IL-12p40 and IL-18 in Crescentic Glomerulonephritis: IL-12p40 is the Key Th1-Defining Cytokine Chain, Whereas IL-18 Promotes Local Inflammation and Leukocyte Recruitment

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Experimental crescentic glomerulonephritis (GN) is characterized by T helper 1 (Th1) directed nephritogenic immune responses and cell-mediated glomerular injury. IL-12p40, the common cytokine chain for both IL-12 and IL-23, is important in the generation and potentially the maintenance of Th1 responses, whereas IL-18 is a co-factor for Th1 responses that may have systemic and local proinflammatory effects. For testing the hypothesis that both endogenous IL-12p40 and endogenous IL-18 play pathogenetic roles in crescentic GN, accelerated anti–glomerular basement membrane GN was induced in mice genetically deficient in IL-12p40 (IL-12p40$^{-/-}$), IL-18 (IL-18$^{-/-}$), or both IL-12p40 and IL-18 (IL-12p40$^{-/-}$IL-18$^{-/-}$). Compared with wild-type C57BL/6 mice, IL-12p40$^{-/-}$ mice failed to make a nephritogenic Th1 response and developed markedly reduced crescent formation and renal leukocytic infiltration, despite renal production of chemoattractants and adhesion molecules. IL-18$^{-/-}$ mice developed an intact antigen-specific systemic Th1 response, a similar degree of crescent formation, but fewer glomeruli affected by other severe histologic changes and fewer leukocytes in glomeruli and interstitium. IL-18 was expressed within diseased kidneys. Local production of TNF, IL-1β, IFN-γ, CCL3 (MIP-1α), and CCL4 (MIP-1β) was reduced in IL-18$^{-/-}$ mice, demonstrating a local proinflammatory role for IL-18. Combined deletion of IL-12p40 and IL-18 did not result in synergistic effects. Consistent with the hypothesis that inflammation leads to fibrosis, all three groups of deficient mice expressed lower levels of intrarenal TGF-β1 and/or α1(I) procollagen mRNA. These studies demonstrate that in severe experimental crescentic GN, IL-12p40 is the key Th1-defining cytokine chain, whereas IL-18 has local proinflammatory roles.

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Crescentic glomerulonephritis (GN) is the most severe and rapidly progressive subset of GN. Kidneys from patients with human crescentic GN demonstrate the presence of effectors of delayed type hypersensitivity (DTH), particularly T cells and macrophages (1–3). These data, with other findings regarding the nature of the immune response in these patients (4), suggest a role for T helper cell 1 (Th1)-directed nephritogenic immune responses in the pathogenesis of crescentic GN. The relevance of these observations has been confirmed in experimental murine crescentic GN (5,6). Severe crescentic glomerular injury is Th1 directed, effector CD4+ cell mediated (6,7), and regulated by endogenous and exogenous Th2 cytokines IL-4 and IL-10 (8–10). Although it is clear that humoral immune mediators are capable of inducing injury (11,12), crescent formation can occur in the absence of autologous antibody (13–15).

Previous work, using anti–IL-12p40 antibodies and IL-12p40$^{-/-}$ mice, has demonstrated that in experimental murine models, glomerular crescent formation is directed by IL-12p40 (7,16–18). IL-12 is a heterodimeric cytokine, consisting of a p40 subunit that binds to the IL-12Rβ1 and a p35 subunit that binds to the IL-12Rβ2. Recently, a related cytokine was cloned, IL-23 (19), also a heterodimer, that shares the IL-12p40 subunit and its binding to IL-12Rβ1 but has a different subunit, the IL-23p19 subunit, that binds to the IL-23R. Although there is some overlap in their biologic effects, studies in experimental autoimmune encephalomyelitis (EAE) have shown that whereas IL-12 primes cells for IFN-γ production, IL-23 has significant proinflammatory effects on both memory T cells and macrophages (20).

IL-18, originally described as IFN-γ–inducing factor (21), both is a co-factor for IFN-γ and other Th1 cytokine production and induces expression of leukocyte chemoattractants and adhesion molecules (16,22). Exogenous IL-18 can enhance immune responses and increase the severity of experimental crescentic GN (16), independent of IL-12p40. Intrarenal IL-18 expression has been documented in murine lupus nephritis

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(23), rat experimental crescentic GN (24), and experimental ischemia reperfusion injury (25), playing a functional role in the last disease (26). However, the role of endogenous IL-18 in experimental crescentic GN, as well as any synergism or differential effects with IL-12p40, is unknown. Using mice that are genetically deficient in IL-12p40, IL-18, or both IL-12p40 and IL-18, we sought to define the relative roles and nature of the contributions of endogenous IL-12p40 and IL-18 in experimental crescentic GN.

Materials and Methods

Experimental Design

IL-12p40$^{-/-}$ mice (27) (C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, MA). IL-18$^{-/-}$ mice (C57BL/6 background) were created as described previously (28). Mice were bred at Monash University (Clayton, Victoria, Australia). Combined IL-12p40– and IL-18–deficient (IL-12p40$^{-/-}$IL-18$^{-/-}$) mice were created by crossing IL-12p40$^{-/-}$ mice with IL-18$^{-/-}$ mice, then interbreeding IL-12p40$^{+/-}$ and IL-18$^{+/-}$ offspring. Progeny were selected as founders for IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. Colony founders, breeders, and experimental animals in the colonies were genotyped by a PCR-based protocol. Figure 1 shows a representative experiment confirming the disruption of the IL-18 gene and/or the absence of the wild-type (WT) IL-12p40 gene in appropriate mice. Anti-mouse glomerular basement membrane (GBM) globulin was prepared as described previously (29).

For inducing crescentic GN, 8- to 10-wk-old male WT C57BL/6 (n = 20), IL-12p40$^{-/-}$ (n = 5), IL-18$^{-/-}$ (n = 16), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 13) mice were sensitized by subcutaneous injection of 500 μg of sheep globulin (SG) in 100 μl of Freund's complete adjuvant (FCA). After 10 d, GN was initiated by intravenous injection of 12 mg of sheep anti-mouse GBM globulin. Renal injury was studied after an additional 10 d. For assessing intrarenal leukocytes, cytokines, and chemokine production, another experiment was performed in WT C57BL/6 (n = 7), IL-12p40$^{-/-}$ (n = 6), IL-18$^{-/-}$ (n = 5), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 7) mice using a similar experimental protocol but antisera from a different sheep. Studies adhered to the National Health and Medical Research Council of Australia guidelines for animal experimentation. Histologic examinations were performed on coded slides. Results are expressed as the mean ± SEM. The significance of differences between groups was determined by ANOVA.

Assessment of Systemic Immune Responses

For experiments in dermal DTH, WT C57BL/6 (n = 4), IL-12p40$^{-/-}$ (n = 5), IL-18$^{-/-}$ (n = 4), and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice (n = 7) and were sensitized with 2 mg of SG in 100 μl of FCA, boosted with 1 mg of SG in 100 μl of FCA after 7 d and after an additional 7 d challenged by intradermal injection of 300 μg of SG in 30 μl of PBS into the ear (30). Horse globulin was injected in the opposite ear as a control. DTH was assessed after 24 h by measuring the difference between the SG and horse globulin injected ear thicknesses using a micrometer. For cytokine production, splenocytes from mice with GN were cultured for 72 h with 10 μg/ml normal sheep IgG. IFN-γ, IL-4, IL-1β, TNF, and GM-CSF in supernatants were measured by ELISA as described previously (16). IL-5 was measured by ELISA using the rat anti-mouse IL-5 mAb TRFK5 (0.5 μg/ml; R&D Systems, Minneapolis, MN) for capture and biotinylated TRFK4 (100 ng/ml; R&D Systems) for detection, with rmIL-5 as a standard (R&D Systems) and streptavidin–horseradish peroxidase (HRP; 1:1000; Chemicon, Boronia, Victoria, Australia). Circulating levels of mouse anti-SG immunoglobulins were measured by ELISA as described previously (31) on serum collected at the end of experiments. For IgG1 assessment (serum dilution 1:100), HRP-conjugated goat anti-mouse IgG1 antibodies (Southern Biotechnology Assoc., Birmingham, AL; 1:4000) were used. For IgG3 (serum dilution 1:50), biotinylated rat anti-mouse IgG3 (R40–82; Pharmingen, San Diego, CA; 2 μg/ml) then streptavidin-HRP complex (Silenus, Victoria, Australia) were the detecting antibodies.

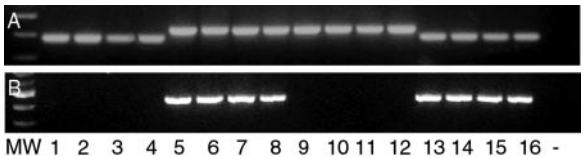

Figure 1. Confirmation of IL-12p40 and IL-18 gene disruption. Lanes 1 to 4, IL-12p40$^{-/-}$ mice; lanes 5 to 8, IL-18$^{-/-}$ mice; lanes 9 to 12, combined IL-12p40$^{-/-}$IL-18$^{-/-}$ mice; and lanes 13 to 16, wild-type (WT) C57BL/6 mice. (A) Presence of the WT IL-18 gene product (291 bp) in IL-12p40$^{-/-}$ and WT mice and the disrupted 320-bp allele in IL-18$^{-/-}$ and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. (B) Absence of an intact IL-12p40 gene (465 bp) is demonstrated in IL-12p40$^{-/-}$ and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice.

Assessment of Glomerular Injury, Leukocyte Accumulation, and Adhesion Molecules

Glomerular crescent formation (two or more layers of cells observed in Bowman's space) was assessed on periodic acid-Schiff–stained paraffin sections. The proportion of glomeruli that were severely affected (crescent formation, evidence of accumulation of cells in Bowman's space that did not satisfy the criteria for crescent formation, >50% of the glomerular tuft affected by necrosis, or severe proliferative changes) was assessed, according to a modification of a previously published method (32). A minimum of 50 glomeruli were assessed in each animal. Tissue sections of periodate lysine paraformaldehyde–fixed kidneys were stained to demonstrate CD4$^+$ cells, CD8$^+$ cells, and Mac-1(CD11b)$^+$ macrophages/neutrophils using a three-layer immunoperoxidase technique. The primary antibodies were GK1.5 (anti-mouse CD4; American Type Culture Collection [ATCC], Manassas, VA), 53-6·7 (anti-mouse CD8; ATCC), and M1/70 (anti-mouse CD11b; ATCC). A minimum of 20 consecutively viewed glomeruli and a minimum of 10 high-power cortical interstitial fields (excluding perivascular regions) were assessed per mouse, and results were expressed as cells per glomerular cross-section (c/gcs) or cells per high-power field (c/hpf). Renal deposition of P-selectin and intercellular adhesion molecule-1 (ICAM-1) was assessed as described previously (33) and scored semiquantitatively—0, background staining; 1, lowest clearly positive staining; 2, moderate staining; and 3, intense deposition—in 20 randomly selected glomeruli and 20 randomly selected tubulointerstitial areas at medium power.

Assessment of IL-18 in Nephritic Kidneys

Tissue sections of periodate lysine paraformaldehyde–fixed kidneys were stained to demonstrate the presence of IL-18, using a polyclonal rabbit anti-mouse IL-18 antibody (a gift of Dr. M. Kurimoto, Fujisaki Institute, Okayama, Japan) at a concentration of 11 μg/ml, followed by HRP-conjugated swine anti-rabbit antibodies (Dako, Glostrup, Denmark; 1:100), then HRP-conjugated rabbit anti-peroxidase globulin (Dako; 1:100), then diaminobenzidine. Sections of spleen were positive controls. Negative controls included substituting normal rabbit immunoglobulins for the primary antibody, preincubating the primary antibody with excess rmIL-18 (16) (final concentration 2 μg/ml; Dr. M. Kurimoto), and immunostaining sections of an IL-18$^{-/-}$ mouse with GN.

Assessment of Intrarenal Chemokine, Cytokine, and Collagen mRNA Expression

Total kidney RNA was extracted with TRIzol reagent (Invitrogen, San Diego, CA) according to the manufacturer's protocol from randomly selected mice from a single experiment ($n = 4$ to 5 for each group with GN). Multiprobes incorporating [α-^{32}P]UTP were transcribed from the template set mCK-5c (RiboQuant System, Pharmingen), a modification of mCK-2 (IL-12p40, IL-4, IL-13, IL-18, IL-1β, IL-10, IFN-γ, and MIF probes; Pharmingen), and a custom template (lymphotoxin-α [LT-α], lymphotoxin-β, TNF, IL-13, IFN-γ, α1(I) procollagen, TGF-β1, and TGF-β3 probes; Pharmingen) using T7 RNA polymerase *in vitro* transcription. After DNase I treatment, riboprobes were isolated by phenol/chloroform extraction and precipitation with 4 M ammonium acetate and ethanol. Incorporation of [α-^{32}P]UTP was determined by Cherenkov activity, probes were diluted to 3.5×10^5 cp/mμl, then added to 20 μg of kidney RNA. Hybridization and isolation were conducted according to the RiboQuant manual. RNA hybrids were separated by electrophoresis (5% polyacrylamide/8 M urea). The gel was dried, then exposed to the phosphoimager imaging plate (FLA-2000; Fuji Photo Film Co., Tokyo, Japan). Image Gauge software (version 3.46; Fuji Photo Film Co.) was used to evaluate the gel image. Gene expression was measured and normalized to the housekeeping gene L32.

For measurement of IFN-γ mRNA by real-time PCR, 1 μg of RNA ($n = 6$ to 7 for each group with GN) was treated with 1 unit of amplification-grade DNase I (Invitrogen) then primed with 500 ng of Oligo(dT)$_{12\text{-}18}$ (Roche, Mannheim, Germany) and reverse transcribed (Super Script II; Invitrogen). Gene-specific primers for murine IFN-γ and murine β-actin were designed (Vector NTI software; Invitrogen). IFN-γ cDNA was amplified using the PCR primers (F 5-GAAAGACAATCAGGCCATCA-3′ and R 5′-TTGCTGTTGCTGAAGAAGGT-3′) to produce a product of 78 bp, whereas the β-actin cDNA was amplified with primers (F 5′-AGGCTGTGCTGTCCCTGTAT-3′ and R 5′-AAGGAAGGCTGGAAAAGAGC-3′) to produce a 388-bp product. Real-time PCR was performed on a Rotor Gene RG-3000 (Corbett Research, Mortlake, New South Wales, Australia) using Faststart DNA master, Sybr Green I (Roche). IFN-γ and β-actin mRNA expression was quantified using serial dilutions of an exogenous standard. IFN-γ levels were normalized to β-actin and expressed as fg IFN-γ/pg β-actin mRNA. PCR products were confirmed by melt-curve analysis.

Assessment of Humoral Immune Reactants in Glomeruli

Immunofluorescence was performed on 4-μm cryostat-cut tissue. Glomerular mouse Ig was evaluated using FITC-conjugated sheep anti-mouse Ig (Silenus) and C3 using FITC-conjugated goat anti-mouse C3 (Cappel, Durham, NC). For both mouse Ig and C3, a single dilution of 1:100 was scored (0 to 3+, based on fluorescence intensity). Sections in which only some glomeruli were positive were graded as 0.5. In addition, quantitative evaluation of the extent of glomerular antibody deposition was made by capturing images of at least 10 randomly selected glomeruli (high power) from each mouse and analyzing mean fluorescence intensity in each glomerular tuft by tracing each tuft, after removing background values (*e.g.*, light emanating from stained section without tissue) for each slide (NIH Image) as previously published (12,30). Values are expressed as arbitrary units of fluorescence per pixel.

Renal Function and Urinary Protein Excretion

Urinary creatinine concentrations were measured by the alkaline picric acid. Serum creatinine concentrations were measured by an enzymatic creatininase assay. Urinary protein excretions were determined by the Bradford method on 24-h urine collections from mice before disease and from each mouse over the final 24 h of the experiment. Urinary protein excretion was expressed by a 24-h value and as a urinary protein to urinary creatinine ratio.

Results

IL-12p40 Is the Key Th1-Defining Cytokine Chain in Antigen-Specific Cell-Mediated Immune Responses

Systemic immune responses to the nephritogenic antigen (SG) were assessed in the presence and the absence of endogenous IL-12p40, IL-18, or both IL-12p40 and IL-18 (Figure 2). IL-12p40$^{-/-}$ mice developed significantly diminished dermal DTH, and a similar trend was evident in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. However, deficiency of IL-18 alone did not result in diminished dermal DTH. Total serum antigen-specific antibody titers were marginally and nonsignificantly decreased in both groups that lacked IL-18 (Figure 2B). The Th2-associated IgG1 subclass was unchanged (Figure 2C). There was a trend to decreased IgG3 in both IL-18–deficient groups of mice (Figure 2D). Antigen-stimulated splenocytes from IL-12p40$^{-/-}$ mice did not produce detectable amounts of IFN-γ (Figure 3A), TNF (Figure 3C), or GM-CSF (Figure 3B), confirming the key role for IL-12p40 in the development of Th1 responses. However, there was no effect on any of these three key proinflammatory cytokines in the absence of IL-18, showing that IL-18 is not required for Th1 cytokine production by immune cells. In IL-12p40$^{-/-}$IL-18$^{-/-}$ mice, cytokine production was low, except in the case of TNF, in which levels seemed paradoxically elevated in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. Deletion of IL-12p40 suppressed IL-4 and IL-5 production (Figure 3, D and E; as previously observed [16,34]). Levels of IL-4 and IL-5 were increased in the absence of IL-18. IL-1β was measured by ELISA, but only one mouse produced measurable levels of IL-1β (sensitivity 3.9 pg/ml).

IL-12p40 Is the Key Cytokine Chain in Glomerular Crescent Formation

Ten days after challenge with sheep anti-mouse GBM globulin, sensitized WT C57BL/6 mice developed severe proliferative and crescentic GN, with significant tubulointerstitial injury (Figures 4, A and E, and 5). Glomerular and tubulointerstitial disease was markedly attenuated in the absence of IL-12p40 (Figure 4, B and F). In the absence of IL-18, histologic renal injury was still severe but qualitatively marginally less so than that seen in WT mice (Figure 4, C and G). IL-12p40$^{-/-}$IL-18$^{-/-}$ mice developed disease that was less severe than in WT mice but more severe than in IL-12p40$^{-/-}$ mice (Figure 4, D and H). Analysis of glomerular crescent formation in two separate experiments (Figure 5, A and B) showed decreased crescent formation in IL-12p40$^{-/-}$ mice but no significant decrease in either IL-18$^{-/-}$ or combined IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. Significant reductions in the proportions of glomeruli severely affected by crescent formation, proliferative changes, or necrosis were evident in all three groups of genetically deficient mice (Figure 5, C and D).

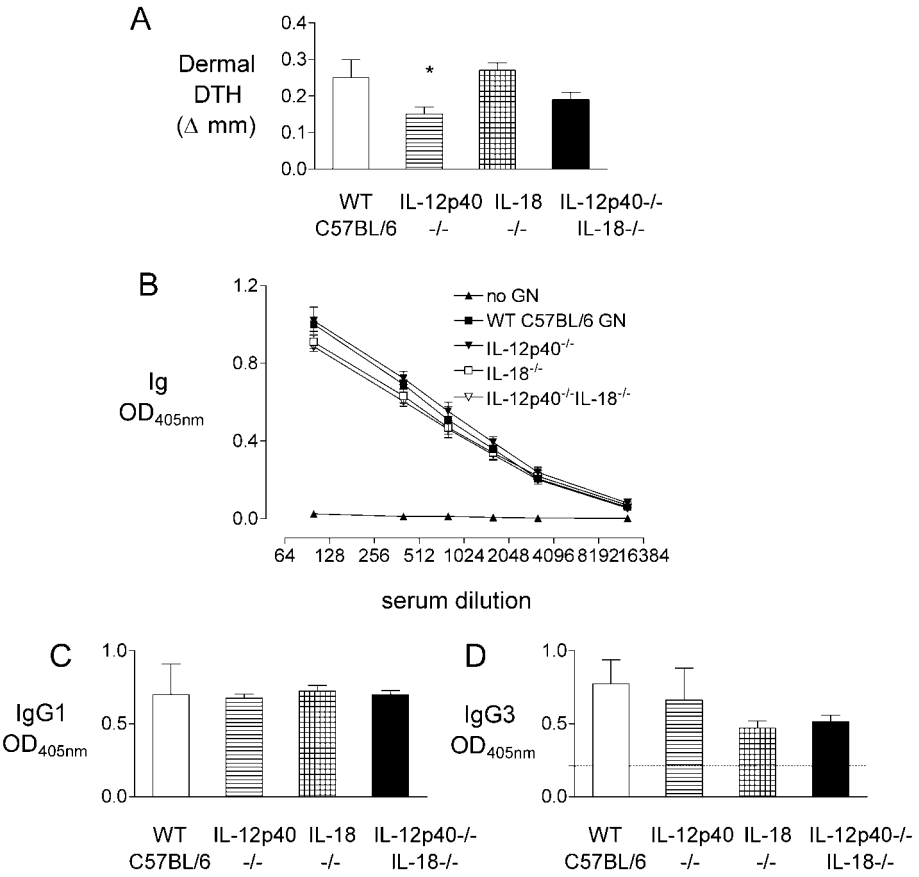

Figure 2. Dermal delayed type hypersensitivity (DTH; A), serum antigen (sheep globulin)-specific antibody responses (B), with the antigen-specific IgG subclasses IgG1 (C) and IgG3 (D) in mice with anti–glomerular basement membrane (GBM) glomerulonephritis (GN), showing reduced dermal DTH particularly in IL-12p40$^{-/-}$ mice but not in IL-18$^{-/-}$ mice, no statistically significant alterations in total antigen-specific Ig, IgG1, or IgG3, although IL-18$^{-/-}$ and IL-12p40$^{-/-}$IL-18$^{-/-}$ groups had a trend to reduced IgG3. *$P < 0.05$ *versus* WT C57BL/6 mice (ANOVA). Results are expressed as the mean ± SEM. Numbers of mice studied are WT C57BL/6 ($n = 4$), IL-12p40$^{-/-}$ ($n = 5$), IL-18$^{-/-}$ ($n = 4$), and IL-12p40$^{-/-}$IL-18$^{-/-}$ ($n = 7$) in A and WT C57BL/6 ($n = 11$ to 20), IL-12p40$^{-/-}$ ($n = 5$), IL-18$^{-/-}$ ($n = 10$ to 16), and IL-12p40$^{-/-}$IL-18$^{-/-}$ ($n = 12$ to 13) in B through D.

Both IL-12p40 and IL-18 Contribute to Leukocyte Accumulation in Inflammatory Renal Disease

In glomeruli of mice with GN, CD4+ cell (Figure 6A) and CD11b(Mac-1)$^+$ cell (Figure 6C) accumulation was reduced in all groups of genetically deficient mice, with the reduction in infiltrate greatest in mice deficient in IL-12p40 (including IL-12p40$^{-/-}$IL-18$^{-/-}$–deficient mice). Changes in CD8$^+$ cell infiltration (Figure 6B) were significant in IL-12p40$^{-/-}$ mice but not in IL-18$^{-/-}$ or IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. Similar findings were present in the interstitium (Figure 6, D through F), although the reduction in CD8$^+$ cells in combined IL-12p40$^{-/-}$ IL-18$^{-/-}$ mice was significant, and reductions in Mac-1$^+$ cells seemed more prominent in IL-18$^{-/-}$ mice.

IL-18 Expression in Kidneys of Mice with Anti-GBM GN

Given the reduced leukocytic infiltrates in IL-18$^{-/-}$ mice despite the lack of a key role for systemic/lymphoid IL-18 to influence Th1 responses, local IL-18 production was examined. IL-18 mRNA was detected in renal tissue from WT C57BL/6 mice with GN (4.1 ± 1.2 AU) by RNAse protection assay. IL-18 mRNA was present to a similar level in IL-12p40$^{-/-}$ mice with GN (4.2 ± 0.3 AU) but was not detected in IL-18 and combined IL-12p40$^{-/-}$IL-18$^{-/-}$–deficient mice. Immunohistochemistry, using a rabbit anti-mouse IL-18 antibody, revealed local accumulation of IL-18 (Figure 7). Staining was abrogated by substituting normal rabbit Ig for the primary antibody (Figure 7B), by preabsorbing the primary antibody with excess rmIL-18 (Figure 7C), or by staining tissue from an IL-18$^{-/-}$ mouse with GN (data not shown).

Intrarenal Chemokine Production and Adhesion Molecule Expression in Mice with GN

To determine whether endogenous IL-18 alters renal chemokine and adhesion molecule expression, we measured intrarenal chemokine mRNA expression by RNAse protection assay (Table 1). There were no significant effects on mRNA expression of the T cell–related chemokines CXCL10 (IP-10), XCL1 (lymphotactin), CCL5 (RANTES), or CCL1 (TCA-3). However, endogenous IL-18 does regulate macrophage and neutrophil chemoattractants. Compared with WT C57BL/6 mice with GN, IL-18$^{-/-}$ mice had significantly reduced CCL3 (MIP-1α) and

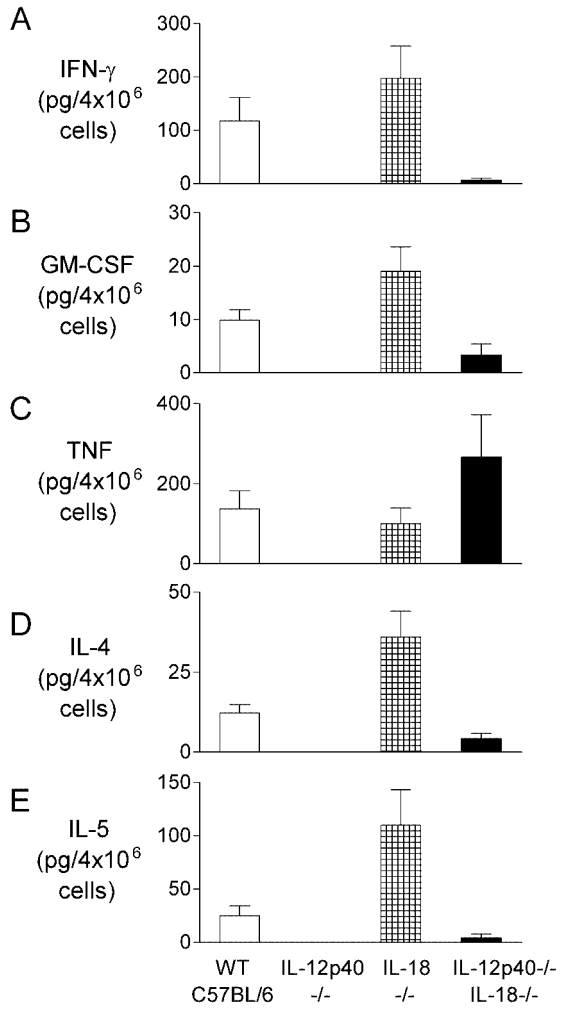

Figure 3. Cytokine production by antigen-stimulated splenocytes from mice developing GN. Cytokine production was measured in culture supernatants (4×10^6 cells/ml) at 72 h by ELISA. IFN-γ (A), GM-CSF (B), TNF (C), IL-4 (D), and IL-5 (E) were undetectable in splenocyte cultures from IL-12p40$^{-/-}$ mice with GN, but in IL-18$^{-/-}$ mice, cytokine production was either similar (IFN-γ, GM-CSF, and TNF) or increased (IL-4 and IL-5). Results are expressed as the mean ± SEM. Numbers of mice studied for are WT C57BL/6 (n = 11 to 14), IL-12p40$^{-/-}$ (n = 5), IL-18$^{-/-}$ (n = 8 to 16), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 8 to 13).

CCL4 (MIP-1β). In IL-12p40$^{-/-}$IL-18$^{-/-}$ mice, reductions in both CCL3 and CCL4 were seen as for IL-18$^{-/-}$ mice, and the reduction in CXCL1 (MIP-2) was significant. In both IL-18$^{-/-}$ and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice, expression of CCL2 (MCP-1) mRNA fell to approximately two thirds of that in WT mice with GN, but this reduction did not reach statistical significance. Semiquantitative assessment of immunofluorescent staining for the adhesion molecules P-selectin and ICAM-1 revealed no significant differences in the expression of either molecule (Table 2), although as previously found (16), a trend to decreased ICAM-1 in IL-12p40–deficient mice (in either IL-12p40$^{-/-}$ or IL-12p40$^{-/-}$IL-18$^{-/-}$ mice) was noted. The minimal interstitial expression of P-selectin was not formally assessed.

Intrarenal Cytokine Production in Mice with GN

Kidneys of WT C57BL/6 mice with GN expressed mRNA for IL-1β, TNF, MIF, IFN-γ, and LT-α (Figure 8). Genetically deficient mice expressed less intrarenal IL-1β and TNF mRNA (not reaching significance in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice for IL-1β mRNA and IL-12p40$^{-/-}$ mice for TNF mRNA). Upregulation of TNF in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice was not seen in the kidney. In contrast to the findings in antigen-stimulated splenocytes (Figure 3A), renal IFN-γ mRNA, measured by real-time PCR, was reduced in IL-18$^{-/-}$ mice but not in IL-12p40$^{-/-}$ mice. LT-α mRNA levels were similar in all groups of mice. Detectable levels of mRNA for the anti-inflammatory cytokine IL-10 were found only in mice that lacked IL-12p40 (IL-12p40$^{-/-}$ and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice), suggesting that IL-12p40 but not IL-18 suppresses this protective cytokine in inflammatory renal disease. Intrarenal MIF mRNA expression was unchanged. IL-4, IL-12p40, IL-13, and lymphotoxin-β mRNA were not detected by RNAse protection assay in any group of mice with GN.

Effects of IL-12p40 and IL-18 on Humoral Mediators of Renal Injury

Autologous antibody and C3 were detected in glomeruli by immunofluorescence (Table 3) and assessed by both semiquantitative scoring of fluorescence intensity (0 to 3+; WT C57BL/6 n = 18, IL-12p40$^{-/-}$ n = 5, IL-18$^{-/-}$ n = 16, and IL-12p40$^{-/-}$ IL-18$^{-/-}$ n = 13). There was little difference in the deposition of Ig in glomeruli by this method (WT C57BL/6 1.5 ± 0.2, IL-12p40$^{-/-}$ 1.6 ± 0.4, IL-18$^{-/-}$ 1.2 ± 0.1, IL-12p40$^{-/-}$IL-18$^{-/-}$ 1.2 ± 0.1), although C3 deposition seemed diminished in the IL-12p40$^{-/-}$IL-18$^{-/-}$ mice (WT C57BL/6 2.1 ± 0.2, IL-12p40$^{-/-}$ 1.6 ± 0.3, IL-18$^{-/-}$ 1.6 ± 0.2, IL-12p40$^{-/-}$IL-18$^{-/-}$ 1.2 ± 0.1; $P < 0.01$ *versus* WT C57BL/6 mice). Quantitative analyses of fluorescence intensity performed from tissues in an additional experiment (Table 3) demonstrated increased mouse Ig in glomeruli in both IL-18$^{-/-}$ and combined IL-12p40$^{-/-}$IL-18$^{-/-}$ mice and no alterations in C3 deposition.

Markers of Progressive Renal Disease: Intrarenal TGF-β and α1(I) Procollagen mRNA

As markers of renal fibrotic potential in mice with GN, renal mRNA expression of the anti-inflammatory but profibrotic cytokine TGF-β1 and mRNA expression for α1(I) procollagen were measured (Figure 9). Intrarenal TGF-β1 mRNA expression was reduced in IL-12p40$^{-/-}$ and IL-18$^{-/-}$ mice (trend in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice). All groups of genetically deficient mice with GN had reduced expression of intrarenal α1(I) procollagen mRNA expression compared with WT C57BL/6 mice with GN. TGF-β3 mRNA was unchanged in all groups.

Functional Indices of Injury in Mice with GN

WT C57BL/6 mice with crescentic GN developed significant proteinuria and moderate renal impairment (Table 4). Although values did not reach statistical significance, both serum creatinine values and urinary protein excretion values reflected the lesser severity of disease in IL-12p40$^{-/-}$ mice, which had

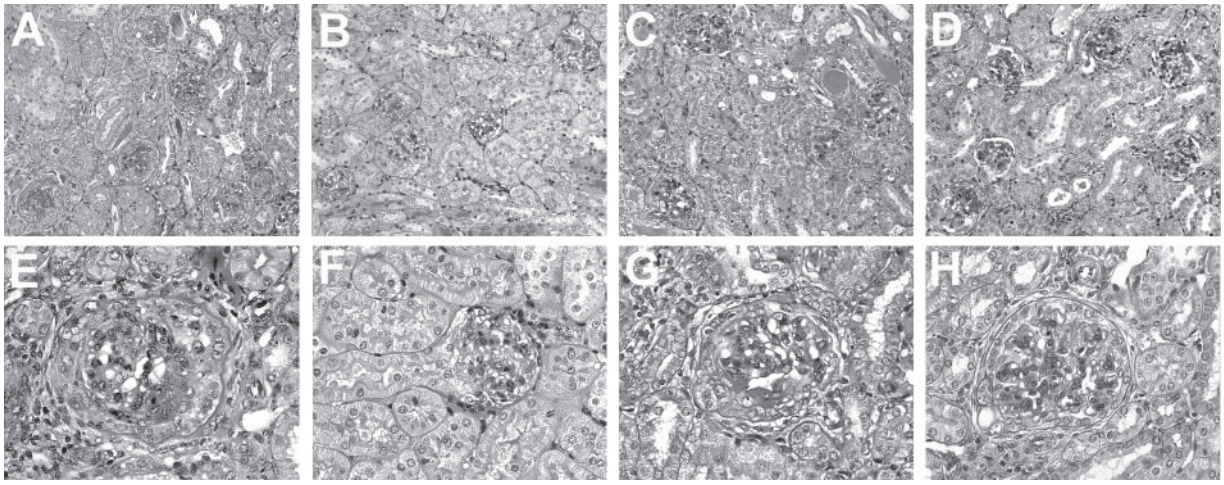

Figure 4. Histologic features of injury in WT C57BL/6, IL-12p40$^{-/-}$, IL-18$^{-/-}$, and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice with GN. Severe proliferative and crescentic GN with significant tubulointerstitial injury was present in WT C57BL/6 mice with GN (A and E). Injury was significantly attenuated in IL-12p40$^{-/-}$ mice (B and F) and marginally less severe in IL-18$^{-/-}$ mice (C and G). Combined IL-12p40$^{-/-}$IL-18$^{-/-}$ mice (D and H) showed an intermediate phenotype between IL-12p40$^{-/-}$ and IL-18$^{-/-}$ groups.

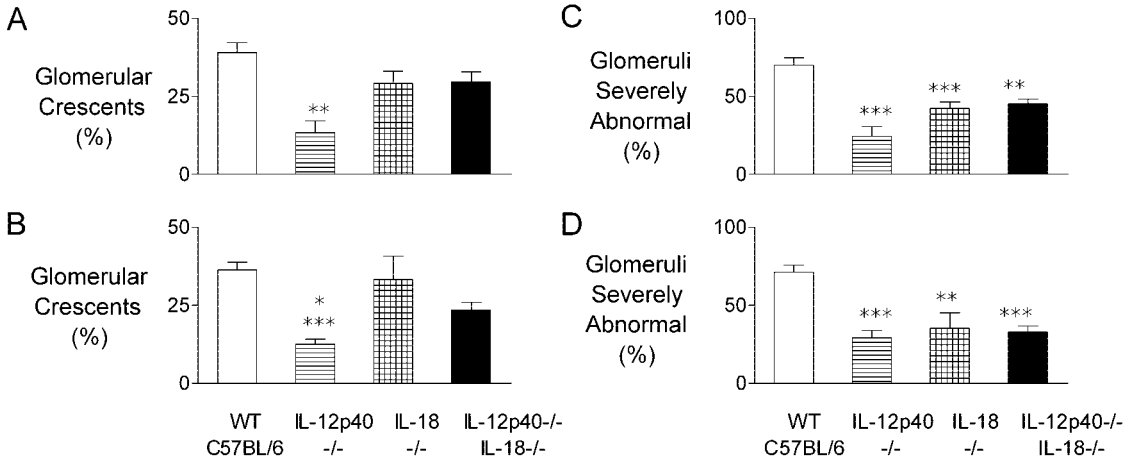

Figure 5. Analysis of glomerular injury in mice with accelerated autologous-phase anti-GBM GN at 10 d. Significant crescent formation seen in C57BL/6 mice was attenuated in the absence of IL-12p40 but not reduced in the absence of IL-18. Crescent formation was intermediate in extent in the absence of both IL-12p40 and IL-18. Severe histologic changes were most prominently reduced in IL-12p40$^{-/-}$ mice but also less common in IL-18$^{-/-}$ and combined IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. A and C, and B and D show analyses of separate experiments. *$P < 0.01$ *versus* IL-18$^{-/-}$; **$P < 0.01$ *versus* WT C57BL/6; ***$P < 0.001$ *versus* WT C57BL/6 (ANOVA). Results are expressed as the mean ± SEM. Numbers of mice studied are WT C57BL/6 ($n = 20$), IL-12p40$^{-/-}$ ($n = 5$), IL-18$^{-/-}$ ($n = 16$), and IL-12p40$^{-/-}$IL-18$^{-/-}$ ($n = 13$) for A and C and WT C57BL/6 ($n = 7$), IL-12p40$^{-/-}$ ($n = 6$), IL-18$^{-/-}$ ($n = 5$), and IL-12p40$^{-/-}$IL-18$^{-/-}$ ($n = 7$) for B and D.

serum creatinine values not significantly different from mice without GN.

Discussion

Glomerular crescent formation indicates severe and rapidly progressive GN. The presence of effector T cells and macrophages in patients with this lesion, together with studies in experimental models of GN, suggests that CD4$^+$ cell-directed, macrophage-mediated effector responses, particularly Th1 responses, are important in the genesis of glomerular crescent formation. We have comprehensively characterized severe glomerular injury in this model. At an effector level, it is dependent on effector CD4$^+$ cells (6,11,14), independent of autologous antibodies (14), complement (11), and CD8$^+$ cells (35). At a molecular level, IL-12p40, IFN-γ, GM-CSF, TNF, and IL-1β are pathogenetic (7,29,36–38), whereas endogenous IL-4 and IL-10 are protective (8,9).

The current studies used mice that were genetically deficient in IL-12p40, the common chain of both IL-12 and the more recently described cytokine IL-23, and/or IL-18 to define the relative roles of these molecules in severe immune renal injury. The key findings from these studies are (*1*) IL-12p40 is the key

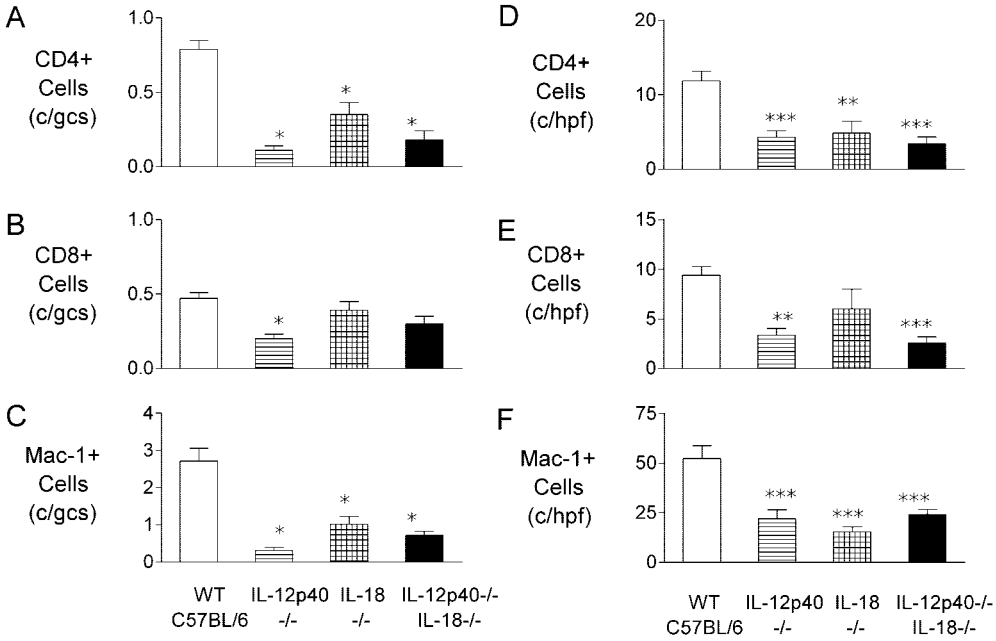

Figure 6. Leukocyte accumulation in mice with accelerated autologous-phase anti-GBM GN at 10 d. (A through C) Values for glomeruli, expressed as cells per glomerular cross section (c/gcs). (D through F) Values for the tubulointerstitium expressed as cells per high-power field (c/hpf). *$P < 0.05$ *versus* WT; **$P < 0.01$ *versus* WT; ***$P < 0.001$ *versus* WT (ANOVA). Results are expressed as the mean ± SEM. Numbers of mice studied are WT C57BL/6 ($n = 7$), IL-12p40$^{-/-}$ ($n = 6$), IL-18$^{-/-}$ ($n = 5$), and IL-12p40$^{-/-}$IL-18$^{-/-}$ ($n = 7$).

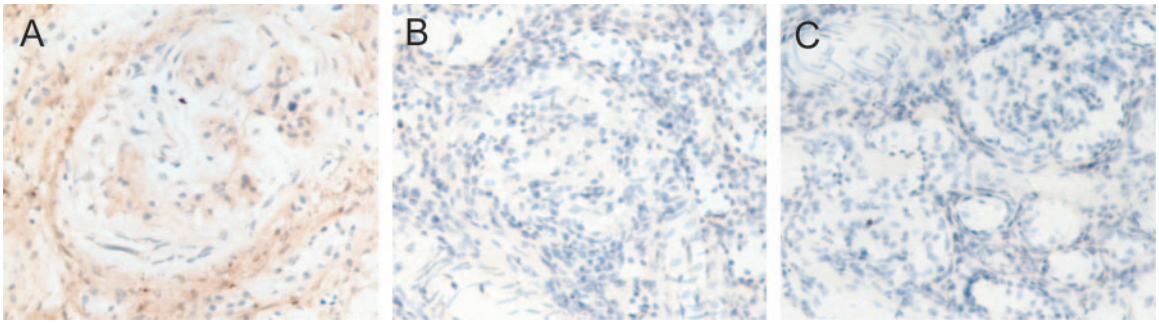

Figure 7. Expression of IL-18 protein in experimental crescentic GN. Mice with crescentic anti-GBM GN showed local expression of IL-18 (A; brown reaction product). Negative controls included the substitution of nonimmune rabbit Ig for the primary antibody (B) and preabsorbing the primary antibody with excess rmIL-18 (C). High-power views using diaminobenzidine (brown reaction product) and hematoxylin counterstain (blue).

cytokine chain in defining Th1 nephritogenic immune responses; (2) whereas IL-18 is not required for systemic Th1 responses that generate Th1 effectors and crescent formation, it plays a role in local inflammation, the severity of glomerular injury, and the production of chemoattractants; (3) deficiency of both of these molecules does not offer additional protection; and (4) reducing inflammation by deleting either of these molecules reduces surrogate markers of progressive renal disease.

This study confirms our (7,16,17) and others' (18) previous work that has shown a key role for IL-12p40 in crescentic GN. IL-12p40$^{-/-}$ mice did not make strong Th1 responses, and glomerular crescent formation, severe glomerular changes, and leukocytic infiltration were significantly inhibited. In addition to IL-12p40's effects on the systemic immune responses, local production of TNF mRNA was reduced in the absence of IL-12p40. Local expression of IL-10 mRNA, which has been shown to be protective in this lesion (9), was increased in the IL-12p40$^{-/-}$ mice. There was only minor, if any, effect on chemoattractants and adhesion molecules, suggesting that effector CD4^{+} cells that are generated in the absence of IL-12p40 lack the appropriate receptors (*e.g.*, CXCR3) to localize to glomeruli. Whereas systemic IFN-γ was reduced in IL-12p40$^{-/-}$ mice, local IFN-γ mRNA production was unchanged, suggesting that intrinsic renal cell–derived IFN-γ may not be induced by IL-12p40 but by IL-18. The potential differential roles of IL-12 and IL-23 in immune renal disease remain to be explored, although current data in EAE suggest both systemic and local roles for IL-23 in inflammatory disease (20).

Table 1. Intrarenal chemokine mRNA levels in mice with glomerulonephritis[a]

	WT C57BL/6	IL-12p40$^{-/-}$	IL-18$^{-/-}$	IL-12p40$^{-/-}$/IL-18$^{-/-}$
CXCL10 (IP-10)	75.8 ± 12.0	83.0 ± 17.4	93.4 ± 31.3	56.8 ± 10.6
XCL1 (lymphotactin)	2.03 ± 0.44	2.88 ± 1.22	1.20 ± 0.32	1.16 ± 0.37
CCL5 (RANTES)	86.4 ± 13.0	54.6 ± 9.9	80.4 ± 22.5	53.6 ± 13.1
CCL1 (TCA-3)	7.89 ± 0.99	7.27 ± 1.04	7.99 ± 2.15	5.61 ± 1.16
CCL2 (MCP-1)	100.3 ± 11.2	78.9 ± 16.5	66.0 ± 19.4	63.1 ± 8.8
CCL3 (MIP-1α)	8.06 ± 1.43	6.22 ± 0.40	2.91 ± 0.48[c,d]	3.96 ± 0.56[b]
CCL4 (MIP-1β)	11.6 ± 1.35	7.53 ± 1.66	4.12 ± 1.03[d]	5.98 ± 0.96[b]
CXCL1 (MIP-2)	17.0 ± 3.4	10.5 ± 3.9	7.73 ± 2.22	5.27 ± 0.68[b]

[a]Results are expressed as the means ± SEM (arbitrary units). WT, wild-type mice. Numbers of mice studied are WT C57BL/6 (n = 4), IL-12p40$^{-/-}$ (n = 4), IL-18$^{-/-}$ (n = 5), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 5).
[b]$P < 0.05$ *versus* WT C57BL/6.
[c]$P < 0.05$ *versus* IL-12p40$^{-/-}$.
[d]$P < 0.01$ *versus* WT C57BL/6 (ANOVA).

Table 2. P-selectin and ICAM-1 expression in mice with glomerulonephritis[a]

	WT C57BL/6	IL-12p40$^{-/-}$	IL-18$^{-/-}$	IL-12p40$^{-/-}$/IL-18$^{-/-}$
Glomerular P-selectin	2.00 ± 0.19	1.77 ± 0.20	1.99 ± 0.15	2.11 ± 0.15
Glomerular ICAM-1	1.99 ± 0.18	1.36 ± 0.23	1.74 ± 0.18	1.50 ± 0.21
Interstitial ICAM-1	2.57 ± 0.12	2.06 ± 0.27	2.40 ± 0.09	2.15 ± 0.17

[a]Results are expressed as the mean ± SEM of the semiquantitative score of immunofluorescence intensity (0 to 3+). Numbers of mice studied are WT C57BL/6 (n = 7), IL-12p40$^{-/-}$ (n = 6), IL-18$^{-/-}$ (n = 5), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 7). ICAM-1, intercellular adhesion molecule-1.

Acute glomerular injury was only modestly attenuated in the absence of IL-18. Although glomerular crescent formation was not significantly reduced in its absence, the proportion of glomeruli that were severely affected was diminished. The current studies dissect this local role of IL-18 in experimental GN. Systemically, in this response, IL-18 is not required for the generation of a Th1 response, in contrast to IL-12p40. Antigen-stimulated IFN-γ, TNF, and GM-CSF production by splenocytes was not reduced in the absence of IL-18, although IL-4 and IL-5 production (as Th2 markers) was increased. Furthermore, IL-18$^{-/-}$ mice made normal dermal DTH responses. The presence of normal antigen-specific systemic Th1 responses means that in IL-18$^{-/-}$ mice (unlike IL-12p40$^{-/-}$ mice), CD4^{+} cells localizing to glomeruli are likely to possess a Th1 phenotype. The localization of these injurious leukocytes requires the expression of the relevant adhesion molecules and chemokines. IL-18 was expressed locally in the kidneys of mice with GN, suggesting that it may upregulate local recruitment. In its absence, chemokine mRNA expression (particularly MIP-1α and MIP-1β) was reduced. Consequently, leukocyte recruitment was attenuated but not abrogated. Although less histologic injury (excluding crescent formation) and proinflammatory cytokine mRNA (TNF, IL-1β, and IFN-γ) was observed, injury remained substantial. There are three likely explanations for this finding: (*1*) Leukocytes, particularly CD4^{+} cells, infiltrating glomeruli in IL-18$^{-/-}$ mice possessed a Th1 proinflammatory phenotype (in contrast to cells from IL-12p40$^{-/-}$ mice); (*2*) numbers of Th1 cells in glomeruli IL-18$^{-/-}$ mice exceeded a threshold number of glomerular leukocytes required to trigger significant crescent formation (observed previously in this model [10]); and (*3*) unlike IL-12p40$^{-/-}$ mice, local mRNA expression of the protective cytokine IL-10 was not upregulated. The current results, highlighting a potential role for local IL-18 production in amplifying leukocyte-mediated immune renal injury, are consistent with other studies showing that IL-18 is produced locally in renal inflammation (23–25) and that its production regulates leukocyte chemoattractants (22,39).

IL-12p40$^{-/-}$IL-18$^{-/-}$ mice did not show additional protection from injury beyond that demonstrated in IL-12p40$^{-/-}$ mice. In IL-12p40$^{-/-}$IL-18$^{-/-}$ mice, key indicators of injury, such as glomerular crescent formation and leukocytic infiltration, were not less than in IL-12p40$^{-/-}$ mice. If anything, values for IL-12p40$^{-/-}$IL-18$^{-/-}$ mice were intermediate (less injury than in IL-18$^{-/-}$ mice but more than in IL-12p40$^{-/-}$ mice), similar to findings in an antigen-induced model of arthritis (40). The reasons for this lack of effect are not clear. Whereas IL-12p40$^{-/-}$IL-18$^{-/-}$ mice usually exhibited the phenotype consistent with the single gene–deficient mouse that showed the lesser inflammation, splenocytes from IL-12p40$^{-/-}$ IL-18$^{-/-}$ mice seemed to produce more TNF than IL-12p40$^{-/-}$ mice (supported by one previously published study [41]). However, renal TNF mRNA expression was not increased in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice. Although combined deficiency of three key Th1 cytokines could have switched glomerular injury to a Th2 predominant pattern in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice, this is unlikely as (*1*) Increased antigen-specific IgG1 levels

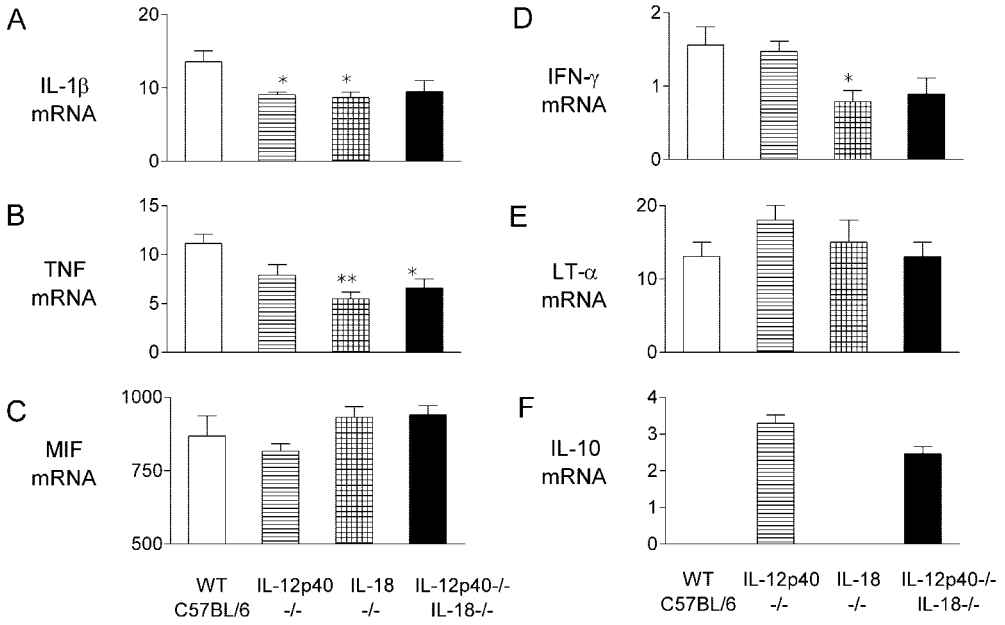

Figure 8. Intrarenal cytokine mRNA in crescentic GN. Genetically deficient mice had lower levels of IL-1β and TNF (values not reaching significance for IL-1β in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice and for TNF in IL-12p40$^{-/-}$ mice). IL-18$^{-/-}$ mice had lower levels of intrarenal IFN-γ, suggesting that IL-18 promotes secretion of this cytokine locally. MIF and LT-α levels were unchanged. IL-10 mRNA could be detected only in mice that were deficient in IL-12p40 (IL-12p40$^{-/-}$ and IL-1240$^{-/-}$IL-18$^{-/-}$ mice). *$P < 0.05$ *versus* WT C57BL/6 mice; **$P < 0.01$ *versus* WT C57BL/6 mice (ANOVA). Results are expressed as the mean ± SEM (arbitrary units). Numbers of mice studied are WT C57BL/6 (n = 4 to 7), IL-12p40$^{-/-}$ (n = 4 to 6), IL-18$^{-/-}$ (n = 5 to 6), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 5 to 6).

Table 3. Ig and complement (C3) deposition in mice developing GN[a]

	Glomerular Ig (AU)	Glomerular C3 (AU)
WT C57BL/6	68 ± 3.4	50 ± 3.2
IL-12p40$^{-/-}$	91 ± 5.9	61 ± 5.2
IL-18$^{-/-}$	103 ± 4.8[c]	59 ± 6.6
IL-12p40$^{-/-}$IL-18$^{-/-}$	96 ± 9.7[b]	53 ± 3.9

[a]Results are expressed as the mean ± SEM of the quantification of Ig deposition by image capture and NIH image analysis of fluorescence from glomeruli (antibody dilution 1:100) for each animal. Numbers of mice studied are WT C57BL/6 (n = 7), IL-12p40$^{-/-}$ (n = 6), IL-18$^{-/-}$ (n = 5), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 7). GN, glomerulonephritis.
[b]$P < 0.05$ *versus* WT C57BL/6 mice (ANOVA).
[c]$P < 0.05$ *versus* WT C57BL/6 mice (ANOVA).

were not observed, (2) systemic IL-4 or IL-5 production was not increased, (3) local increases in IL-4/IL-13 mRNA were not observed, and (4) an increased neutrophil infiltrate (that can occur in deviant Th2 immune responses) was not seen. The finding of a modest increase of mouse IgG in glomeruli (at least by one method of measurement) suggests a component of increased humoral injury, perhaps relating to higher antigen-induced production of IL-4 and IL-5. It has been shown that severe injury in this model is independent of autologous antibody (6,14), meaning that in this context, humoral immunity is unlikely to be the key determinant of glomerular injury.

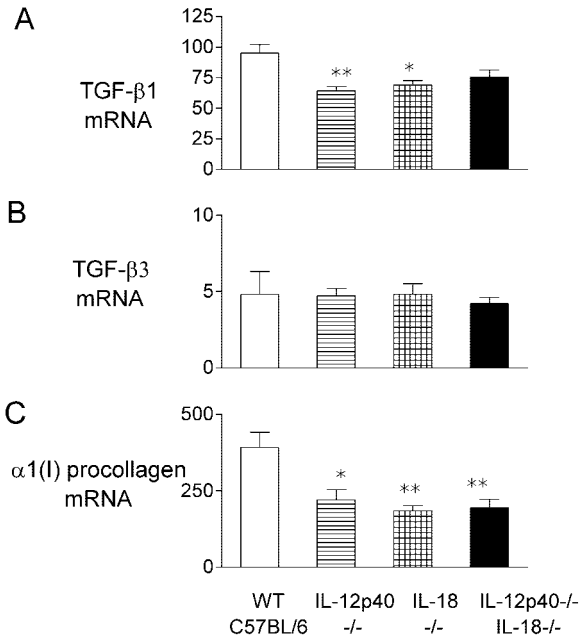

Figure 9. Intrarenal expression of TGF-β1, TGF-β3, and type 1 procollagen (α1 chain) mRNA in mice with GN. Cytokine-deficient mice exhibited reduced expression of TGF-β1 and α1(I) procollagen mRNA, except for the IL-12p40$^{-/-}$IL-18$^{-/-}$ group, in which TGF-β1 mRNA expression did not reach statistical significance. TFG-β3 mRNA expression was similar in all groups of mice. *$P < 0.05$ *versus* WT C57BL/6 mice; **$P < 0.01$ *versus* WT C57BL/6 mice (ANOVA). Results are expressed as the mean ± SEM (arbitrary units). Numbers of mice studied are WT C57BL/6 mice (n = 5), IL-12p40$^{-/-}$ (n = 5), IL-18$^{-/-}$ (n = 5), and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice (n = 5).

Table 4. Renal function and urinary protein excretion in C57BL/6 WT, IL-12p40$^{-/-}$, IL-18$^{-/-}$, and IL-12p40$^{-/-}$IL-18$^{-/-}$ mice with GN[a]

	Serum Creatinine (μmol/L)	Urinary Protein (mg/24 h)	Urinary Protein:Creatinine (mg/μmol)
No GN	11.1 ± 0.8	1.2 ± 0.8	ND
WT C57BL/6 GN	19.4 ± 1.2[b]	7.9 ± 1.1	3.0 ± 0.6
IL-12p40$^{-/-}$ GN	16.1 ± 1.5	4.8 ± 1.0	1.3 ± 0.2
IL-18$^{-/-}$ GN	18.0 ± 1.8[c]	5.6 ± 1.0	3.1 ± 0.6
IL-12p40$^{-/-}$/IL-18$^{-/-}$ GN	18.5 ± 0.9[c]	5.1 ± 0.4	1.9 ± 0.2

[a]Results are expressed as the mean ± SEM. Numbers of mice studied for serum creatinine are No GN (n = 8) WT C57BL/6 (n = 20), IL-12p40$^{-/-}$ (n = 4), IL-18$^{-/-}$ (n = 16), and IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 13) and for proteinuria are No GN (n = 13) WT C57BL/6 (n = 19), IL-12p40$^{-/-}$ (n = 5), and IL-18$^{-/-}$ (n = 16), IL-12p40$^{-/-}$IL-18$^{-/-}$ (n = 13).
[b]$P < 0.01$ *versus* No GN.
[c]$P < 0.05$ *versus* No GN (ANOVA).

Progression of inflammatory renal disease is characterized not only by ongoing inflammation but also by the production of profibrotic cytokines, such as TGF-β1 and matrix proteins, for example type I collagens. Renal TGF-β1 and α1(I) procollagen mRNA was reduced in all groups (TGF-β1 in IL-12p40$^{-/-}$IL-18$^{-/-}$ mice not reaching significance). It has been suggested that Th2 responses are profibrotic (42), but the current studies demonstrate that in renal inflammation, deletion of key Th1 cytokines does not drive immune responses toward pathogenetic Th2 responses or seem to induce a local profibrotic state. Rather, the dampening of renal inflammation in mice deficient in IL-12p40 and/or IL-18 is associated with decreased production of mRNA for prototypic markers of renal fibrosis, suggesting that in this lesion, dampening ongoing inflammation will lead to reduced progressive renal fibrosis.

In summary, these studies demonstrate that IL-18 plays local proinflammatory roles in immune renal inflammation, whereas IL-12p40 is the key cytokine chain in nephritogenic Th1 responses that lead to crescentic GN.

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