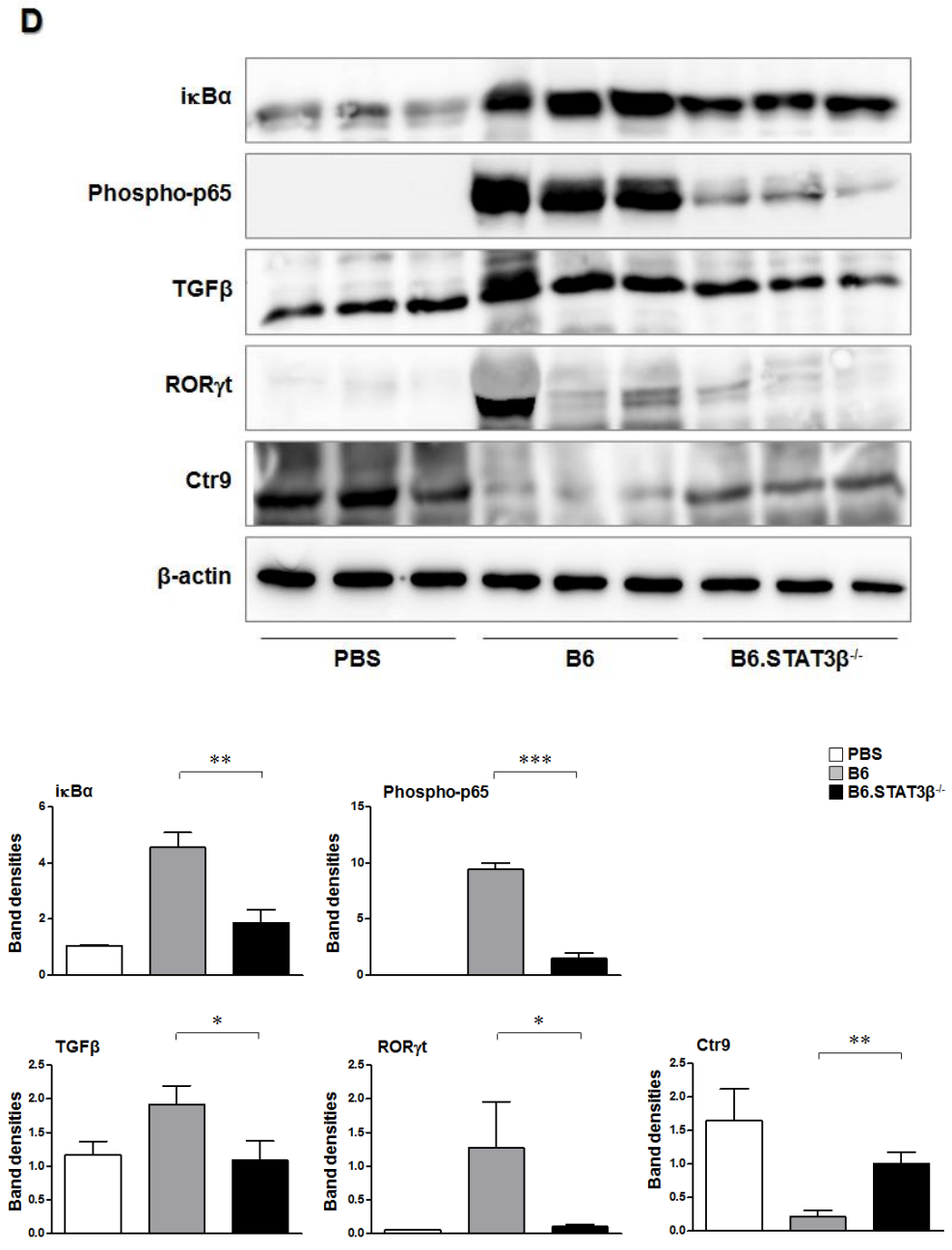
In wild-type and STAT3β knockout mice with anti-GBM GN, the expression of Ctr9 in the kidney showed an inverse relationship with renal injury and Th17 activity. Figure 2A shows immunohistochemical labeling of Ctr9 in the kidney obtained from each animal group. In wild-type mice injected with PBS, Ctr9 was moderately expressed in the glomerulus and surrounding tubulointerstitium, although the expression was highly variable among individual mice. Wild-type mice injected with anti-GBM IgG did not express Ctr9 in the glomerulus, whereas STAT3$\beta^{-/-}$ mice injected with anti-GBM IgG expressed moderate amount of Ctr9 in the glomerulus. Changes in mRNA abundances of Ctr9 were consistent with those in protein expression in the kidney (Figure 2B), and inversely correlated with IL-17 content in the kidney (Figure 2C). On immunoblotting, anti-GBM IgG upregulated IκBα, phosphorylated NF-κB p65 subunit (phospho-p65), TGFβ, and RORγt; and downregulated Ctr9 in wild-type mice. STAT3β knockout reversed these changes, showing a significant decrease in inflammatory mediators and RORγt and an increase in Ctr9 (Figure 2D). These results raised the question that Ctr9 might be directly repressed by STAT3. To address this question, I examined previously published chromatin immunoprecipitation with deep sequencing (ChIP-seq) datasets from two independent studies (5, 8) for genome-wide binding sites of STAT3 in Th17 cells. There was no evidence of STAT3 binding to the promoter region of Ctr9 (Figure 2E). Although the dissociation of PAFc from the gene body of IL-6-activated genes requires JAK2 activity (36), STAT3 does not directly activate or repress the transcription of Ctr9. In summary, STAT3β knockout

reduces Th17 activation and mitigates renal injury due to anti-GBM IgG. The expression of Ctr9 has a negative correlation with Th17 activation, although its transcription is not directly regulated by STAT3.

Figure 2. Ctr9 is upregulated in B6.STAT3β$^{-/-}$ mice with anti-GBM GN.




Ctr9 was moderately expressed in the glomerulus of wild-type mice injected with PBS. Center. B6 mice injected with anti-GBM IgG did not show Ctr9 expression in the glomerulus. Right. Compared with B6 mice, B6.STAT3β$^{-/-}$ mice had a moderate but significant increase in Ctr9 expression in the glomerulus and tubulointerstitium. (B) Renal mRNA abundances of Ctr9 which is normalized by GAPDH in each animal group, $^{**}P < 0.01$, $^{***}P < 0.005$. (C) IL-17 concentrations in the whole-kidney homogenate from each animal group, $^{*}P < 0.05$.




Immunoblotting of whole-kidney homogenates for iκBα, phosphorylated p65, TGFβ1 RORγt, and Ctr9. Each experimental group has three animals. α-actin was

used as a loading control. The bar graph for each protein represents band densities relative to β-actin (***$P < 0.005$).

**E**

***Il17a***

**Durant et al.** — STAT3 wild-type mice

**Hirahara et al.**
- STAT3: negative control
- STAT3: IL-6
- STAT3: IL-27

***Stat3***

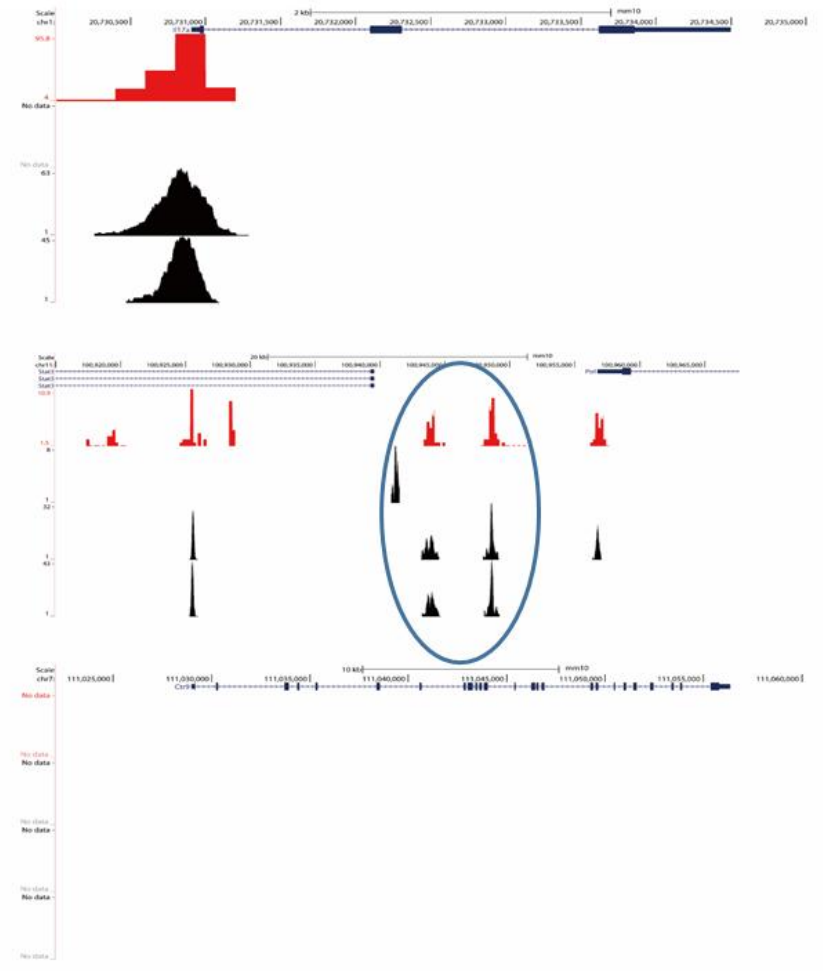
**Durant et al.** — STAT3 wild-type mice

**Hirahara et al.**
- STAT3: negative control
- STAT3: IL-6
- STAT3: IL-27

***Ctr9***

**Durant et al.** — STAT3 wild-type mice

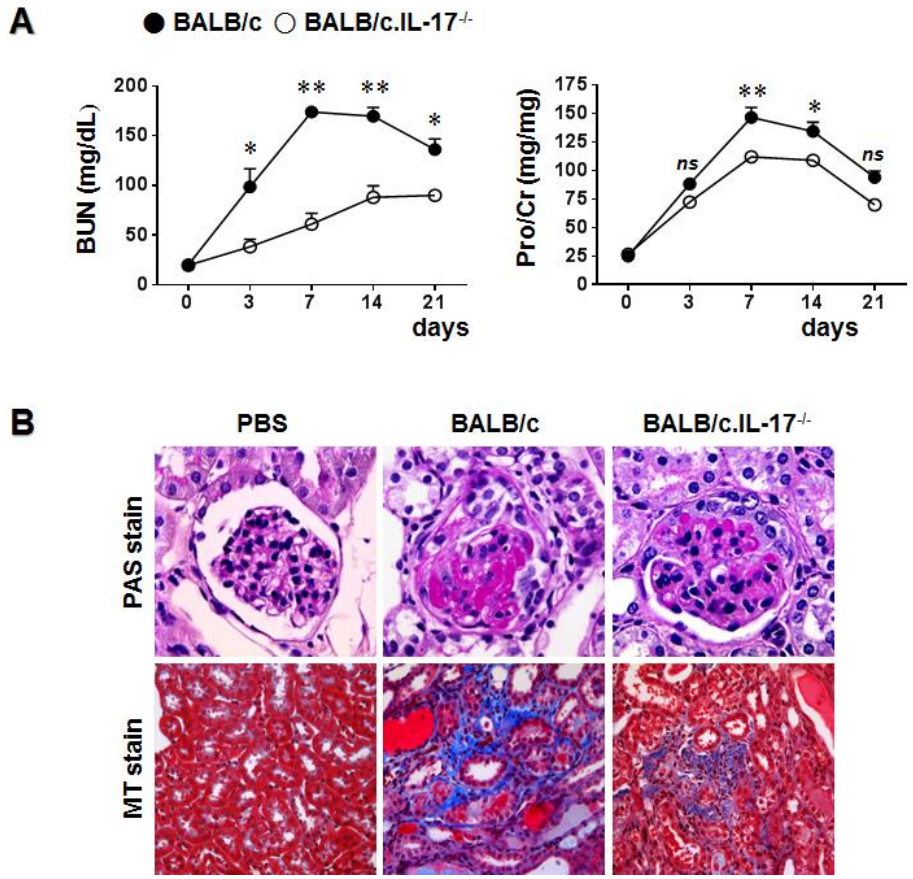
**Hirahara et al.**
- STAT3: negative control
- STAT3: IL-6
- STAT3: IL-27



A snapshot of the UCSC genome browser (mouse genome, mm10) showing STAT3-binding sites for IL-17A (Il17a), STAT3 (Stat3) and Ctr9 (Ctr9) in mouse Th17 lymphocytes. Using chromatin immunoprecipitation with deep sequencing (ChIP-seq), Durant et al. and Hirahara et al. independently probed the mouse genome for genome-wide STAT3 binding sites in Th17 lymphocytes. In Hirahara's data, T lymphocytes were treated with vehicle, IL-6, or IL-27. While there are strong signals for STAT3 binding in the promoter region of Il17a and Stat3 (blue circle for Stat3), there is no binding signal for STAT3 in the promoter region of Ctr9.

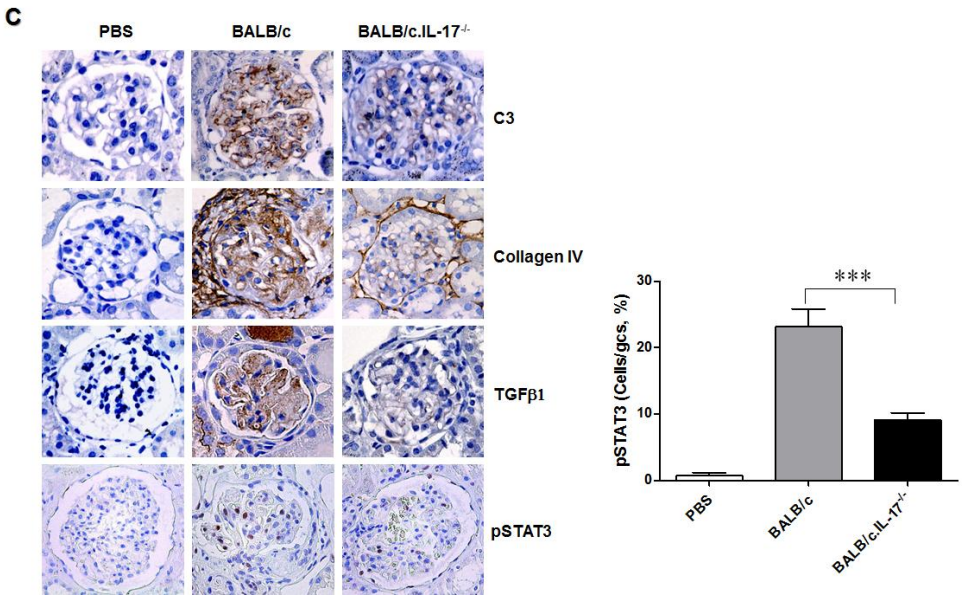
These findings were largely reproduced in BALB/c mice and BALB/c.IL-17$^{-/-}$ mice. Similar to the previous experiment, BALB/c (wild-type) mice injected with anti-GBM IgG developed significant renal damage by day 7 whereas BALB/c.IL-17$^{-/-}$ (IL-17$^{-/-}$) mice were protected (Figure 3A). Histologically, wild-type BALB/c mice injected with anti-GBM IgG showed crescentic GN with tubulointerstitial inflammation and fibrosis, whereas these changes were attenuated in IL-17$^{-/-}$ mice (Figure 3B). Immunohistochemical staining revealed C3 deposition along the GBM, collagen IV deposition in the glomerulus and surrounding tubulointerstitium, and increases in TGFβ and pSTAT3 in wild-type mice (Figure 3C). Quantitative real-time PCR showed that pro-inflammatory and Th1-related cytokines [monocyte chemoattractant protein-1 (MCP-1), IL-1β, interferon γ (IFNγ) and tumor necrosis factor α (TNFα)] were downregulated and Th2-related cytokines (IL-10 and IL-13) upregulated in the kidneys of the IL-17$^{-/-}$ mice (Figure 3D), suggesting a shift in T cell differentiation. Furthermore, IL-17 knockout decreased Th17-promoting cytokines (IL-6, IL-12p40, and IL-23p19) and increased IL-27, a negative regulator of Th17 response (19) (Figure 3E), suggesting that IL-17 in anti-GBM GN might further enhance Th17 differentiation by upregulating IL-6 and IL-23 and downregulating IL-27. In line with what I have observed in STAT3β$^{-/-}$ mice, IL-17$^{-/-}$ mice exhibited higher Ctr9 and lower STAT3 and IL-17 receptor expression than their wild-type counterparts (Figure 3F).

Figure 3. IL-17 knockout protects against renal injury caused by anti-GBM GN.

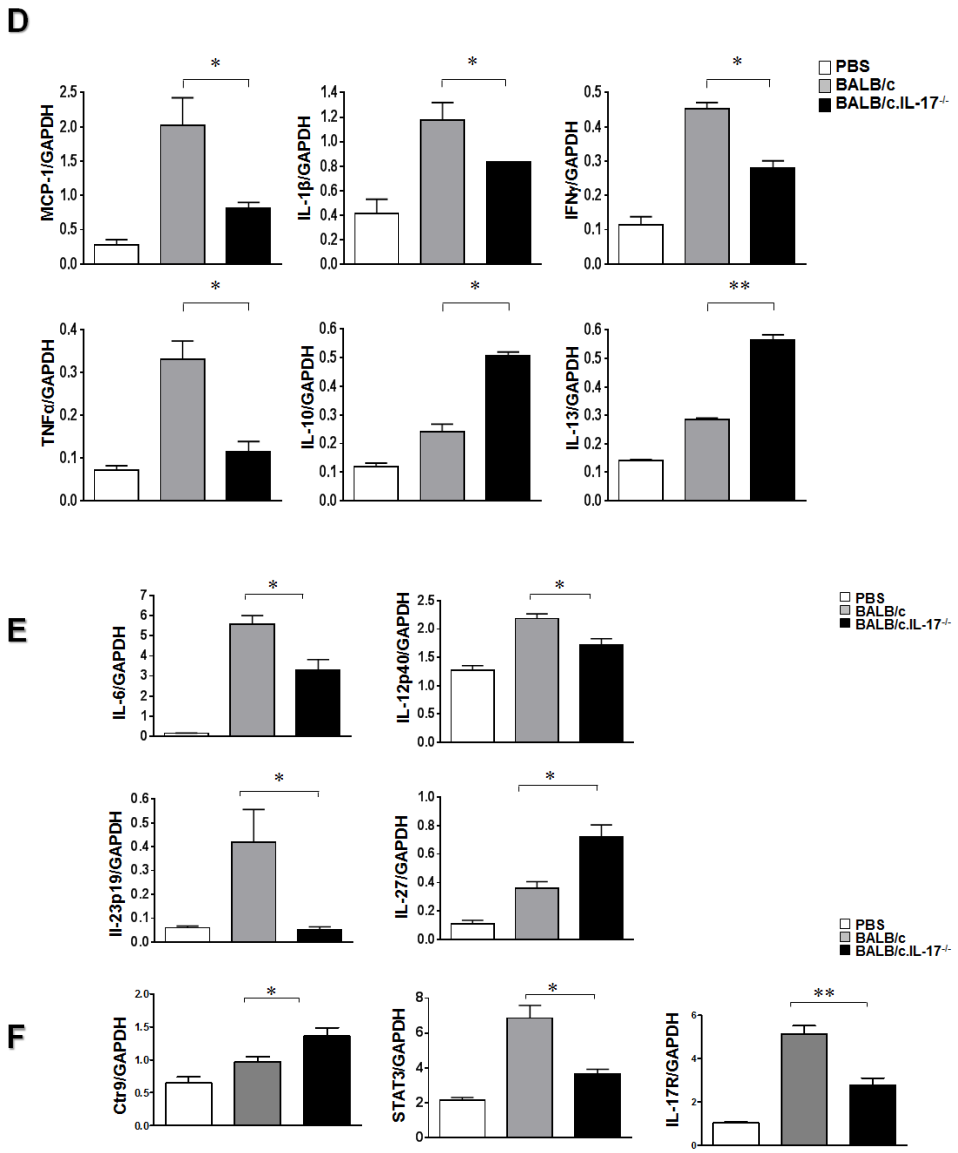



(A) Renal injury was significantly attenuated in BALB/c.IL-17$^{-/-}$ mice (n = 8 per group; ns, no significance; *$P < 0.05$, **$P < 0.01$) 7 days after the injection of anti-GBM IgG. (B) PAS and MT staining of kidney sections from BALB/c mice and BALB/c.IL-17$^{-/-}$ mice injected with PBS or anti-GBM IgG (original magnification 400x).

Abbreviations: BUN, blood urea nitrogen; Pro/Cr, random urine protein-to-creatinine ratio; PAS, periodic acid-Schiff; MT, Masson's trichrome.




Immunohistochemical staining of kidney sections from BALB/c and BALB/c.IL-$17^{-/-}$ mice for complement 3, type IV collagen, TGFβ, and phosphorylated STAT3.




(D, E) Quantitative real-time PCR for intrarenal cytokines in BALB/c and BALB/c.IL-17$^{-/-}$ mice. (F) Quantitative real-time PCR for intrarenal Ctr9, STAT3, and IL-17 mRNAs in BALB/c and BALB/c.IL-17$^{-/-}$ mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

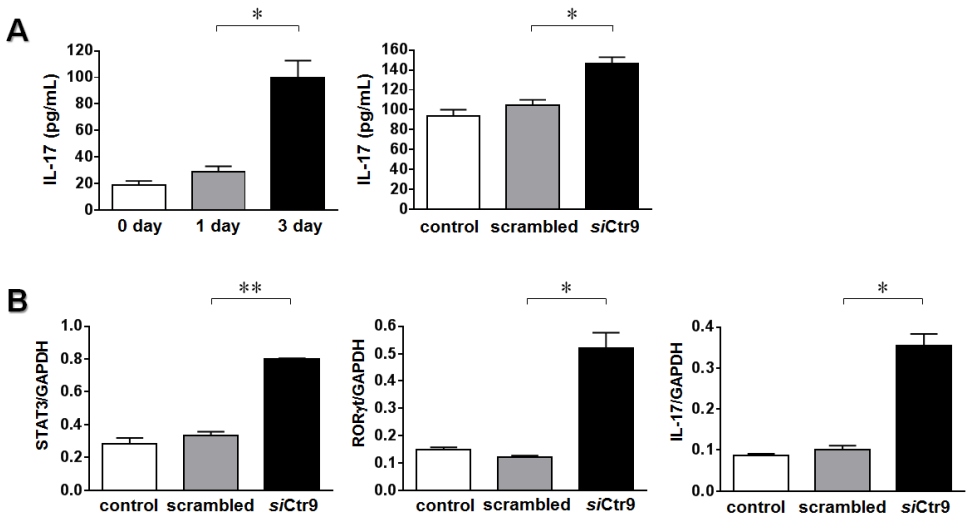
In summary, disruption of Th17 pathway ameliorated anti-GBM IgG-induced renal injury, and Ctr9 was a negative correlate of Th17 activity. In the following sections, I investigated the role of Ctr9 in T cells and mesangial cells.

## Knockdown of Ctr9 in intrarenal lymphocytes promotes Th17 differentiation

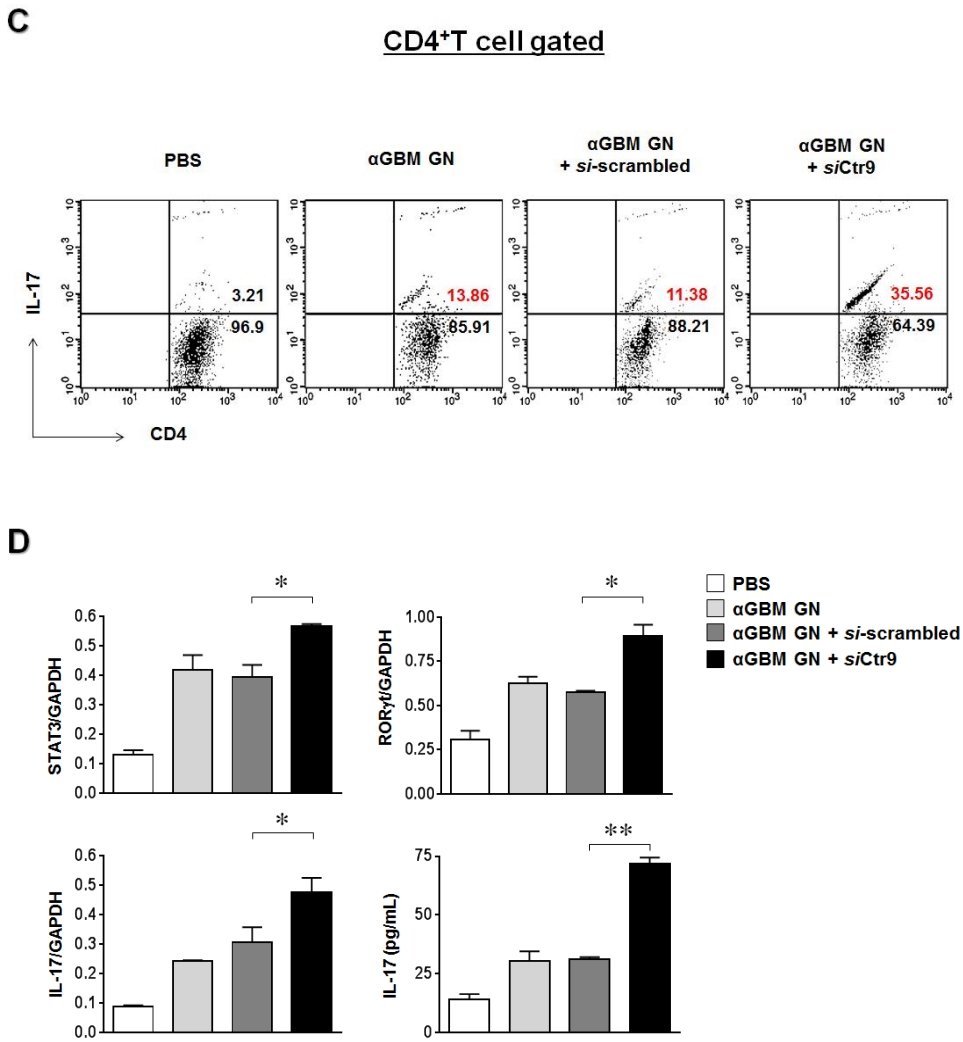
I further investigated the role of Ctr9 in regulation of Th17 cells in anti-GBM GN by knocking down Ctr9 in intrarenal lymphocytes. In a preliminary experiment, we assessed the efficacy of Ctr9-siRNAs using naïve CD4+ T cells isolated from spleens of wild-type C57BL/6 mice. When the naïve CD4+ T cells were cultured under a Th17-differentiating condition (in the presence of TGFβ1, IL-6, anti-CD3, and anti-CD28), differentiation into Th17 cells as determined by IL-17 production was the strongest at day 3 (Figure 4A, left). Next, we cultured naïve CD4+ T cells under the same Th17-differentiating condition and treated them with Ctr9-siRNAs or scrambled siRNAs for 3 days. Compared with scrambled siRNAs, Ctr9-siRNAs significantly reduced the production of IL-17 (Figure 4A, right) and mRNA abundances of STAT3, RORγt, and IL-17 (Figure 4B). This preliminary experiment established Ctr9-siRNAs as an effective means for disrupting Ctr9 in Th17 cells. Using this strategy, we isolated CD4+ lymphocytes from the kidney with anti-GBM GN and introduced scrambled- or Ctr9-siRNAs. Consistent with the previous findings in transgenic animals, the proportion of Th17 cells (CD4+ IL-

17+ cells) increased in the kidney of wild-type C57BL/6 mice with anti-GBM GN, compared with PBS-treated controls (Figure 4C, first and second panels). Compared with scrambled siRNAs, Ctr9-siRNAs significantly increased the proportion of Th17 lymphocytes (Figure 4C, third and fourth panels; 12.14 ± 1.19% for scrambled siRNA, 26.24 ± 4.69% for Ctr9 siRNA, n = 3 per each group, P = 0.04). Similar to the preliminary data from spleen-derived naïve T cells, Ctr9-siRNAs upregulated STAT3, RORγt, and IL-17 mRNAs and IL-17 production in intrarenal lymphocytes of anti-GBM GN (Figure 4D). In summary, knockdown of Ctr9 enhanced differentiation of Th17 cells, indicating that Ctr9 directly regulates Th17 activity in anti-GBM GN.

Figure 4. siRNA-mediated knockdown of Ctr9 leads to further activation of Th17 cells in anti-GBM GN.




(A) Left, IL-17 production from splenocytes grown under Th17-differentiating condition at day 0, 1, and 3. Right, IL-17 production from splenocytes treated with vehicle, scrambled siRNAs, or Ctr9-siRNAs on day 3 (*$P < 0.05$). (B) Relative mRNA abundances of STAT3, RORγt, and IL-17 in splenocytes treated with vehicle, scrambled siRNAs, or Ctr9-siRNAs (*$P < 0.05$, **$P < 0.01$).



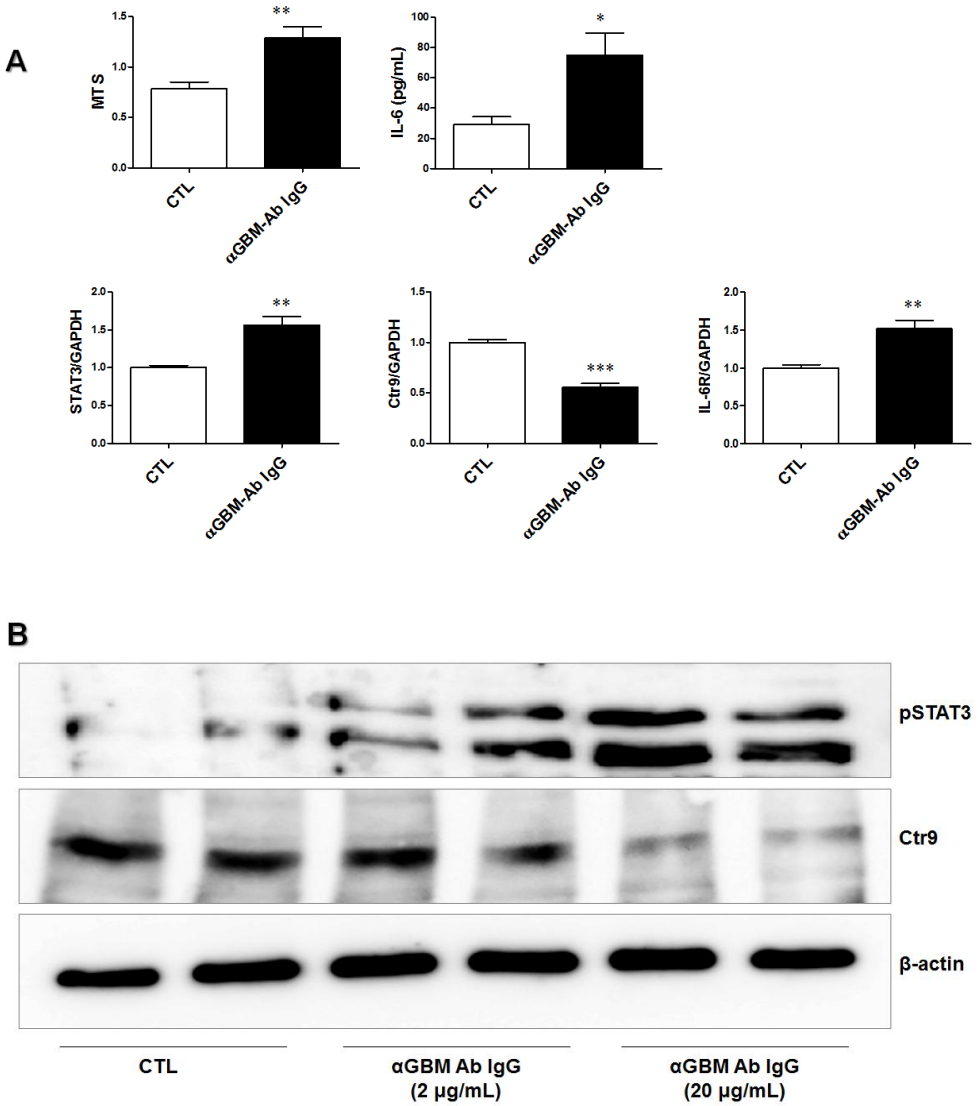

(C) Representative flowcytometry images for T lymphocytes isolated from the kidney of wild-type mice injected with vehicle (PBS), anti-GBM IgG, anti-GBM IgG + scrambed siRNAs, or anti-GBM IgG + Ctr9-siRNAs at day 3. (D) GAPDH normalized mRNA abundances of STAT3, RORγt, and IL-17 in intrarenal lymphocytes at day 3 after the induction of anti-GBM GN (*$P < 0.05$, **$P < 0.01$).

## Anti-GBM IgG and IL-17 repress Ctr9 expression in mesangial cells

Mesangial cell proliferation is frequently observed in anti-GBM GN. Anti-GBM IgG can bind to mesangial cells and stimulate the production of pro-inflammatory cytokines and chemokines, and mesangial cells can proliferate in response to anti-GBM IgG (6, 13, 26). Observing that Ctr9 is moderately expressed in glomeruli of untreated wild-type mice, changes in the expression of mesangial Ctr9 in response to anti-GBM IgG were investigated. In primary mouse mesangial cells, anti-GBM IgG (2 μg/mL) caused an increase in cell proliferation, along with upregulation of IL-6, IL-6 receptor, and STAT3, and downregulation of Ctr9 (Figure 5A). These changes are similar to the whole kidney and intrarenal lymphocytes. Immunoblotting showed that anti-GBM IgG increased pSTAT3 and decreased Ctr9 protein abundances in a concentration-dependent manner (Figure 5B). I further tested if an inflammatory environment rich in IL-17 can induce mesangial cell proliferation. Mouse mesangial cells were grown in the presence of recombinant TGFβ (5 ng/mL), IL-17 (50 ng/mL), or both. A combination of TGFβ and IL-17 induced cell proliferation in mesangial cells (Figure 5C). Interestingly, TGFβ and IL-17 synergistically induced IL-6 in mesangial cells, suggesting that activation of Th17 cells in anti-GBM GN might be further assisted by mesangial cells. TGFβ and IL-17, alone or in combination, decreased Ctr9 and increased pSTAT3 and IL-17 receptor expression (Figure 5D-F). Upregulation of IL-17 receptors in response to IL-17 suggests autocrine action of IL-17 on mesangial cells. In addition, STAT3, RORγt, and IL-23 receptor increased and Ctr9 decreased in mRNA abundances,

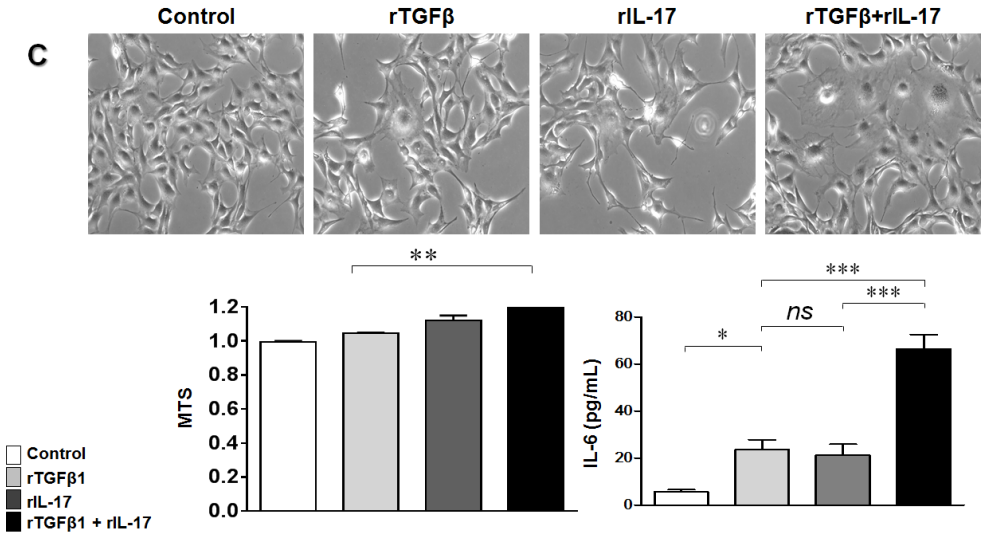
suggesting that IL-6 produced by mesangial cells might act on mesangial cells in an autocrine or paracrine manner (Figure 5G). In summary, either anti-GBM IgG or IL-17 plus TGFβ can induce Th17-differentiating cytokines and repress Ctr9 in mesangial cells.

Figure 5. Anti-GBM IgG or IL-17 represses Ctr9 in mesangial cells.

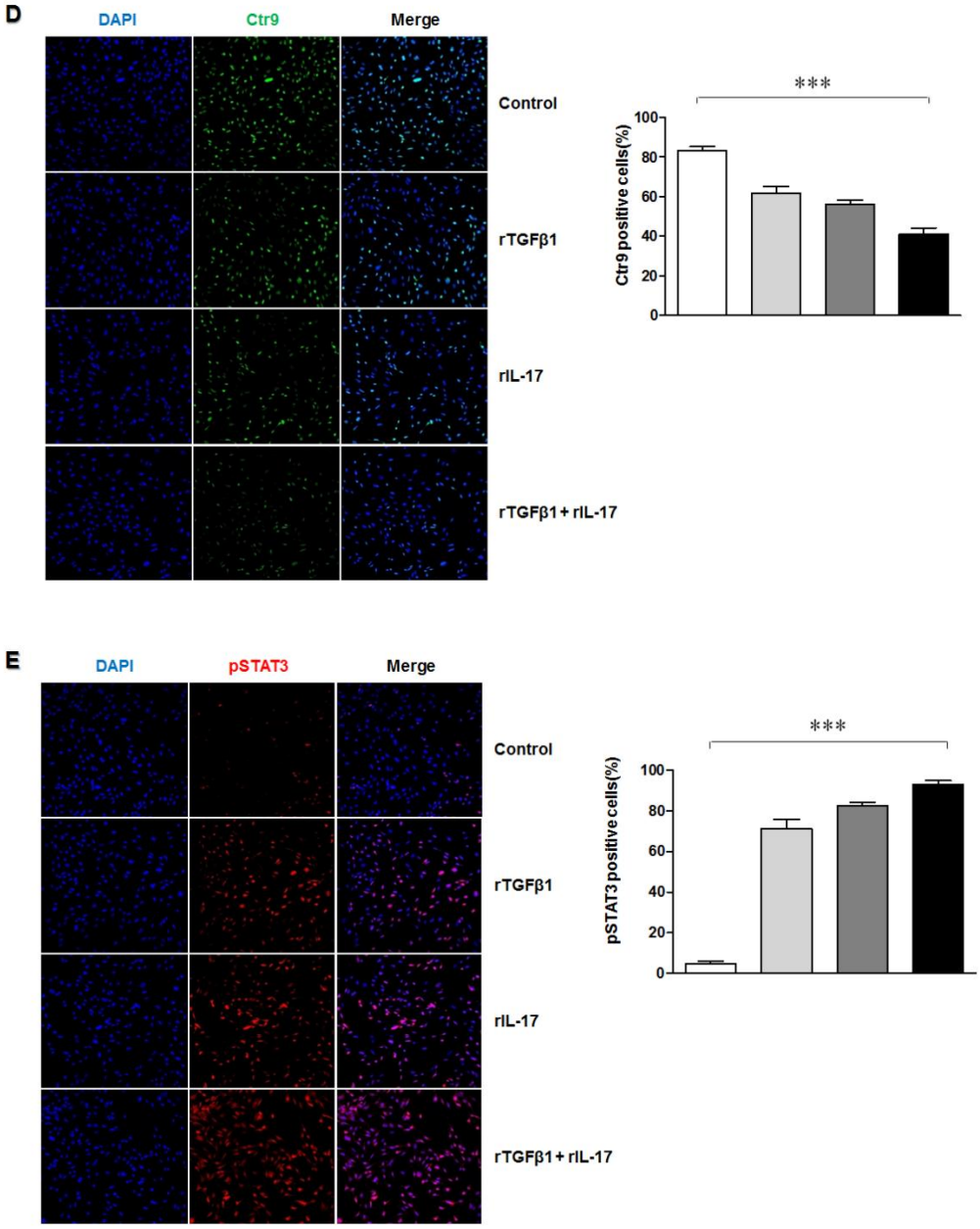



(A) Cell proliferation, production of IL-6, and expression of IL-6 receptors, STAT3, and Ctr9 in mouse primary mesangial cells treated with vehicle or rabbit anti-GBM IgG (2 μg/mL). (B) Immunoblotting for phosphorylated STAT3 (pSTAT3) and

Ctr9 in mouse mesangial cells treated with vehicle or varying concentrations of anti-GBM IgG.




Cell proliferation and IL-6 production in mouse mesangial cells grown in the presence of TGFβ, IL-17, or both (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).


D
DAPI
Ctr9
Merge
Control
rTGFβ1
rIL-17
rTGFβ1 + rIL-17
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Ctr9 positive cells(%)
100
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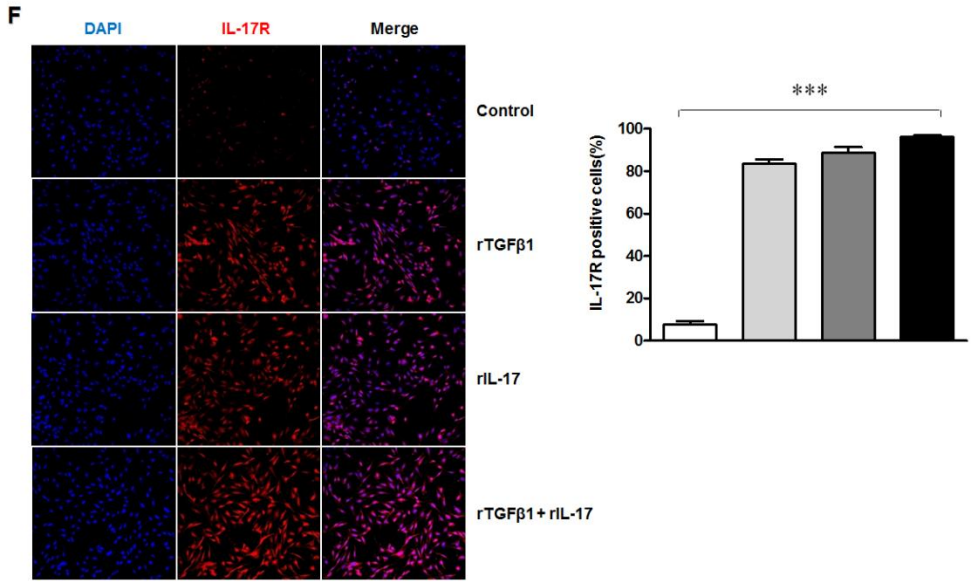
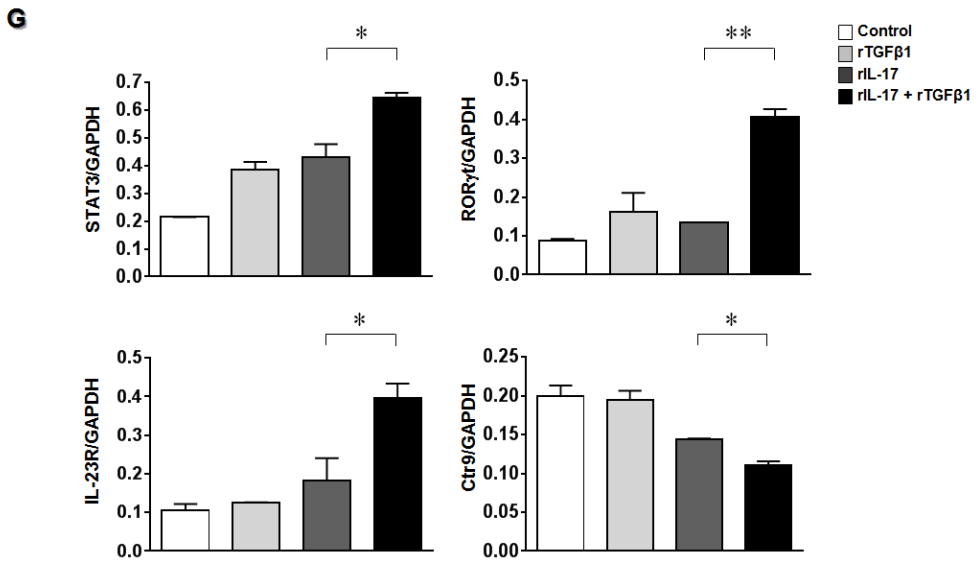



(D-F) Immunofluorescence microscopy images of mouse mesangial cells for Ctr9 (D), phosphorylated STAT3 (E), and IL-17 receptor (F). ***$P < 0.005$.

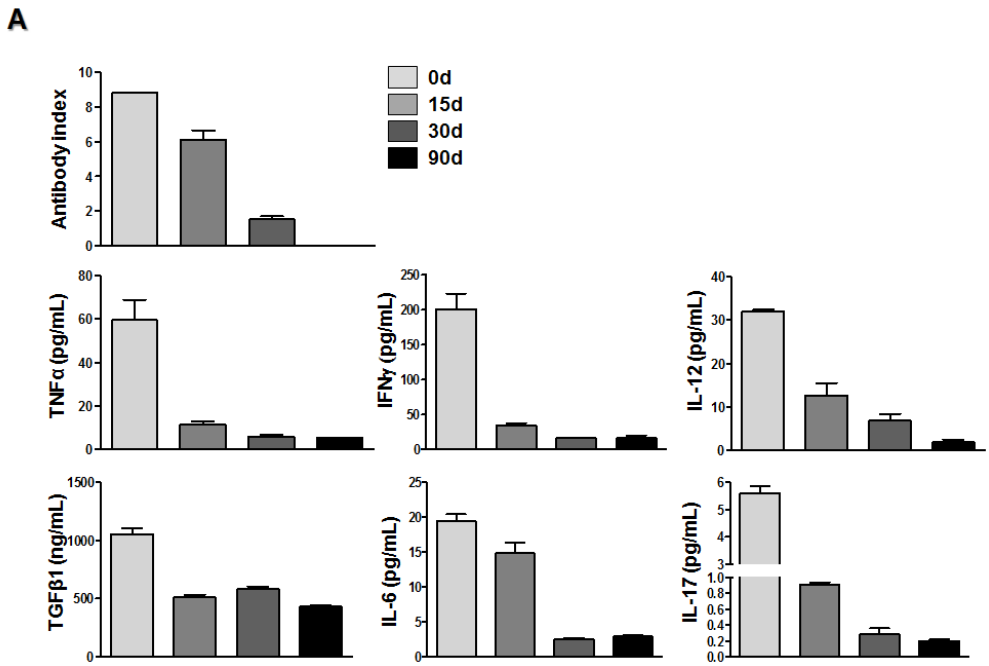



Quantitative real-time PCR for STAT3, RORγt, IL-23 receptor, and Ctr9 (*$P$ < 0.05, **$P$ < 0.01).

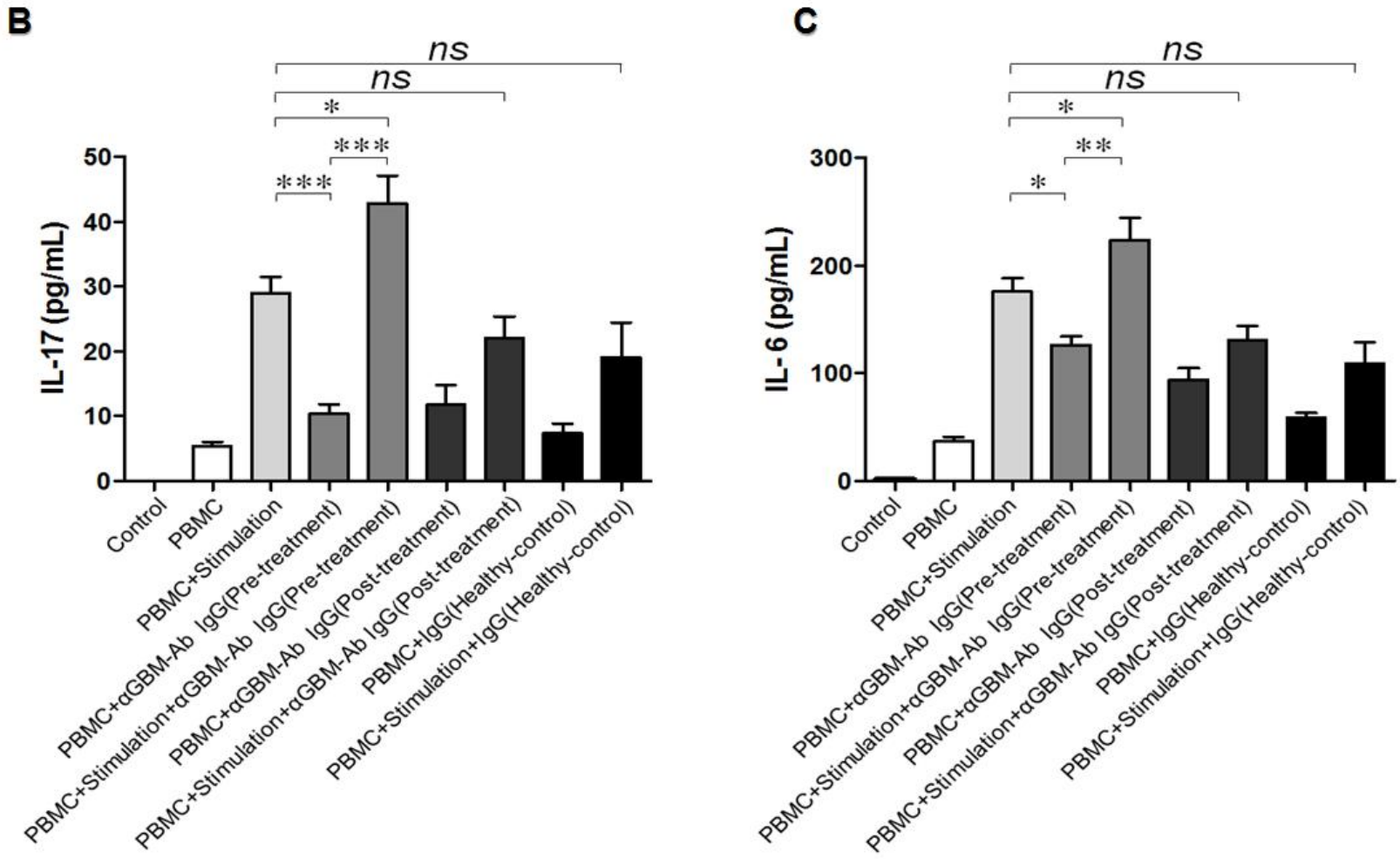
To further investigate the interactions between anti-GBM IgG, Th17 cells, and mesangial cells, I treated a co-culture system of human primary mesangial cells and peripheral-blood mononuclear cells (PBMCs) with Th17-activating cytokines (recombinant IL-6, TGFβ1, and IL-23), anti-GBM IgG obtained from a patient with anti-GBM GN, or both. The titers of anti-GBM Ab serially obtained from this patient showed a positive correlation with Th17-related and pro-inflammatory cytokines; plasmapheresis and immunosuppressive therapy gradually reduced the concentrations of circulating anti-GBM IgG and other pro-inflammatory cytokines over 90 days (Figure 6A). Mesangial cells alone did not produce IL-17 (Figure 6B, Control); PBMCs produced only a small amount of IL-17 in the absence of cytokine stimulation (Figure 6B, PBMC). IL-17 production from PBMCs was strongly induced by Th17-activating cytokines (Figure 6B, PBMC + stimulation) and further induced when pre-treatment (i.e. before the start of plasmapheresis and immunosuppression) anti-GBM IgG was added (Figure 6B, PBMC + stimulation + αGBM-Ab IgG). Anti-GBM IgG and Th17-activating cytokines additively increased the production of IL-6 from mesangial cells (Figure 6C, PBMC + stimulation + αGBM-Ab IgG). The post-treatment anti-GBM IgG isolated 30 days after the start of treatment elicited weaker response from mesangial cells and/or PBMCs, suggesting a concentration-dependent effect. Ctr9 in mesangial cells was repressed by Th17-activating cytokines and further repressed by anti-GBM IgG (Figure 6D). In summary, anti-GBM IgG and Th17-activating cytokines

cooperatively induce IL-17 in PBMCs and IL-6 in mesangial cells and repress Ctr9 in mesangial cells.

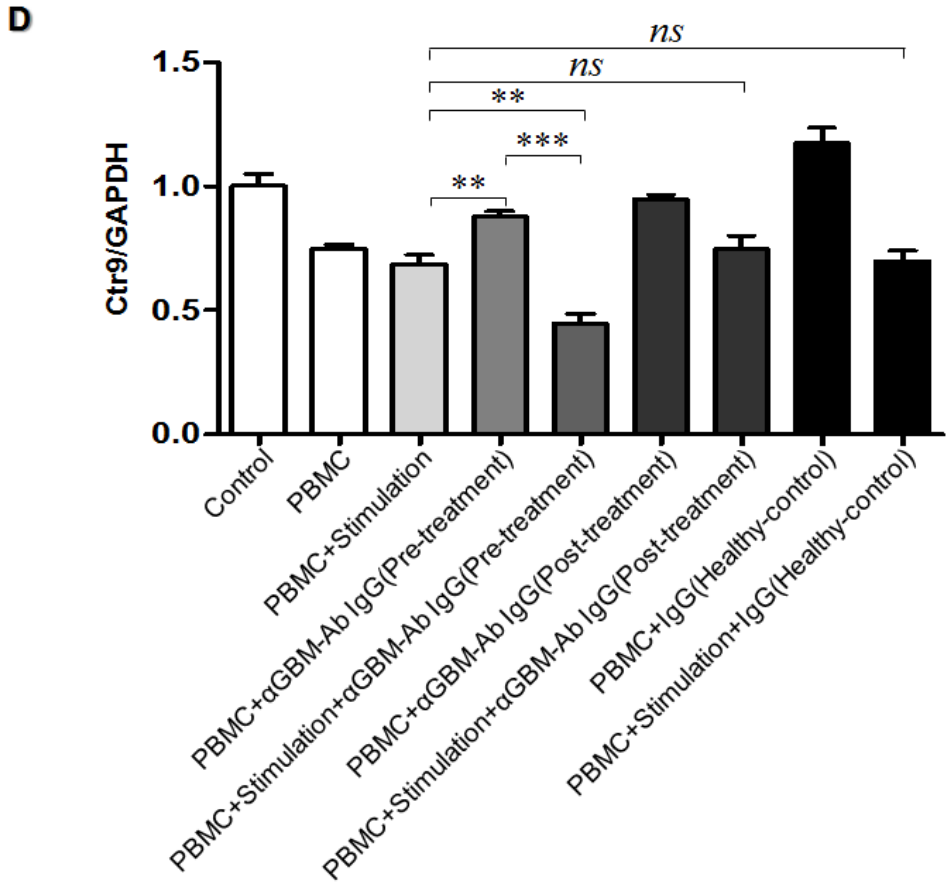
Figure 6. Anti-GBM IgG and Th17 environment additively induce IL-17 and IL-6 and repress Ctr9 in human primary mesangial cells.




Time courses of anti-GBM antibody titers and serum concentrations of Th17-related cytokines in a patient with anti-GBM GN. Serum samples were obtained at day 0 (prior to therapy), day 15, 30, and 90 after the initiation of therapy.


B
IL-17 (pg/mL)
50
40
30
20
10
0
ns
ns
*
***
***
Control
PBMC
PBMC+Stimulation
PBMC+αGBM-Ab IgG(Pre-treatment)
PBMC+Stimulation+αGBM-Ab IgG(Pre-treatment)
PBMC+αGBM-Ab IgG(Post-treatment)
PBMC+Stimulation+αGBM-Ab IgG(Post-treatment)
PBMC+IgG(Healthy-control)
PBMC+Stimulation+IgG(Healthy-control)
C
IL-6 (pg/mL)
300
200
100
0
ns
ns
*
**
*
Control
PBMC
PBMC+Stimulation
PBMC+αGBM-Ab IgG(Pre-treatment)
PBMC+Stimulation+αGBM-Ab IgG(Pre-treatment)
PBMC+αGBM-Ab IgG(Post-treatment)
PBMC+Stimulation+αGBM-Ab IgG(Post-treatment)
PBMC+IgG(Healthy-control)
PBMC+Stimulation+IgG(Healthy-control)

IL-17 (B) and IL-6 (C) production from human primary mesangial cells treated with various combinations of peripheral blood mononuclear cells (PBMCs), Th17 stimulation (rTGFβ1 + IL-6 + IL-23), anti-GBM IgG obtained from the same patient as in (A), and total IgG obtained from a healthy subject (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

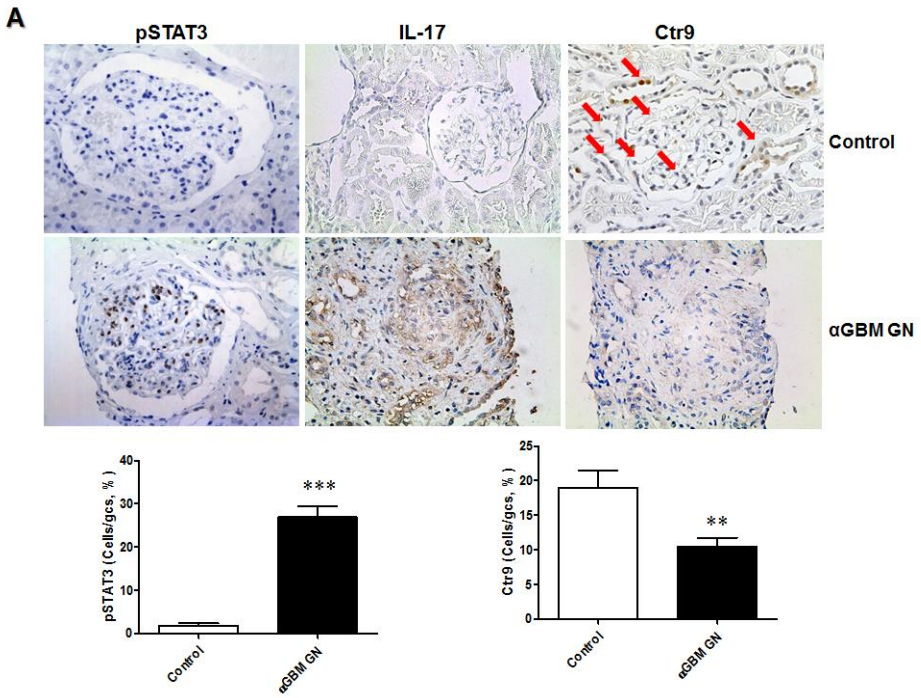



The mRNA abundances of Ctr9 in human primary mesangial cells as measured by quantitative real-time PCR (**$P < 0.01$, **$P < 0.005$).

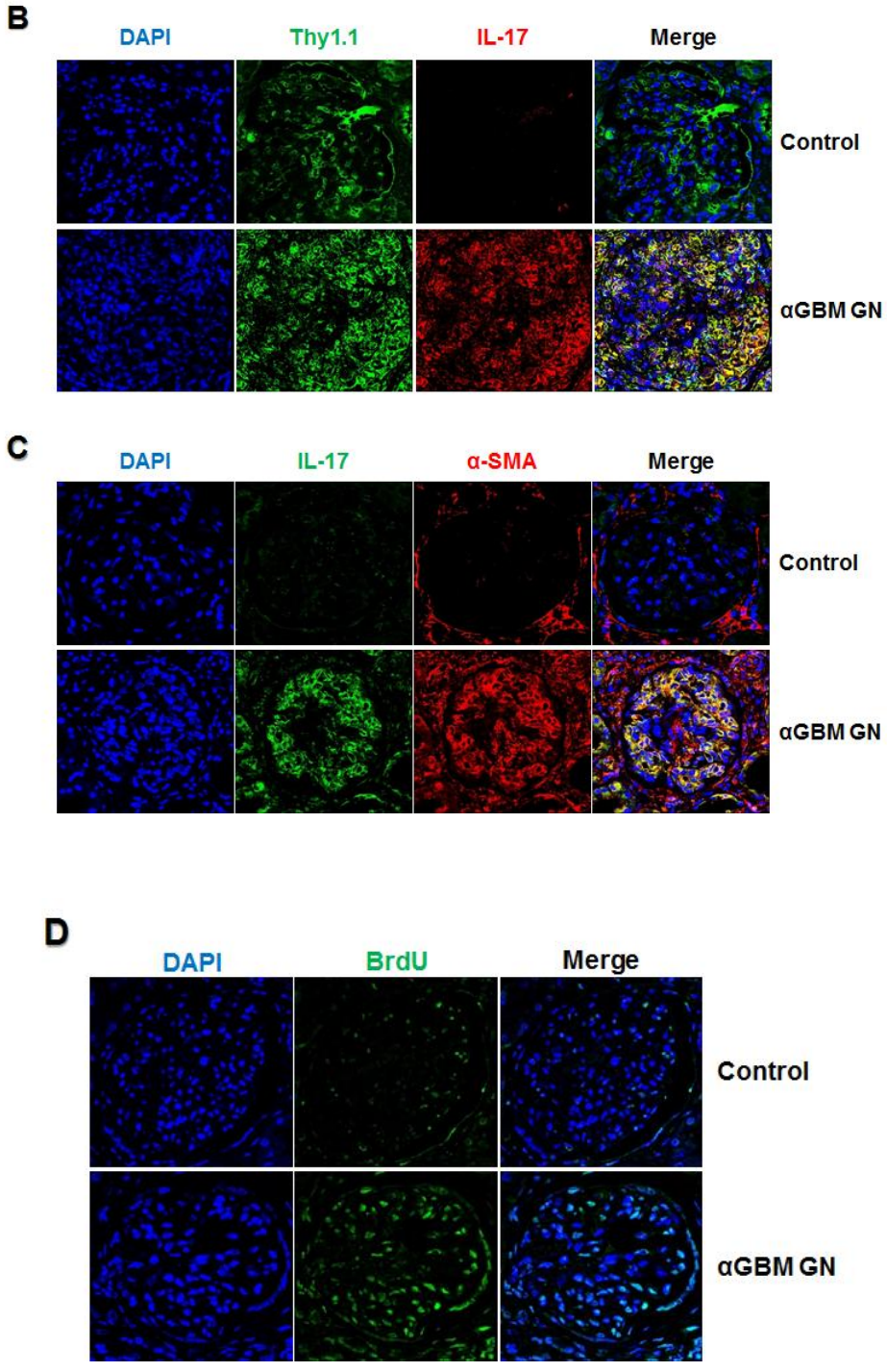
## Expression of Ctr9 in human kidneys with anti-GBM GN

Finally, I examined the activity of Th17 pathway in renal biopsies obtained from patients with anti-GBM GN (n = 4) and normal subjects (n = 3) using immunohistochemistry and quantitative real-time PCR. Clinical characteristics and renal functions of the patients and normal subjects are summarized in Table 3. On light microscopy, the expression of pSTAT3 was increased in the glomerulus of patients with anti-GBM GN and it was mainly observed in cellular crescents (Figure 7A, left). The expression of IL-17 was more diffuse than that of pSTAT3, showing significant upregulation in tubular epithelia as well as glomeruli (Figure 7A, center). Ctr9 expression was almost absent in anti-GBM GN (Figure 7A, right), recapitulating its negative correlation with Th17 pathway. On immunofluorescence microscopy, IL-17 expression showed a significant overlap with a mesangial-cell marker Thy1.1 (Figure 7B) and a proliferation marker α-smooth muscle actin (Figure 7C). Consistent with the pathophysiology of anti-GBM GN, cell proliferation was mainly seen in parietal epithelial cells and mesangial cells in bromodeoxyuridine staining (Figure 7D). In quantitative real-time PCR, intrarenal expression of STAT3α, STAT3β, and IL-17 mRNAs was increased and Ctr9 was decreased in anti-GBM GN (Figure 7E). Taken together, Ctr9 was a negative correlate of Th17 activity in human anti-GBM GN.

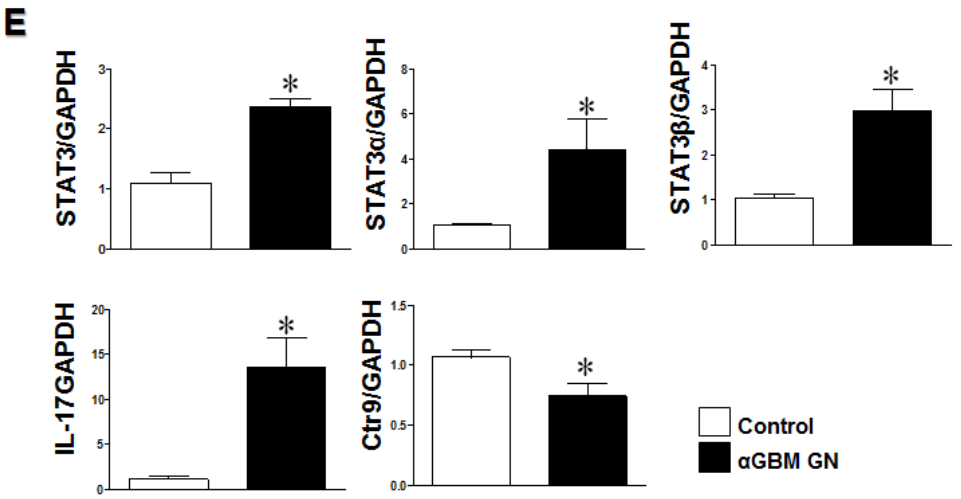
Figure 7. Ctr9 and Th17 activity in human anti-GBM GN.




Representative images of human kidney tissues obtained from normal subjects and patients with anti-GBM GN (original magnification 400x). In normal kidneys, Ctr9 expression was observed in the nuclei of glomerular and tubular epithelial cells (arrows). The percentages of phosphorylated STAT3 (pSTAT3)- and Ctr9-positive cells are shown in bar graphs (**$P < 0.01$, ***$P < 0.005$.


B
DAPI
Thy1.1
IL-17
Merge
Control
αGBM GN
C
DAPI
IL-17
α-SMA
Merge
Control
αGBM GN
D
DAPI
BrdU
Merge
Control
αGBM GN

(B) Immunofluorescence images of a glomerulus probed for Thy1.1 and IL-17 expression, (C) for IL-17 and α-smooth muscle actin, and (D) for bromodeoxyuridine (original magnification 600x).




(E) Relative mRNA abundances of STAT3, STAT3α, STAT3β, IL-17, and Ctr9 mRNAs in human kidney tissues obtained from 4 patients with anti-GBM GN and 3 normal controls (*$P < 0.05$, **$P < 0.01$).

# DISCUSSION

In this study, the role of renal Ctr9 was investigated in anti-GBM GN, focusing on relationship between Ctr9 and Th17-driven inflammation. Our findings could be summarized as follows: (1) Ctr9 is expressed at a moderate level in the normal kidney, especially in mesangial cells; (2) Th17-dominant inflammation in anti-GBM GN represses the expression of Ctr9; (3) disruption of Th17 differentiation increases the expression of Ctr9; (4) inhibition of Ctr9 in intrarenal T cells enhances differentiation of Th17 cells; (5) Ctr9 is normally expressed in mesangial cells and repressed by anti-GBM IgG and/or IL-17, probably through upregulation of IL-6; (6) anti-GBM IgG and Th17 cells cooperatively induce IL-17 and IL-6 and repress Ctr9 in mesangial cells; (7) and the expression patterns of Ctr9 in humans are consistent with those in knockout mice and cell cultures.

The relationship between Ctr9 and Th17-driven inflammation was initially focused in anti-GBM GN. When STAT3β or IL-17 knockout mice were injected with anti-GBM IgG, renal inflammation and the expression of Th17 transcripts significantly decreased, and Ctr9 increased relative to wild-type mice with anti-GBM GN. Although Ctr9 is repressed by Th17-driven inflammation, this does not mean that Ctr9 is directly repressed by STAT3 or IL-17. For STAT3, any binding of STAT3 at the promoter of Ctr9 gene was not found (Figure 2E). Since IL-6 initiates Th17 differentiation and directly represses Ctr9 expression (35), it is more likely that low IL-6 production in STAT3β-/- mice led to a decrease in repression

of Ctr9. IL-17 knockout essentially reproduced the protective effect of STAT3β knockout, consistent with prior knowledge on the role of IL-17 in organ-specific autoimmune disorders (1, 12). Similar to STAT3β$^{-/-}$ mice, the relative increase in Ctr9 mRNAs seen in IL-17 knockout mice with anti-GBM GN was likely to be mediated by a decrease in expression of IL-6. In contrast to previous studies that have reported an anti-inflammatory role of STAT3β (17, 37), our study suggests that STAT3β might function as an independent transcription factor capable of inducing transcription of the IL-6 target genes. Notably, disruption of STAT3β protected against renal injury due to anti-GBM IgG, as shown by reduction in cellular crescents and extent of renal injury and by a dramatic decrease in the deposition of anti-GBM IgG along the GBM. These findings indicate that STAT3β plays a critical role in the initiation of glomerular injury in anti-GBM GN, probably helping autoantibody bind to the GBM.

It was demonstrated that, albeit indirectly, the inhibitory role of Ctr9 in Th17 differentiation in anti-GBM GN. Although low efficiency of delivery and off-target effects of siRNAs prevented us from trying to inject Ctr9-siRNAs or Ctr9 overexpression vectors into mice with anti-GBM GN, *in vitro* differentiation experiments with splenocytes and intrarenal lymphocytes illustrated that Ctr9 represses Th17 differentiation in the context of autoimmune inflammation. This finding is consistent with a previous report that Ctr9 inhibition enhances Th17 differentiation and worsens inflammation in collagen-induced arthritis, a model of autoimmune joint inflammation (35). Disruption of Ctr9 in intrarenal lymphocytes

increased the proportion of Th17 cells and the mRNA abundances for Th17-related transcription factors STAT3 and RORγt. This up-regulation of STAT3 and RORγt mRNAs likely resulted from an increase in Th17 differentiation. Another explanation for Ctr9 knockout-induced up-regulation of STAT3 is that Ctr9 might inhibit the transcription of Stat3 gene at baseline and dissociate from Stat3 upon IL-6 stimulation, in the same way as Ctr9 regulates the transcription of IL-17. STAT3 itself is a target of IL-6, and Stat3 has a STAT3-binding element in its promoter sequence. In Th17 cells, STAT3 binds to the promoter of Stat3 (Figure 2E).

Aside from its role in Th17 lymphocytes, Ctr9 in mesangial cells may have a distinct role in regulating cell proliferation and production of pro-inflammatory cytokines in response to anti-GBM IgG or Th17 cytokines. In the experiments with STAT3β and IL-17 knockout mice, it was noted that Ctr9 was moderately expressed in normal mouse kidneys, mainly in the mesangial area. Consistent with this finding, cultured mouse and human mesangial cells expressed a moderate degree of Ctr9 at baseline. This moderate *in vivo* and *in vitro* expression of Ctr9 suggests that Ctr9 in mesangial cells plays a constitutive role such as inhibition of cell proliferation. Ctr9 potentially functions as a tumor-suppressor gene in some cancers, as a recent study has reported Ctr9 mutations in families with Wilms' tumors (7). In this regard, downregulation of Ctr9 in mesangial cells may be biologically significant in GNs with mesangial cell proliferation. However, a recent study on estrogen receptor-positive breast cancer cells has demonstrated that loss

of Ctr9 leads to a decrease in cell proliferation, calling into question the role of Ctr9 as a tumor-suppressor gene (39). Certainly, future research on Ctr9 as an inhibitor or facilitator of cell proliferation will be of great interest.

Interestingly, downregulation of Ctr9 by anti-GBM IgG or IL-17 seems to be mediated by IL-6. Similar to its regulation in Th17 cells, downregulation of Ctr9 in mesangial cells was inversely related to IL-6 production. Given the target specificity of Ctr9 and the autocrine/paracrine action of IL-6, it is tempting to argue that anti-GBM IgG and IL-17 represses Ctr9 via upregulation of IL-6. These findings are consistent with the *in vivo* experiments with mice deficient in Th17 activation, in which IL-6 production in response to anti-GBM IgG was significantly decreased in STAT3β and IL-17 knockout mice compared with wild-type counterparts.

Co-culture of PBMCs and mesangial cells further illustrated the complex nature of Th17-driven inflammation in anti-GBM GN. It developed in Anti-GBM IgG and Th17-activating cytokines (i.e. IL-6, TGFβ, and IL-23) additively increased the production of IL-17 from PBMCs and IL-6 from mesangial cells, and decreased the expression of Ctr9 in mesangial cells. Our *in vivo* and *in vitro* studies suggest that Ctr9 and IL-17 might reciprocally regulate each other in T lymphocytes and mesangial cells in anti-GBM GN. In T cells, Ctr9 constitutively represses the transcription of IL-17 and is released from the gene body of Il17a upon IL-6 binding (35); IL-17 binds to mesangial cells to induce IL-6 and repress Ctr9. Although IL-17 may directly repress the transcription of Ctr9, it would be very

difficult to distinguish the effect of IL-17 from that of IL-6 *in vivo* because IL-17 can induce IL-6 in many different cell types including Th17 cells (12) and IL-6 in turn induces differentiation of Th17 cells. More studies are needed to further address the mechanism of Ctr9 action in mesangial cells responding to the pro-inflammatory milieu in anti-GBM GN.

In this study, we used mesangial cells for the main player of renal injury in anti-GBM GN, although renal injury of anti-GBM GN started from endothelial basement membrane damage. Human kidney mesangial cells play an active role in the inflammatory response to glomerular injury. They contribute to phagocyte infiltration of the glomerulus because they express intracellular adhesion molecule-1 (41) and vascular cell adhesion molecule-1 (42) or secrete osteopontin, and a glycoprotein with cell adhesive properties which plays a role in macrophage infiltration and fibronectin (43, 44). Moreover, they have evolved prominent proinflammatory features as a result of the cross-communication with invading immune cells (45). From these, most of previous studies dealing with autoimmune crescentic GN investigated the pathogenesis by using mesangial cells (13, 22-25). Although the main target of injury was not explored in this study, cascade of renal inflammation could be regulated in the mesangial cells in anti-GBM GN. Further researches focusing glomerular endothelial cells or glomerular endothelial cell-mesangial cell cross-talk may contribute to understanding our study findings more elaborately.

In conclusion, Ctr9 is normally expressed in both T cells and mesangial cells, and is associated with Th17-driven inflammation suppression in anti-GBM GN. In T cells, Ctr9 inhibits differentiation of Th17 cells. In mesangial cells, anti-GBM autoantibodies and/or IL-17 repress Ctr9, probably through IL-6 upregulation. It is likely that Ctr9 in T lymphocytes and mesangial cells transcriptionally modulates transcription of IL-6-activated genes in both cell types and thereby participates in an interaction network encompassing cells and cytokines involved in the pathogenesis of anti-GBM GN. This is the first study to examine the association between Ctr9 and Th17 driven renal inflammation in anti-GBM GN. Further study should be considered to clarify whether Ctr9 has an impact on Th17 pathway as a negative regulator in anti-GBM GN.

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# 국문 초록

T helper 17 (Th17) 림프구는 항기저막사구체신염에서 신장의 염증을 악화시키며, signal transducer and activator of transcription 3 (STAT3)는 IL-6 와 TGF$\beta$을 통해 Th17 세포를 활성화시킨다. Clin three requiring 9 (Ctr9)는 RNA polymerase-associated factor complex (PAFc)의 한 구성 요소로서, IL-6/STAT3 에 관련된 유전자의 전사를 조절하는 역할을 한다. 본 연구에서는 항기저막사구체신염에서 Ctr9 과 Th17 연관 신장 염증과의 관계에 대하여 조사해 보고자 하였다.

생쥐에서 STAT3$\beta$ 혹은 IL-17 를 knockout 시켰을 때, 항기저막항체에 의한 신장 손상이 감소하였다. 이 현상은 retinoic acid receptor-related orphan receptor $\gamma$t (ROR$\gamma$t), IL-17, 인산화 STAT3, 그리고 다양한 항 염증성 사이토카인의 감소와 연관되어 있었다. 이 때 Ctr9 은 STAT3$\beta$ 혹은 IL-17 knockout 시킨 생쥐에서 정상에 비해 증가하였고, Th17 세포 관련 전사체와 음의 상관관계를 가지고 있었다. Small interfering RNA (siRNA)를 통해 신장 내 림프구의 Ctr9 을 knockdown 시켰을 때, Th17 연관 전사체의 발현이 증가하여, Ctr9 이 Th17 경로의 발현 감소와 관련되어 있음을 확인하였다. 흥미롭게도 Ctr9 은 사람과 생쥐의 메산지움 세포에서 모두 발현되고 있었으며, 항기저막항체를 주입하는 경우 발현이 감소되었다. 메산지움 세포에서 Ctr9 은 항기저막항체와 Th17 경로 연관 사이토카인을 함께 투여하였을 때, 각각을 단독으로 투여했을 때보다 더욱 현저히 감소하였다. 이와 같은

맥락으로 항기저막사구체신염 환자의 신장 조직에서도 Ctr9 전사체 발현이 감소되어 있고, 인산화 STAT3 와 IL-17 의 발현이 증가되어 있었다.

본 연구에서는 항기저막사구체신염에서 Ctr9 이 Th17 경로와 음의 상관 관계를 가지고 있고, 메산지움 세포에 항기저막항체를 투여하였을 때 IL-17 의 발현이 감소함을 확인하였다. 이 연구는 항기저막사구체신염에서 Ctr9 의 발현과 과 Th17 연관 신장 염증과의 관계를 밝힌 최초의 연구이다. 항기저막사구체신염에서 Ctr9 이 Th17 경로에 직접적으로 영향을 미치는 전사조절인자인지를 확인하기 위해서는 추가적인 연구가 필요할 것으로 생각된다.

**주요어**: 항기저막사구체신염, Ctr9, STAT3, Th17 경로

**학 번**: 2012-30559