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The IL-27 Receptor Has Biphasic Effects in Crescentic Glomerulonephritis Mediated Through Th1 Responses

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Despite its initially defined role as a T-helper type 1 cell (Th1)–inducing cytokine, interleukin-27 (IL-27) has complex roles *in vivo*. The role of IL-27 receptor (IL-27R) was defined in experimental crescentic glomerulonephritis induced by a foreign antigen, sheep globulin, which is planted in glomeruli. This lesion is dependent on a Th1 effector cellular response. Twenty-one days after the administration of sheep anti-mouse glomerular basement membrane antibody, wild-type mice developed histologic and functional inflammatory renal injury. Injury was attenuated in the absence of IL-27R α chain (IL-27Rα), the unique component of the IL-27R complex. In contrast to the attenuated renal injury on day 21, *Il27ra*$^{-/-}$ mice exhibited enhanced systemic immune responses, including Th1 responses, with increased IL-2–dependent interferon-γ (IFN-γ) production. However, earlier in the development of the nephritogenic immune response, IFN-γ production was decreased, with reduced early immune responses translating into attenuated renal injury. Having demonstrated decreased early Th1 systemic immune responses, followed by enhanced nephritogenic Th1 immune responses, renal injury was studied at later time points. On days 28 and 35 after injection of the nephritogenic antigen, renal injury was enhanced in *Il27ra*$^{-/-}$ mice compared with wild-type mice in an at least partially IFN-γ–dependent manner. In Th1-dependent autoinflammatory lesions, IL-27Rα has a biphasic role *in vivo*, initially pathogenic, but ultimately playing a protective role by regulating immune responses and attenuating disease. *(Am J Pathol 2011, 178:580–590; DOI: 10.1016/j.ajpath.2010.10.013)*

Glomerulonephritis (GN) is a common cause of renal disease, including end-stage renal failure. Whereas Igs play an important role in some types of GN, crescentic GN features the prominent involvement of cell-mediated immune effectors. Previously, we demonstrated that murine crescentic GN is driven by cell-mediated nephritogenic T-helper type 1 cell (Th1) immune responses.[1,2] In this model, renal injury is induced by a foreign antigen (sheep globulin) planted in glomeruli by systemic injection of sheep anti-mouse glomerular basement membrane (GBM) globulin. This antigen induces systemic immune responses and is also planted in glomeruli. Cell-mediated effector responses play an important role in the resultant injury. Severe crescentic injury is effector CD4$^+$ cell dependent[2,3] and is independent of autologous antibodies.[3] The Th1 cytokines and transcription factors T-bet,[4] interleukin-12 (IL-12),[5] IL-18,[6] and interferon-γ (IFN-γ)[2,7] mediate severe disease. Conversely, the endogenous Th2 cytokines IL-4 and IL-10 limit disease,[8,9] and administration of IL-4 and/or IL-10 lessens glomerular injury.[8,10,11]

Interleukin-27 is a member of the IL-6/IL-12 family consisting of two subunits: IL-27p28 and an Epstein-Barr virus–induced protein 3.[12] The IL-27p28 subunit shares homology with IL-12p35, and Epstein-Barr virus–induced protein 3 shares homology with IL-12p40. Interleukin-27 binds to a heterodimeric receptor consisting of a unique α chain, IL-27Rα (also known as WSX-1 and T-cell cytokine receptor), and to the gp130 chain. The IL-27Rα/gp130 complex is the only known

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receptor for IL-27. Although IL-27Rα is unique, the gp130 chain is used by several other cytokines, including IL-6 and IL-11.[13] High expression of IL-27Rα has been identified on $CD4^+$ T cells, natural killer cells, and macrophages.[14,15]

Initial studies suggested that IL-27 is responsible for directing host immune responses toward a Th1 proinflammatory phenotype. T-bet was induced after stimulating undifferentiated T cells with IL-27,[16,17] IL-27 induced IFN-γ production after T-cell stimulation,[12,17,18] and cells from *Il27ra*$^{-/-}$ mice produced less IFN-γ after stimulation.[14] Experimental models of infection performed in *Il27ra*$^{-/-}$ mice suggested early Th1 deficiency[15,19,20] and a later proinflammatory state with protection from infection but overproduction of inflammatory mediators.[21–23] Further evidence supporting a role for endogenous IL-27 in immunoprotection was demonstrated in murine experimental hepatitis[24] and experimental autoimmune encephalomyelitis.[25] In experimental systemic lupus erythematosus, *Il27ra*$^{-/-}$ mice developed skewed immune responses with a shift in renal injury to membranous lupus nephritis.[26] However, subsequent studies suggested that overexpression of WSX-1 protected mice from autoimmune disease.[27]

To define an *in vivo* role for IL-27Rα in Th1-dependent crescentic GN, we examined the effects of administering sheep anti-mouse GBM antibody to C57BL/6J wild-type (WT) and *Il27ra*$^{-/-}$ mice on a C57BL/6J background at 21 days (the standard experimental end point). In *Il27ra*$^{-/-}$ mice, kidney injury was attenuated despite enhancement of systemic nephritogenic immune responses. However, systemic immune responses at an earlier time point, day 3, demonstrated an early selective deficit in IFN-γ production in *Il27ra*$^{-/-}$ mice. Analyses of renal injury at extended time points (28 and 35 days) showed enhanced kidney injury in *Il27ra*$^{-/-}$ mice, demonstrating a protective role for IL-27Rα later in disease.

Materials and Methods

Experimental Design and Statistics

WSX-1$^{-/-}$ (*Il27ra*$^{-/-}$) mice on a C57BL/6J background backcrossed more than nine times[15] were bred at Monash Medical Centre (Melbourne, VIC, Australia). C57BL/6J WT mice were obtained from Monash Animal Services (Melbourne, VIC, Australia). Sheep anti-mouse GBM antibody was generated as previously described.[7] Autologous phase anti-GBM GN was induced in age-matched 8- to 10-week-old male mice after the i.v. injection of 15 mg of sheep anti-mouse GBM antibody (day 0). Immune responses and/or renal injury were measured on days 3, 21, 28, and 35. In the experiments performed on days 3, 21, 28, and 35, the numbers of mice used for experiments were as follows: 6, 13, 6, and 13 WT mice and 6, 14, 6, and 8 *Il27ra*$^{-/-}$ mice, respectively. For studies of immune responses (day 21) with *ex vivo* cytokine inhibition, six WT and seven *Il27ra*$^{-/-}$ mice were studied. For studies assessing renal injury after *in vivo* anti–IFN-γ antibody administration, eight WT and seven *Il27ra*$^{-/-}$ mice were used. Beginning 14 days after sheep anti-mouse globulin administration, mice were injected (i.p.) with 500 μg of anti–IFN-γ antibody (clone R46A2) every second day until experiments ended on day 28. Studies were performed in accordance with National Health and Medical Research Council of Australia guidelines and were approved by the Monash University Animal Ethics Committee. Results are expressed as mean ± SEM. For statistical analysis, the unpaired *t*-test was used (GraphPad Prism; GraphPad Software Inc., San Diego, CA). A $P < 0.05$ was considered statistically significant.

Assessment of Renal Injury

Glomerular abnormalities were assessed on PAS-stained, Bouin's-fixed, 3-μm-thick, paraffin-embedded sections using coded slides. Glomerular crescent formation was defined as two or more layers of cells in Bowman's space (in ≥50 glomeruli per mouse). Semiquantitative analysis of tubulointerstitial damage was performed on these sections as previously described.[4] From each animal, 10 randomly selected cortical medium-power fields were examined. Injury was defined as tubular dilatation, tubular atrophy, sloughing of tubular epithelial cells, or thickening of the basement membrane. Each cortical field was allocated a score according to the amount of injury observed in the tubulointerstitial field (0, no tubulointerstitial damage; 1, <25%; 2, 25% to 50%; 3, 51% to 75%; and 4, >75% of the tubulointerstitial field damaged). To examine glomerular and interstitial extracellular matrix accumulation at 28 days, 4-μm-thick sections were stained with picrosirius red (Sigma-Aldrich, St. Louis, MO).[28] Collagen deposition was assessed under polarized light and was quantitated by means of NIH *Image* analysis (Scion Image; Scion Corp., Frederick, MD). A minimum of 25 glomeruli and 15 high-power (magnification, ×400) fields were assessed per animal; vascular, periglomerular, and perivascular areas were excluded. Results are expressed as the percentage of the glomerulus or interstitial cortical area affected. Urine samples were collected using metabolic cages for 24 hours before the end of the experiments. Proteinuria was determined using a modified Bradford assay and is expressed as milligrams per 24 hours.[4] Serum creatinine measurements were recorded after termination of the experiment using an alkaline picric acid method and an autoanalyzer.

Renal Leukocyte Accumulation and Immunohistochemical Analysis

Kidney sections were fixed in periodate lysine paraformaldehyde for 4 hours, washed with 20% sucrose solution, and then frozen in liquid nitrogen. Tissue sections were cut, and a three-layered immunoperoxidase technique was used to stain for $CD4^+$ T cells, $CD8^+$ T cells, and macrophages. The primary antibodies used were GK1.5 for $CD4^+$ T cells (anti-mouse CD4; American Type Culture Collection, Manassas, VA), 53–6.7 for $CD8^+$ T

cells (anti-mouse CD8; American Type Culture Collection), and FA/11 for macrophages (anti-mouse CD68; provided by Dr. G. Koch, Cambridge, England). The secondary antibody used was rabbit anti-rat biotin (BD Biosciences, San Jose, CA). A minimum of 20 consecutively viewed glomeruli and 10 interstitial sections were assessed per animal. Results are expressed as cells per glomerular cross section or cells per high-power field when examining interstitial sections for $CD4^+$ and $CD8^+$ T cells previously described.[4] When examining interstitial macrophage accumulation, sections were scored according to the area covered by macrophages (1, 0% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, 76% to 100%).

Intrarenal Cytokine mRNA Expression

For measurement of T-bet, tumor necrosis factor (TNF), IL-1β, forkhead box P3 (FoxP3), GATA-3, and RORγ by means of RT-PCR, 500 ng of RNA was treated with 1 U of amplification-grade DNase I (Invitrogen, Melbourne, VIC, Australia), primed with random primers (Applied Biosystems, Foster City, CA), and reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Gene-specific oligonucleotide primers designed using the Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) were synthesized by Invitrogen as previously described.[4] A Rotor Gene RG-3000 (Corbett Research, Mortlake, VIC, Australia) using Power SYBR Green PCR Master Mix (Applied Biosystems) was used to perform RT-PCR. PCR products were confirmed using melting curve analysis, and mRNA expression was quantified using serial dilutions of an exogenous standard. Primer sequences used were as previously described.[4,29] *T-bet*, *TNF*, *IL-1β*, *FoxP3*, *GATA-3*, and *IL-10* expression was standardized to *18S* (housekeeping gene) before being expressed as a fold increase relative to WT mice with GN. gp130, IL-27Rα, IL-27p28, and Epstein-Barr virus–induced protein 3 mRNA are expressed as fold change relative to the housekeeping gene 18S.

Antigen-Stimulated Splenocyte Cytokine Production and Circulating Antigen-Specific Antibody Titers

Using an aseptic technique, a single-cell suspension of splenocytes (4×10^6 cells/ml) was cultured in RPMI/10% fetal calf serum with protein G–purified normal sheep IgG (10 μg/ml) at 37°C for 72 hours. Interferon-γ, IL-2, IL-4, and IL-17A concentrations were measured by means of enzyme-linked immunosorbent assay as previously described.[6] The following antibodies were used: rat anti-mouse IFN-γ (R4-6A2; BD Pharmingen, San Diego, CA), biotinylated rat anti-mouse IFN-γ (XMG1.2; BD Pharmingen), rat anti-mouse IL-4 (11B11; American Type Culture Collection), biotinylated rat anti-mouse IL-4 (BVD6; Dnax, Palo Alto, CA), rat anti-mouse IL-2 (JES6-1A12; Dnax), biotinylated anti-mouse IL-2 (JES6-5H4; Dnax), anti-mouse IL-10 (18141D; BD Pharmingen), and biotinylated rat anti-mouse IL-10 (18152D; BD Pharmingen). For IL-17A concentrations, an enzyme-linked immunosorbent assay (DuoSet; R&D Systems, Minneapolis, MN) was used. For *ex vivo* IL-2 inhibition studies, 10 μg of anti–IL-2 (JES6-1A12) or 10 μg of nonimmune rat IgG were added to cultured splenocytes at the beginning of the culture period. For IL-10 production by $CD4^+$ cells at 21 days, 2×10^6 spleen cells were cultured with protein G–purified normal sheep IgG (5 μg/ml) for 24 hours. Cells were stimulated with phorbol myristate acetate (1 mg/ml) and ionomycin (0.5 mg/ml) blocked with brefeldin A (1 mg/ml) and then were labeled with allophycocyanin-Cy7–conjugated anti-CD4 and allophycocyanin–anti–IL-10 (all from eBioscience Inc., San Diego, CA) and analyzed using a BD FACS Canto (BD Biosciences). Enzyme-linked immunosorbent assay was used to detect circulating serum antigen-specific IgG titers,[11] with serial dilutions of sera (1:50 to 1:3200). For IgG1, sera were tested at a dilution of 1:100, and for IgG3, at 1:50 using biotinylated rat anti-mouse antibodies (BD Pharmingen). Results are expressed as $OD_{450} \pm$ SEM.

Proliferation, Apoptosis, and Activation of $CD4^+$ T Cells and $CD19^+$ B Cells

For the assessment of proliferation and activation using flow cytometry, spleens were obtained from WT and *Il27ra*$^{-/-}$ mice on day 3 after injection of sheep anti-mouse GBM globulin. To measure proliferation, 1 mg of bromodeoxyuridine (BrdU; Sigma-Aldrich) was injected i.p. 48, 36, 24, and 12 hours before the end of the experiments. Proliferation, apoptosis, and activation were assessed by analyzing intracellular BrdU incorporation (results are expressed as the percentage of $CD4^+$ or $CD19^+$ cells that are $BrdU^+$).[4] The antibodies used were allophycocyanin-Cy7–conjugated anti-CD19, phosphatidylethanolamine-conjugated anti-CD4, and fluorescein isothiocyanate–conjugated anti-BrdU with DNase (BD Pharmingen).

Results

Histologic and Functional Renal Injury Is Attenuated in Il27ra$^{-/-}$ *Mice on Day 21*

Initial studies focused on injury and immune responses in mice 21 days after intravenous injection of sheep anti-mouse GBM antibodies. Day 21 of disease (after development of a mature immune response to sheep globulin and when significant crescentic glomerular injury has developed) is the standard time point used to study this model. By 21 days, genetically intact C57BL/6J WT mice had developed glomerular crescent formation, tubulointerstitial injury, and functional renal injury (Figure 1). Crescentic glomerular injury and tubulointerstitial damage were attenuated in *Il27ra*$^{-/-}$ mice (Figure 1, A and B). Serum creatinine levels were less elevated in *Il27ra*$^{-/-}$ mice (Figure 1C), but proteinuria was not significantly different between the two groups (Figure 1D). The mean $\pm$ SEM serum creatinine value in WT mice without GN

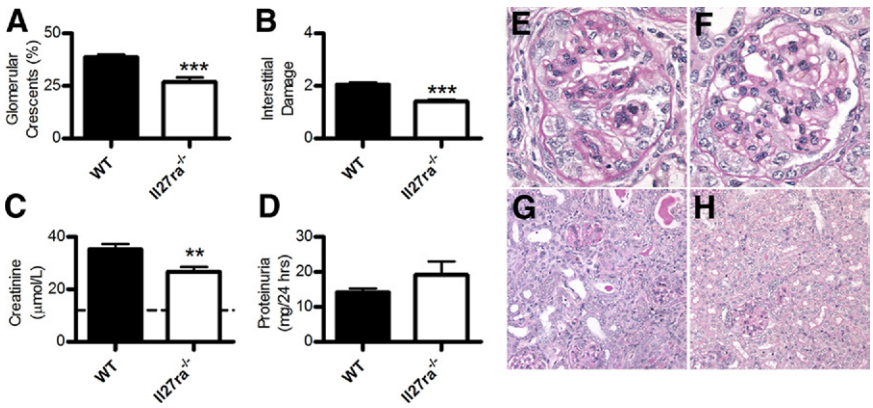

Figure 1. Histologic and functional injury in wild-type (WT) and *Il27ra*$^{-/-}$ mice 21 days after injection of sheep anti-mouse GBM globulin. **A:** On day 21, *Il27ra*$^{-/-}$ mice had developed fewer glomerular crescents. **B:** Tubulointerstitial injury was diminished in the absence of the IL-27Rα chain. **C:** Functional injury demonstrated less renal impairment (lower serum creatinine levels) in *Il27ra*$^{-/-}$ mice than in WT control mice. The dashed horizontal line represents the mean serum creatinine values in untreated WT mice. **D:** There was no change in 24-hour urine protein excretion. **E–H:** Representative kidney sections demonstrating histologic injury in WT and *Il27ra*$^{-/-}$ mice 21 days after the administration of sheep anti-mouse GBM antibody. Glomerular renal injury is demonstrated at high power in WT (**E**) and *Il27ra*$^{-/-}$ (**F**) mice and interstitial injury in WT (**G**) and *Il27ra*$^{-/-}$ (**H**) mice. Original magnification: ×400 (**E** and **F**); ×200 (**G** and **H**). Error bars represent SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT mice with GN.

(12.0 ± 1.7 μmol/L) is represented by the dashed line in Figure 1C. Representative kidney sections demonstrating glomerular injury in the presence and absence of IL-27Rα are shown in Figure 1, E and F, and representative photomicrographs of interstitial injury are shown in Figure 1, G and H.

Cellular Effectors, Transcription Factors, and Proinflammatory Cytokines in Kidneys of Mice with GN on Day 21

Compared with WT mice with GN, at 21 days, there were more CD4$^+$ T cells in glomeruli of *Il27ra*$^{-/-}$ mice. Glomerular macrophage and CD8$^+$ T-cell numbers were unaltered (Figure 2A). A similar pattern was evident in the tubulointerstitial infiltrate (Figure 2B). Compared with WT mice with GN, intrarenal mRNA expression of the key Th1-specific transcription factor (T-bet) was decreased in *Il27ra*$^{-/-}$ mice (Figure 2C), as was expression of TNF mRNA and IL-1β mRNA (Figure 2, D and E), proinflammatory cytokines with known functional roles in promoting renal injury in this model.[30,31] There was no difference in intrarenal mRNA expression of GATA-3, the key Th2 transcription factor, although expression of RORγ, the key Th17 transcription factor, was increased in *Il27ra*$^{-/-}$ mice (Figure 2, F and G). Intrarenal FoxP3 mRNA expression was unchanged in the absence of IL-27Rα (Figure 2H). Intrarenal IL-10 mRNA expression was not different between the groups [WT versus *Il27ra*$^{-/-}$: 1.0 ± 0.2 versus 0.8 ± 0.3 (mean ± SEM fold change with WT normalized to 1.0)].

Systemic Immune Response to the Nephritogenic Antigen Are Enhanced in Il27ra$^{-/-}$ *Mice, and Enhanced IFN-γ Is IL-2 Dependent*

On day 21, systemic T-cell responses assessed by antigen-stimulated splenocyte cytokine were enhanced in *Il27ra*$^{-/-}$ mice. Compared with WT mice, IFN-γ and IL-2 production was increased in *Il27ra*$^{-/-}$ mice (Figure 3A and B). At this time point, IL-4 production was also increased in *Il27ra*$^{-/-}$ mice compared with WT mice (Figure 3C), and there was a trend toward increased IL-10 production in *Il27ra*$^{-/-}$ mice (Figure 3D), but IL-17A production was unaltered in the absence of a functional IL-27R (Figure 3E). Flow cytometry of IL-10 production by CD4$^+$ cells confirmed that IL-27Rα was not playing a major role in regulating IL-10 from CD4$^+$ cells (mean ± SEM: 0.60% ± 0.27% and 0.59% ± 0.33% of CD4$^+$ cells producing IL-10 in WT and *Il27ra*$^{-/-}$ mice, respectively). To determine whether IL-2 was responsible for the enhanced IFN-γ production in *Il27ra*$^{-/-}$ mice, IL-2 was neutralized in splenocyte cultures. Anti–IL-2 antibodies reduced IFN-γ production in *Il27ra*$^{-/-}$ mice (mean ± SEM: rat IgG, 765 ± 280; anti–IL-2 antibodies, 152 ± 22 pg/ml; $P < 0.05$) but had little effect on IFN-γ production in WT mice (mean ± SEM: rat IgG, 326 ± 210; anti–IL-2 antibodies, 233 ± 98 pg/ml). Compared with WT mice, *Il27ra*$^{-/-}$ mice exhibited increased levels of circulating antigen-specific mouse IgG levels (Figure 4A). Production of antigen-specific IgG1 but not of the IgG3 subclass was increased in *Il27ra*$^{-/-}$ mice compared with WT mice (Figure 4, B and C).

Early Systemic IFN-γ Production Is Decreased in Il27ra$^{-/-}$ *Mice*

Reduced renal disease in *Il27ra*$^{-/-}$ mice at day 21, in the face of heightened T- and B-cell responses, led us to hypothesize that early in the development of nephritogenic immunity, Th1 responses would be diminished in *Il27ra*$^{-/-}$ mice. Systemic T-cell immune responses in WT and *Il27ra*$^{-/-}$ mice were analyzed 3 days after the administration of sheep anti-mouse GBM globulin. Whereas splenic T- and B-cell proliferation on day 3 was unaltered (BrdU$^+$ incorporation; Figure 5 A and B), antigen-stimulated IFN-γ production was decreased in *Il27ra*$^{-/-}$ mice compared with WT mice (Figure 5C), demonstrating impaired early development of Th1 responses. There was a trend toward reduced IL-2 levels in *Il27ra*$^{-/-}$ mice (Figure 5D), whereas splenocyte IL-4, IL-10, and IL-17A production was comparable between WT and *Il27ra*-deficient mice (Figure 5, E–G). Therefore, there is a delay in the development of systemic Th1 responses in *Il27ra*$^{-/-}$ mice after injection of sheep anti-mouse GBM globulin.

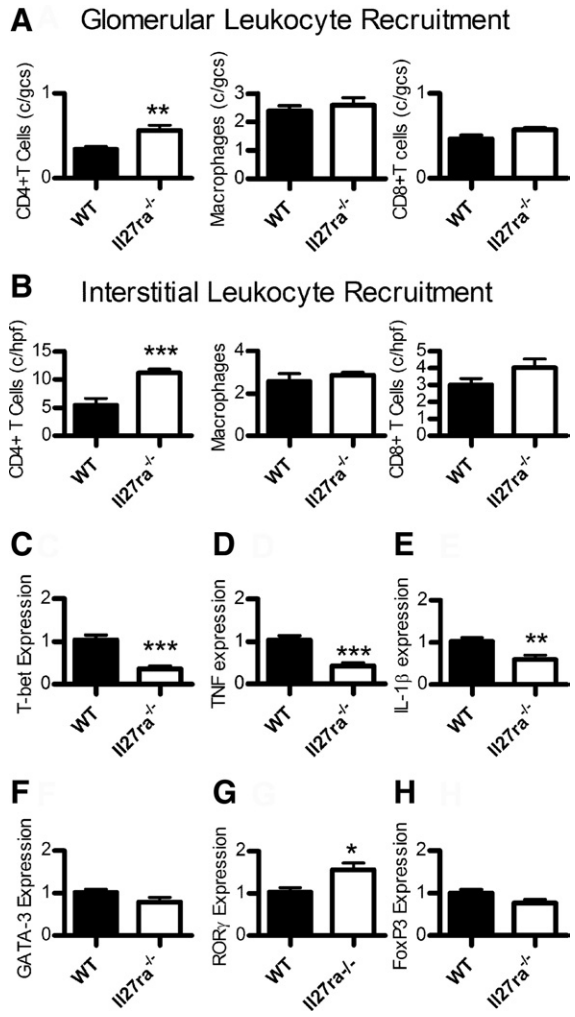

Figure 2. Effectors of cell-mediated renal injury in WT and *Il27ra*$^{-/-}$ mice. **A:** At day 21, there were more CD4$^+$ T cells in glomeruli of *Il27ra*$^{-/-}$ mice than of WT mice with GN but no differences in glomerular macrophage accumulation or glomerular CD8$^+$ T-cell recruitment. **B:** Increased interstitial CD4$^+$ T cells but not macrophages or CD8$^+$ T cells were seen in *Il27ra*$^{-/-}$ mice compared with WT mice. Intrarenal mRNA expression of proinflammatory mediators was decreased in *Il27ra*$^{-/-}$ mice, including expression of T-bet (**C**), TNF (**D**), and IL-1β (**E**). There was no difference in mRNA expression of GATA-3 (**F**), whereas RORγ expression (**G**) was increased in *Il27ra*$^{-/-}$ mice. **H:** A trend toward decreased intrarenal FoxP3 mRNA expression was seen in *Il27ra*$^{-/-}$ mice ($P = 0.07$). Error bars represent SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT mice with GN.

Histologic and Functional Renal Injury Is Exacerbated in Il27ra$^{-/-}$ *Mice on Day 28, Which Is at Least Partially IFN-γ Dependent*

Nephritogenic immunity in *Il27ra*$^{-/-}$ mice demonstrated an early Th1 deficit, but increased systemic responses were apparent by day 21. Therefore, renal injury was assessed in WT and *Il27ra*$^{-/-}$ mice at later time points. By 28 days after administration of anti-GBM antibodies, the crescentic renal injury and interstitial disease present in WT mice was augmented in the absence of IL-27Rα (Figures 6 and 7). Compared with WT mice, *Il27ra*$^{-/-}$ mice exhibited more glomerular crescent formation (Figure 6A), more tubulointerstitial injury (Figure 6B), increased renal impairment (Figure 6C), and increased proteinuria (Figure 6D). Representative images of glomerular injury in WT mice (Figure 7A) and *Il27ra*$^{-/-}$ mice (Figure 7B) and interstitial injury in WT mice (Figure 7C) and *Il27ra*$^{-/-}$ mice (Figure 7D) are shown. There were no differences in cytokine production or humoral responses on day 28 between the groups.

Renal fibrosis results from chronic inflammation. To quantitate chronic injury, kidney sections were assessed for glomerular and interstitial matrix accumulation by using picrosirius red staining. Glomerular (but not interstitial) matrix deposition was increased in *Il27ra*$^{-/-}$ mice compared with WT controls (analyzed using NIH *Image*; Figure 7, E–J). Increased numbers of CD4$^+$ T cells and macrophages were present in glomeruli of *Il27ra*$^{-/-}$ mice, but glomerular CD8$^+$ T-cell numbers were unchanged (Figure 8A); numbers of all three leukocyte subsets were increased in the interstitium (Figure 8B). Intrarenal mRNA expression of T-bet, TNF, and IL-1β had increased in *Il27ra*$^{-/-}$ mice so that by day 28 there was no decrease in *Il27ra*$^{-/-}$ mice (Figure 8, C–E). There was no difference in intrarenal GATA-3 or RORγ mRNA expression (Figure 8, F and G). Intrarenal FoxP3 expression was increased in *Il27ra*$^{-/-}$ mice compared with WT mice (Figure 8H). There was a trend toward increased intrarenal IL-10 mRNA expression in *Il27ra*$^{-/-}$ mice (mean ± SEM: WT

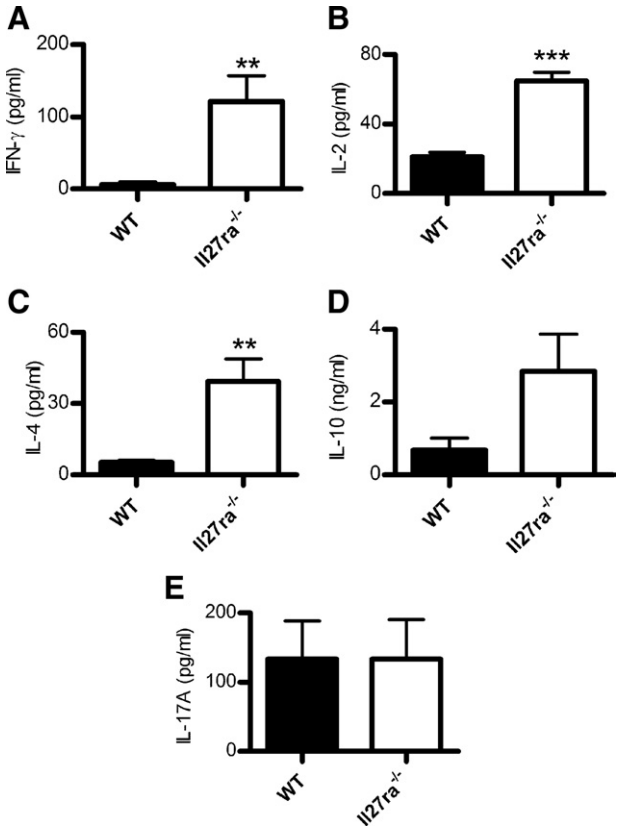

Figure 3. Systemic immune responses to the nephritogenic antigen in WT and *Il27ra*$^{-/-}$ mice on day 21. Antigen-stimulated IFN-γ (**A**) and IL-2 (**B**) production was increased in *Il27ra*$^{-/-}$ mice compared with WT mice. **C:** Antigen-stimulated IL-4 production was enhanced. **D:** A trend toward increased IL-10 production was seen in *Il27ra*$^{-/-}$ mice ($P = 0.08$). **E:** Production of IL-17A was similar in the presence and absence of the IL-27Rα chain. Error bars represent SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT mice with GN.

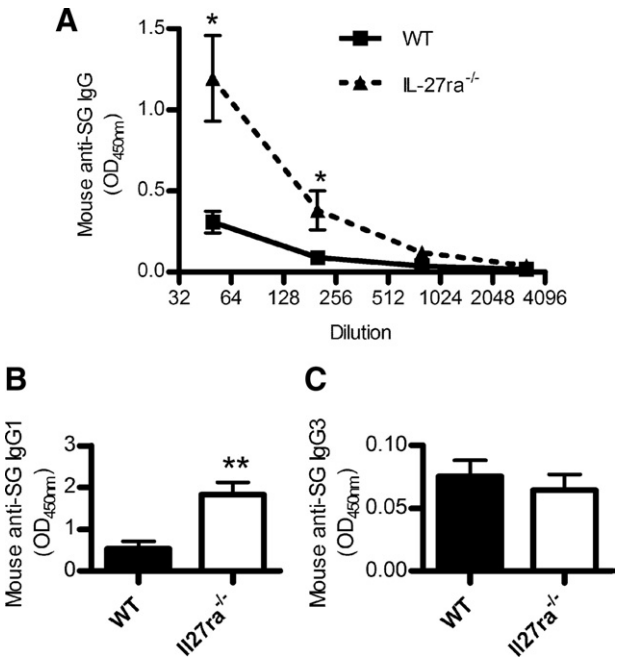

Figure 4. Antigen-specific serum antibody titers in WT and *Il27ra*$^{-/-}$ mice. Serum antigen-specific IgG titers (**A**) and IgG1 subclass titers (**B**) were increased in *Il27ra*$^{-/-}$ mice with GN. **C:** There was no difference in IgG3 production between the two groups. Error bars represent SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT mice with GN.

versus *Il27ra*$^{-/-}$ mice, 1.0 ± 0.2 versus 1.6 ± 0.1 fold increase; $P = 0.05$). To test the hypothesis that the increased disease in *Il27ra*$^{-/-}$ mice at day 28 was mediated by increased IFN-γ production, anti–IFN-γ monoclonal antibodies were administered to *Il27ra*$^{-/-}$ mice during days 14 to 28. Neutralizing IFN-γ reduced injury to that seen in WT mice (Figure 9), with similar glomerular crescent formation, interstitial injury, serum creatinine levels, and proteinuria.

Histologic and Functional Renal Injury Is Enhanced in Il27ra$^{-/-}$ *Mice on Day 35*

Renal disease in WT and *Il27ra*$^{-/-}$ mice was studied 35 days after administration of sheep anti-mouse GBM globulin. The increased disease found in the absence of the IL-27Rα at day 28 was confirmed by the findings at day 35. Compared with WT mice, *Il27ra*$^{-/-}$ mice showed increased histologic and functional renal injury (Figure 10, A–H). By this time point, glomerular and interstitial collagen deposition was increased in *Il27ra*$^{-/-}$ mice (Figure 10, I–N). Collectively, these results demonstrate that renal disease and chronic glomerular and interstitial injury increase in *Il27ra*$^{-/-}$ mice later in the course of experimental crescentic GN.

Expression of IL-27 and Its Receptor Chains in Kidneys across Time

Intrarenal expression of the two chains of IL-27 and its receptor (IL-27Rα and gp130) were assessed on days 0, 21, 28, and 35 in WT mice (Table 1). Expression of intrarenal IL-27p28 mRNA was increased at day 21 and had fallen by days 28 and 35. Epstein-Barr virus–induced protein 3 mRNA was not detected in kidneys ($<10^{-9}$ of 18S mRNA). Both IL-27Rα (unique to the IL-27 receptor) and gp130 (shared by the IL-6 cytokine family) were detected in kidneys of mice with GN. Expression of gp130 was increased at day 21 and by day 35 had fallen; similar trends were evident in IL-27Rα mRNA expression.

Discussion

In planted antigen models of crescentic GN, CD4^{+} T cells initiate the nephritogenic immune response[32] and

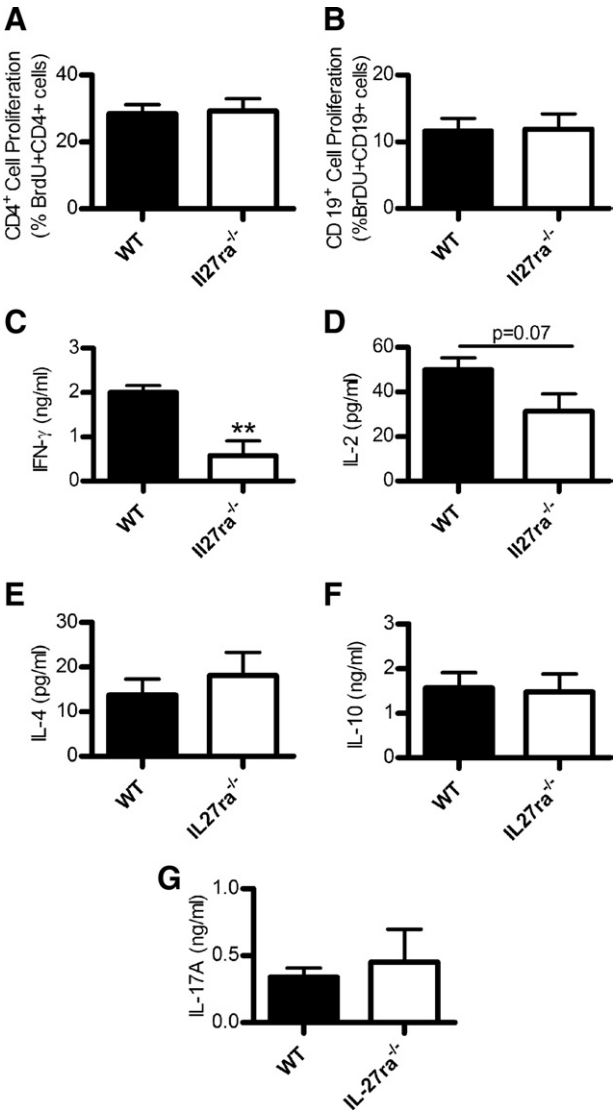

Figure 5. Systemic immune responses in WT and *Il27ra*$^{-/-}$ mice 3 days after i.v. sheep anti-mouse GBM globulin. Deficiency of the IL-27Rα chain did not affect CD4^{+} T-cell proliferation (**A**) or CD19^{+} B-cell proliferation (**B**) (flow cytometric analysis of *in vivo* BrdU incorporation). Antigen-stimulated splenocyte IFN-γ production was decreased in the absence of the IL-27Rα chain (**C**) and there was a trend toward reduced IL-2 production (**D**), but antigen-stimulated IL-4 (**E**), IL-10 (**F**), and IL-17A (**G**) levels were similar in WT and *Il27ra*$^{-/-}$ mice. Error bars represent SEM. $^{**}P < 0.01$ versus WT mice with GN.

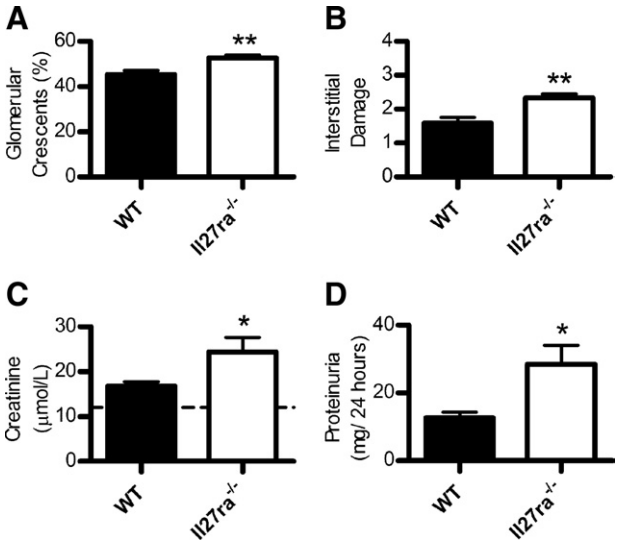

Figure 6. Histologic and functional renal injury in WT and *Il27ra*$^{-/-}$ mice with GN on day 28. **A:** Crescentic injury was enhanced in *Il27ra*$^{-/-}$ mice compared with WT controls. **B:** Similarly, tubulointerstitial disease was increased in *Il27ra*$^{-/-}$ mice. Functional injury was also enhanced in *Il27ra*$^{-/-}$ mice with increased serum creatinine levels (**C**) and urinary protein excretion (**D**). The dashed horizontal line in part C represents serum creatinine values in untreated WT mice. Error bars represent SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT mice with GN.

act as important effector cells in disease.[2,3] Previously, we demonstrated that a CD4^{+} Th1 phenotype drives crescentic injury and subsequent renal impairment[5,7–9] and that mice lacking T-bet, the key Th1 transcription factor, developed attenuated renal disease.[4] Therefore, the present studies set out to determine the role of the IL-27/IL-27R interactions in experimental crescentic GN. Decreased early nephritogenic systemic Th1 immune responses in *Il27ra*$^{-/-}$ mice were associated with reduced kidney injury after 21 days. However, by day 21, systemic immune responses, in particular Th1 responses, had increased in *Il27ra*$^{-/-}$ mice and were more intense than immune responses seen in WT mice. The enhanced systemic Th1 immune response was associated with enhanced kidney injury in *Il27ra*$^{-/-}$ mice at later time points, where histologic kidney injury is more severe and chronic.

Studies examining IL-27 in host immune responses established its role in the initial commitment of naive CD4^{+} T cells to Th1 differentiation *in vitro* and *in vivo*.[33] Consistent with these studies, we found that in *Il27ra*$^{-/-}$ mice, the injection of sheep anti-mouse GBM antibody resulted in less systemic IFN-γ, the signature Th1 cytokine, at an early time point. These results confirmed that IL-27Rα was required to initiate and maintain early nephritogenic systemic Th1 immune responses to sheep globulin. Histologic and functional renal injury was decreased in *Il27ra*$^{-/-}$ mice on day 21. In this autologous phase of injury, effector CD4^{+} cells in the kidney direct injury in a delayed-type hypersensitivity–like manner. Intrarenal mRNA expression of T-bet and the proinflammatory cytokines TNF and IL-1β was decreased in *Il27ra*$^{-/-}$ mice with GN. Whereas there was an increase in glomerular CD4^{+} cells in *Il27ra*$^{-/-}$ mice, there was no change in

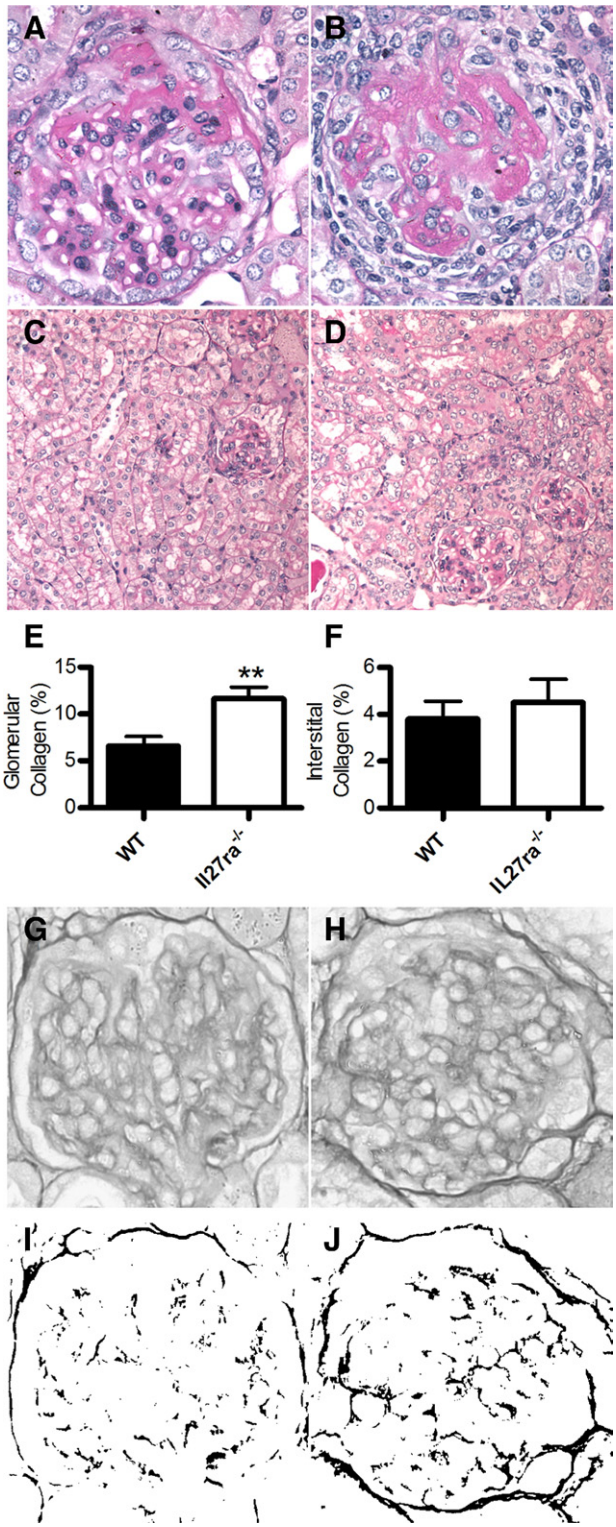

Figure 7. Representative sections demonstrating glomerular histologic injury in WT and *Il27ra*$^{-/-}$ mice 28 days after the administration of sheep anti-mouse GBM antibody. Glomerular renal injury is demonstrated at high power in WT (**A**) and *Il27ra*$^{-/-}$ (**B**) mice and interstitial injury in WT (**C**) and *Il27ra*$^{-/-}$ (**D**) mice. Original magnification: ×400 (**A** and **B**); ×200 (**C** and **D**). **E–J:** Glomerular collagen accumulation in mice with GN at day 28. **E:** Chronic glomerular changes were increased in *Il27ra*$^{-/-}$ mice compared with WT control. **F:** There was no significant increase in interstitial collagen accumulation. Representative high-power glomerular sections of WT (**G**) and *Il27ra*$^{-/-}$ (**H**) mice under standard conditions and after NIH Image analysis; WT (**I**) and *Il27ra*$^{-/-}$ (**J**) mice showing 6% and 12% collagen deposition, respectively. Original magnification, ×400 (**G-J**). Error bars represent SEM. $^{**}P < 0.01$ versus WT mice with GN.

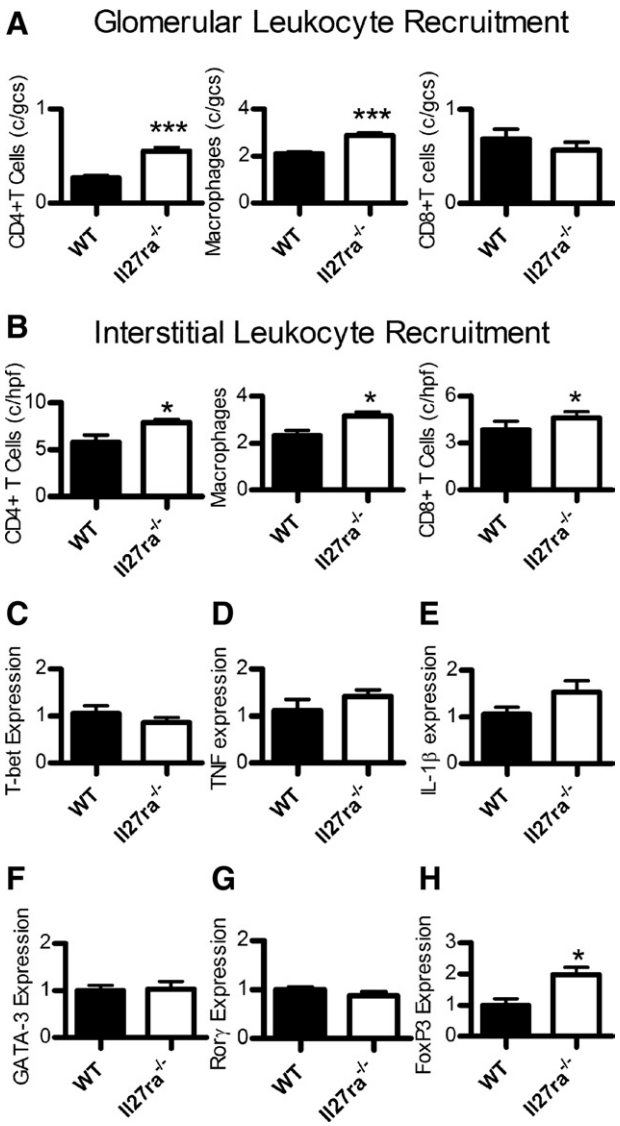

Figure 8. Effectors of cell-mediated renal injury in WT and *Il27ra*$^{-/-}$ mice with GN after 28 days. **A:** Compared with WT mice with GN, *Il27ra*$^{-/-}$ mice demonstrated more glomerular CD4$^+$ T cells and macrophages, but glomerular CD8$^+$ T cell numbers were similar. **B:** Increased numbers of CD4$^+$ T cells, macrophages, and CD8$^+$ T cells were seen in the interstitium of *Il27ra*$^{-/-}$ mice. Intrarenal mRNA expression of T-bet (**C**), TNF (**D**), and IL-1β (**E**) was no longer reduced in the absence of the IL-27Rα chain compared with WT mice, whereas no difference was seen in GATA-3 (**F**) or RORγ (**G**) mRNA expression. **H:** Intrarenal FoxP3 mRNA expression was increased in *Il27ra*$^{-/-}$ mice. Error bars represent SEM. $^*P < 0.05$, $^{***}P < 0.001$ versus WT mice with GN.

glomerular macrophage recruitment. It is not likely that these cells are regulatory T cells (Tregs) because there was no increase in intrarenal FoxP3 mRNA in *Il27ra*$^{-/-}$ mice. Whereas IL-27 can act on naive CD8$^+$ cells, augmenting the generation of effector CD8$^+$ cells,[34] severe renal injury in this model is CD8$^+$ independent,[35] and IL-27R deficiency did not affect CD8$^+$ cell infiltrates.

The decrease in renal injury on day 21 resulted from the early decreased nephritogenic systemic Th1 response seen in *Il27ra*$^{-/-}$ mice (day 3). Given the known role of IFN-γ in this model,[2,7,36] it is likely that reduced nephritogenic Th1 responses (splenocyte IFN-γ production) in the absence of IL-27Rα was responsible for attenuated proinflammatory cytokine production and diminished renal injury on day 21. In a less rapidly progressive model of renal injury, the MRL/*lpr* mouse, driven by systemic autoimmunity, the absence of IL-27R resulted in immune deviation with a change in the pattern of renal injury. WSX-1$^{-/-}$ MRL/*lpr* mice exhibited enhanced survival with less renal injury and developed membranous nephropathy, correlating with a skewed autoimmune re-

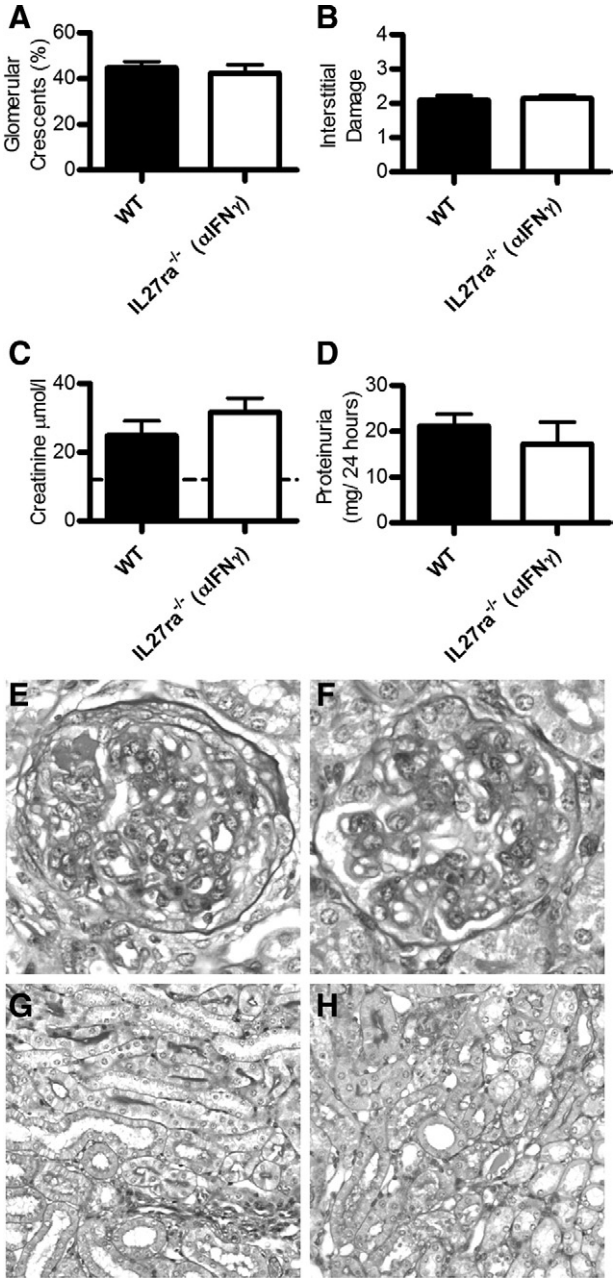

Figure 9. Renal injury in WT and *Il27ra*$^{-/-}$ mice after neutralizing IFN-γ, 28 days after the administration of sheep anti-mouse GBM globulin. Compared with WT mice treated with control, *Il27ra*$^{-/-}$ mice treated with anti–IFN-γ (from day 14) demonstrated no change in histologic (**A** and **B**) or functional (**C** and **D**) renal injury. Error bars represent SEM. **E-H:** Representative sections demonstrating similar histologic renal injury in WT mice and anti–IFN-γ antibody-treated *Il27ra*$^{-/-}$ mice. Glomerular renal injury is demonstrated at high power in WT (**E**) and anti–IFN-γ–treated *Il27ra*$^{-/-}$ (**F**) mice and interstitial injury in WT (**G**) and anti–IFN-γ–treated *Il27ra*$^{-/-}$ (**H**) mice. Original magnification: $\times$400 (**E** and **F**); $\times$200 (**G** and **H**).

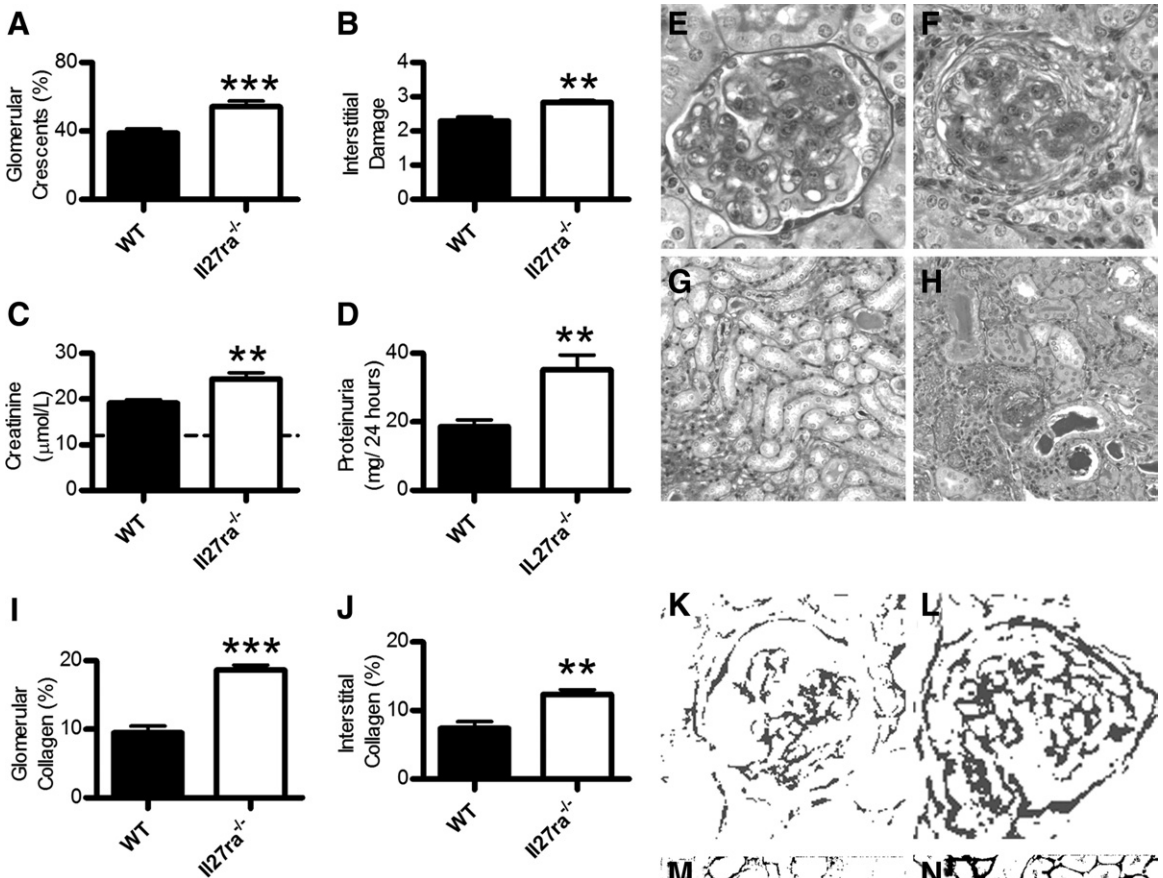

Figure 10. Renal injury in WT and *Il27ra*$^{-/-}$ mice 35 days after the administration of sheep anti-mouse globulin. Assessment of renal injury after 35 days demonstrated increased glomerular crescents (**A**) and interstitial injury (**B**). Serum creatinine levels (**C**) and proteinuria (**D**) were also increased in *Il27ra*$^{-/-}$ mice. **E–H:** Representative sections demonstrating increased injury at day 35 in the absence of IL-27Rα. Glomerular renal injury is demonstrated at high power in WT (**E**) and *Il27ra*$^{-/-}$ (**F**) mice and interstitial injury in WT (**G**) and *Il27ra*$^{-/-}$ (**H**) mice. Original magnification: ×400 (**E** and **F**); ×200 (**G** and **H**). Glomerular (**I**) and interstitial (**J**) matrix deposition was more intense in *Il27ra*$^{-/-}$ mice than in WT controls. Representative sections of WT (**K**) and *Il27ra*$^{-/-}$ (**L**) mice (glomerular) and WT (**M**) and *Il27ra*$^{-/-}$ (**N**) mice (tubulointerstitial) after NIH Image analysis. Original magnification: ×400 (**K** and **L**); ×200 (**M** and **N**). Error bars represent SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT mice with GN.

sponse, displaying a Th2 phenotype.[26] In the present studies, increased production of IL-4 and IL-10 on day 21 may have contributed to the decreased injury observed at this time point because endogenous and exogenous IL-4 and IL-10 limit injury in planted foreign antigen models of GN.[8,10,11]

Table. Intrarenal mRNA Expression of IL-27 and Its Receptor Chains in GN

	Day 0	Day 21	Day 28	Day 35
IL-27p28	2.9 ± 0.9	17.8 ± 2.8*	4.4 ± 0.6	1.9 ± 0.5
gp130	1504 ± 500	5761 ± 868*	3404 ± 300	261 ± 34†
IL-27Rα	80 ± 12	100 ± 8	44 ± 6	43 ± 6†

There was a rise in mRNA (significant for IL-27p28 and gp130) at day 21, followed by a fall to (or in the case of IL-27Rα below) baseline by day 35. Data are expressed as mean ± SEM fold change relative to the housekeeping gene 18S ($\times 10^{-7}$). The numbers of animals analyzed at day 0, 21, 28, and 35 are 4, 7, 6, and 13, respectively. Kidney mRNA Epstein-Barr virus–induced protein 3 expression was not detected ($<10^{-9}$ of 18S mRNA).

*$P < 0.001$ versus day 0.

†$P < 0.05$ versus day 0.

Whereas IL-27Rα is important in early Th1 responses, later IFN-γ production is not IL-27 dependent because the Th1 deficit in *Il27ra*$^{-/-}$ mice is transient in this and other experimental models.[15] The anti-inflammatory properties of IL-27 predominate in some experimental systems. In models of intracellular infection,[21,22,37] experimental autoimmune encephalomyelitis,[25,38] and experimental hepatitis,[24] the absence of IL-27R ultimately resulted in exaggerated systemic immune response and enhanced organ injury. In the present studies, after the early decrease in IFN-γ production, systemic nephritogenic Th1 immune responses were enhanced in *Il27ra*$^{-/-}$ mice on day 21. Production of IL-2 and IL-2–dependent IFN-γ was increased, leading to increased injury by day 28 and continuing at day 35. Mechanisms potentially explaining the enhanced immune responses, in particular the increased IFN-γ expression, in the absence of IL-27Rα include increased IL-2 production,[22] increased IL-17A production,[25] decreased IL-10 production,[39,40] and decreased Treg function.[41] In the present studies, most

increased injury seen at later time points in the absence of IL-27Rα can be explained by increases in IL-2 and IFN-γ expression. Neutralizing IL-2 in *Il27ra*$^{-/-}$ splenocyte cultures reduced IFN-γ production, and *in vivo* neutralization of IFN-γ in *Il27ra*$^{-/-}$ mice from days 14 to 28 reduced injury to levels seen in WT mice. These findings are consistent with published data showing that in the absence of IL-27–mediated events, an initial impairment in Th1 responses is followed by intact or even enhanced IL-2 and IFN-γ production and increased inflammatory abnormalities.[15] Interleukin-27 may suppress the production of IL-17A by Th17 cells.[38] Both Th1 and Th17 responses can induce glomerular injury,[42] and IL-17A is pathogenic in a model similar to that used in our studies.[43] However, in the present studies, IL-17A production was unaltered in *Il27ra*$^{-/-}$ mice on days 3 and 21. Although intrarenal RORγ mRNA expression was increased on day 21 in *Il27ra*$^{-/-}$ mice, glomerular disease was less in *Il27ra*$^{-/-}$ mice at that time point.

Other possible mechanisms by which IL-27 could have affected crescentic GN include alterations in anti-inflammatory cytokines and pathways. Although IL-27 promotes IL-10 production in other systems[39,40] and endogenous IL-10 protects mice from anti-GBM GN,[9] the enhanced renal injury in the present studies cannot be attributed to decreased IL-10 production. In *Il27ra*$^{-/-}$ mice at day 21, splenocyte IL-10 expression was increased, CD4^{+} cell IL-10 production was unchanged, and intrarenal IL-10 mRNA expression was unchanged on day 21 and was not decreased on day 28. Previously, we demonstrated a protective role for IL-4,[10,11] and whereas systemic IL-4 production was increased in *Il27ra*$^{-/-}$ mice, intrarenal GATA-3 expression was not increased. Interleukin-27 may affect regulatory cells, and whereas *Il27ra*$^{-/-}$ mice have normal numbers of CD4^{+}CD25^{+} Tregs,[44] some populations of Tregs express high levels of IL-27Rα, and IL-27 can inhibit the induction of selected subgroups of Tregs.[41] However, our findings suggest that changes in FoxP3 Tregs are not responsible for altered renal injury in *Il27ra*$^{-/-}$ mice. Intrarenal FoxP3 expression was not increased in WT mice after 21 days but had increased in *Il27ra*$^{-/-}$ mice by 28 days, consistent with previous findings of increased FoxP3 expression in inflamed organs in other experimental models[45] and in human kidney transplant biopsy samples.[46]

We hypothesized that enhanced systemic Th1 immune responses by day 21 would ultimately result in more severe renal disease, so renal disease was studied at two later time points: days 28 and 35. In WT mice, histologic disease and proteinuria, but not serum creatinine level, tended to increase across time, similar to previous findings in a similar model.[7,47] Consistent with enhanced systemic immune responses on day 21, renal injury was enhanced in *Il27ra*$^{-/-}$ mice by day 28, confirmed by studies at day 35. In addition to enhanced histologic and functional injury in *Il27ra*$^{-/-}$ mice on days 28 and 35, effector cells including CD4^{+} T cells and macrophages in glomeruli were increased on day 28. These results confirm an initial immunostimulatory role followed by an immunoregulatory role for IL-27Rα in this Th1-dependent model of crescentic GN. Last, given the propensity for ongoing inflammation to result in fibrosis, in the absence of IL-27R, glomerular and interstitial (day 35) collagen deposition was increased, implying a poorer ultimate outcome in the absence of IL-27R.

In conclusion, the present studies in a Th1-dependent model of severe crescentic GN show that endogenous IL-27 promotes early Th1 responses but later regulates Th1 systemic nephritogenic immunity. In *Il27ra*$^{-/-}$ mice, the initial decreased systemic Th1 response leads to attenuated kidney injury with decreased intrarenal proinflammatory cytokine production. The later increases in systemic nephritogenic immune responses in *Il27ra*$^{-/-}$ mice resulted in enhanced renal injury and fibrosis later in the disease process.

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