Experimental Autoimmune Anti–Glomerular Basement Membrane Glomerulonephritis: A Protective Role for IFN-γ

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Abstract. IL-12 and IFN-γ play key roles in murine lupus and planted antigen models of glomerulonephritis. However, their roles in renal organ–specific autoimmunity are unknown. To establish the roles of endogenous IFN-γ and IL-12 in experimental autoimmune anti–glomerular basement membrane (GBM) glomerulonephritis (EAG), EAG was induced in normal C57BL/6 mice (WT), IL-12p40–deficient (*IL-12p40*−/−) mice, and IFN-γ–deficient (*IFN*-γ−/−) mice by immunization with α3-α5(IV)NC1 heterodimers. At 13 wk, WT mice developed EAG with linear mouse anti-GBM antibody deposition, histologic injury, proteinuria, and mild tubulointerstitial disease. Compared with WT mice, *IL-12p40*−/− mice had decreased histologic injury and trends to decreased leukocyte infiltrates. In contrast, 40% (4 of 10) of *IFN*-γ−/− mice developed significant crescent formation and focal or diffuse interstitial infiltrates (WT, 0 of 8). Compared with WT and/or *IL-12p40*−/− mice, *IFN*-γ−/− mice developed increased injury: histologic injury, total glomerular cell numbers, leukocytes in glomeruli, and renal expression of P-selectin and intercellular adhesion molecule 1. All groups developed similar serum anti–α3-α5(IV)NC1 antibodies and glomerular Ig deposition, but *IFN*-γ−/− mice had decreased anti–α3-α5(IV)NC1 IgG2a. Therefore, *IFN*-γ−/− mice developed increased cellular reactants despite a potentially less damaging antibody response. Dermal delayed-type hypersensitivity was increased in α3-α5(IV)NC1 immunized *IFN*-γ−/− mice and was suppressed by recombinant murine IFN-γ. CD4+ cells from draining nodes of immunized *IFN*-γ−/− mice showed increased proportions of proliferating CD4+ cells but similar numbers of apoptotic cells. These studies demonstrate that in renal organ–specific autoimmunity, IL-12 is pathogenetic but IFN-γ is protective. They lend weight to the hypothesis that depending on the context/severity of the nephritogenic immune response IFN-γ has different effects.

Glomerulonephritis (GN), or immune-mediated glomerular injury, is an important cause of renal disease. Although some forms of GN occur in the absence of immune reactants in glomeruli, most forms of GN are characterized by immune reactivity to a variety of self- or foreign antigens. The nature of the immune response to antigens is determined by the strength of and nature of T cell reactivity to these antigens (1). Antigen-specific CD4+ cells direct immune responses by providing help to B cells for antibody production, by activating CD8+ cells, and by accumulating at sites of injury and recruiting innate effectors such as macrophages. The realization that different subsets of CD4+ cells (T helper [Th]) cells exist and are defined by their cytokine production has helped explain the variable nature and direction of antigen-specific effector responses (2).

Two of the key cytokines involved in Th1 responses are IL-12 and IFN-γ. IL-12 is a heterodimer composed of p40 and p35 subunits (3) and produced by antigen-presenting cells to direct uncommitted T cells to the Th1 phenotype. Recent studies have demonstrated that another cytokine that is important in Th1 responses, IL-23, also uses the p40 subunit with a p19 subunit (4). IFN-γ is a product of Th1 cells and promotes macrophage activation and the IgG subclass switching to opsonizing and complement fixing subclasses (5).

Models of severe crescentic GN induced by planted foreign immunoglobulins (so called autologous-phase anti–glomerular basement membrane [GBM] GN) are directed by endogenous IL-12 and IFN-γ (6–9). Some important models of GN complicating systemic autoimmunity (*e.g.*, lupus nephritis in MRL/lpr and NZB/W mice) are also IL-12 (10) and IFN-γ mediated (11–14). IFN-γ has been reported to induce proliferative nephritis in patients with rheumatoid arthritis or systemic lupus erythematosus (15,16). Recent evidence comparing disease phenotype and immune responses in autoimmune anti-GBM (experimental autoimmune glomerulonephritis [EAG]) supports a role for Th1 responses and cell-mediated effectors in EAG (17–19). In autoimmune tubulointerstitial nephritis,

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IFN-γ had antiproliferative effects on renally derived T cell clones but converted a nonnephritogenic clone into a nephritogenic clone (20). However, in several models of organ-specific autoimmunity, for example, experimental autoimmune encephalomyelitis, IL-12 was pathogenetic, whereas IFN-γ was found to have a protective effect (21–24), and models of solid organ transplantation show a protective role for IFN-γ (25–27).

The hallmark of human anti-GBM disease is the production of autoantibodies targeted to the noncollagenous (NC1) domain of the α3 chain of type IV collagen, found in the GBM (28–30). Although long considered a classic antibody-mediated disease, the role of cell-mediated immune responses in anti-GBM GN has attracted more attention recently, and it is likely that both humoral and cellular responses contribute to injury in this disease (17–19,31). The current studies use a model of EAG in mice induced by immunization with α3(IV)NC1, the target antigen in human anti-GBM GN, to address the roles of IFN-γ and IL-12 in renal organ–specific autoimmunity. EAG was induced in genetically normal C57BL/6 mice and in genetically deficient mice on a C57BL/6 background that were deficient in either IL-12 (and IL-23, *via IL-12p40* gene targeting) or IFN-γ, and the resultant renal disease and immune responses were assessed.

Materials and Methods

Experimental Design

EAG was induced by subcutaneously immunizing 4- to 6-wk-old male mice with 25 μg of α3-α5(IV)NC1 heterodimers in 100 μl of Freund's complete adjuvant (FCA) at the base of the tail. Four weeks later, mice were boosted with 20 μg of heterodimers in FCA injected into the flank. Renal injury and immune responses were studied 13 wk after the first immunization. The immunogen, the α3-α5(IV)NC1 heterodimer, was isolated from bovine testis basement membrane, which is enriched in the α3(IV) chain of type IV collagen compared with GBM (32). The α3(IV)NC1 domain, isolated by collagenase digestion of basement membrane, exists mainly as a heterodimer composed of the α3 and α5 NC1 domains (33). This α3-α5(IV)NC1 heterodimer was purified by C18 reverse-phase chromatography (32) followed by treatment with 6 M GuHCl and by gel-filtration chromatography on a TSK SW column to resolve the dimer from monomers (34). The following mice were studied: genetically normal C57BL/6 mice (WT; $n = 8$; Monash University Animal Services, Clayton, VIC, Australia), IL-12p40-deficient mice (*IL-12p40*−/−; $n = 9$) (35) on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME; bred at Monash University), and IFN-γ deficient mice (*IFN*-γ−/−; $n = 10$) (5) on a C57BL/6 background (Jackson Laboratories; bred at Monash University). Separate groups of C57BL/6 mice and *IFN*-γ−/− mice (both $n = 7$) were immunized once, and T cell responses were studied at 4 wk (see below). Histologic assessments were performed on coded slides, and results are expressed as the mean ± SEM. The significance of differences between groups was determined by ANOVA, followed by the Neumann-Kiels *post hoc* test, except for calculating the significance of development of severe disease in some *IFN*-γ−/− mice (χ^2) and for experiments in leukocyte proliferation and apoptosis using only WT and *IFN*-γ−/− mice (unpaired *t* test).

Assessment of Renal Histology, Leukocyte Infiltration, and Adhesion Molecule Expression

Kidney tissue was fixed in Bouin fixative and embedded in paraffin, and 3-μm tissue sections were cut and stained with periodic acid Schiff reagent. The proportion of glomeruli affected was determined by examining a minimum of 50 glomeruli per mouse for abnormalities according to a previously published method (36). Abnormalities included crescent formation (two or more layers of cells in Bowman's space), segmental proliferation, necrosis or hyalinosis, or capillary wall thickening. Total glomerular cell nuclei were counted in a minimum of 20 glomeruli per mouse, and results are expressed as cells per glomerular cross-section.

For assessment of leukocytes in kidneys, tissue was fixed in periodate lysine paraformaldehyde for 4 h, washed (7% sucrose), then frozen. Tissue sections (6 μm) were stained to demonstrate CD4+ cells, CD8+ cells, macrophages, and neutrophils using a three-layer immunoperoxidase technique, as described previously (37,38). The primary monoclonal antibodies were GK1.5 anti-mouse CD4 (American Type Culture Collection [ATCC], Manassas, VA); 53-6.7 anti-mouse CD8 (ATCC); M1/70 anti–Mac-1, which recognizes macrophages and neutrophils (ATCC); and RB6-8C5 anti–Gr-1, which recognizes neutrophils (DNAX, Paulo Alto, CA). At least 50 glomeruli were assessed per animal, and results are expressed as cells per 50 glomerular cross-sections. A minimum of 50 high- power fields within the cortical tubulointerstitium were assessed for leukocytes, excluding glomeruli, periglomerular, and perivascular regions. In *IFN*-γ−/− mice with dense focal infiltrates, cell numbers could not be counted, so these areas were avoided. Results are expressed as cells per 50 high-power fields.

For renal deposition of P-selectin and intercellular adhesion molecule 1 (ICAM-1), snap-frozen tissue sections (6 μm) were used. For P-selectin, sections were blocked with 10% sheep serum, then incubated with polyclonal rabbit anti-human P-selectin (that cross-reacts with mouse P-selectin (38) 10 μg/ml, 1 h), followed by FITC-sheep anti-rabbit IgG (DakoCytomation, Glostrup, Denmark; 1:50). For ICAM-1, sections were incubated with 10% normal rat serum, then PE-hamster anti-mouse CD54 (anti-mouse ICAM-1, 3E2; Pharmingen, San Diego, CA; 1:60, 1 h). P-selectin and ICAM-1 expression was scored semiquantitatively from 0 to 3 as follows: 0, background staining; 1, the 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.

Titers of Serum Antigen-Specific Ig and Glomerular Deposition of Mouse Ig

Titers of mouse anti-GBM were measured by ELISA on serum collected at the end of experiments. Plates were coated with 10 μg/ml bovine α3-α5(IV)NC1, washed, blocked (1% BSA), washed, then incubated with mouse serum (1:100, 1:400, and 1:800, 1 h, 37°C). Bound mouse Ig was detected with horseradish peroxidase–conjugated sheep anti-mouse Ig (Amersham, Little Chalfont, UK; 1:2000). 2,2'-Azino-di-3-ethylbenzthiazoline sulfonate (0.1 M; ABTS, Boehringer Mannheim, Mannheim, Germany) substrate solution was added, and the absorbance was read at 405 nm. For serum IgG1 and IgG2a subclass measurements, plates were coated as above, washed, blocked (2% casein), then incubated with mouse serum (1:100 for IgG1, 1:50 for IgG2a, 2 h at room temperature). Bound IgG1/IgG2a were detected with 2 μg/ml biotinylated rat anti-mouse IgG1 or IgG2a (Becton Dickinson Pharmingen), then biotinylated mouse anti-avidin antibody (Sigma, Castle Hill, NSW, Australia), then 1.1 μg/ml ExtrAvidin-peroxidase (Sigma). 3,3',5',5-Tetremethlyben-

zidine (Sigma) was added, and the reaction stopped with 0.5 M H_2SO_4 and absorbance was read at 450 nm.

For renal deposition of mouse Ig, IgG1 and IgG2a snap-frozen tissue sections (6 μm) were stained using FITC-sheep anti-mouse Ig (Silenus, Hawthorn, Victoria, Australia; 1:100). Fluorescence intensity was assessed semiquantitatively (0 to 3+). For assessment of glomerular deposition of IgG1 and IgG2a subclasses, FITC-rat anti-mouse IgG1 (Pharmingen; 1:100) and FITC-rat anti-mouse IgG2a (Pharmingen; 1:50) were used.

Proteinuria and Serum Creatinine

Urinary protein concentrations were determined by the Bradford method (39) on timed 24-h collections at baseline and 1 d before the end of experiments. Baseline urinary protein excretion was similar in all three strains of mice. Serum creatinine concentrations at the completion of experiments (week 13) were measured by an enzymatic creatininase assay.

Assessment of Dermal Delayed-Type Hypersensitivity in IFN-γ −/− Mice

Three groups of mice (female, 6 to 9 wk) were immunized with 25 μg of bovine α3-α5(IV)NC1 in 100 μl of FCA at the base of the tail. Mice then received intraperitoneal injections daily, for 4 wk, of either recombinant murine IFN-γ (rmIFN-γ; 1 ng [10 units] in 100 μl of 0.1% BSA/PBS; Chemicon, Temecula, CA) or 100 μl of 0.1% BSA/PBS (vehicle alone). The following groups of mice were studied: C57BL/6 WT mice ($n = 4$) that received vehicle alone, *IFN-γ−/−* mice ($n = 5$) that received vehicle alone, and *IFN-γ−/−* mice ($n = 5$) that received rmIFN-γ. After 3 wk and 5 d, mice were challenged with 25 μg of bovine α3-α5(IV)NC1 in 30 μl of PBS injected subcutaneously in one footpad and 100 μg of collagenase solubilized renal basement membrane (RBM) preparation intradermally into the ear pinna. RBM was prepared using a modification of a previously published method (40). The RBM preparation was derived from kidney cortex of C57BL/6 mice, homogenized, then centrifuged. After washing, sonication, and lysis, membranes were collected by centrifugation and then digested with type I collagenase (Sigma). An equivalent dose of BSA in 30 μl of PBS was injected in both the contralateral footpad and the contralateral ear to serve as an appropriate control. Delayed-type hypersensitivity (DTH) was assessed using a micrometer at 24 and 48 h (footpads) and at 48 h (ears) by measuring the difference between the collagen-injected foot pads or ears and the BSA-injected sides in each mouse.

Assessment of Leukocyte Proliferation and Apoptosis

C57BL/6 WT mice ($n = 7$) and *IFN-γ−/−* mice ($n = 7$) were immunized with 25 μg of bovine α3-α5(IV)NC1 in 100 μl of FCA at the base of the tail. After 3 wk and 5 d, mice received an intraperitoneal injection of 0.8 mg of 5-bromo-2'-deoxyuridine (BrdU; Sigma) in PBS and then given drinking water that contained BrdU (0.8 mg/ml for 48 h). One mouse was not given BrdU to act as an appropriate flow cytometric control. Mice were killed humanely, and inguinal lymph nodes were removed. Single-cell suspensions were stained with anti–CD4-PE (Pharmingen) or anti–CD8-APC (Pharmingen). Cells were washed, fixed, and permeabilized (1% paraformaldehyde/PBS/1% Tween 20). After washing, pelleted cells were incubated with anti–BrdU-FITC/DNase (Becton Dickinson; 30 min, room temperature). Labeled cells were washed twice before being analyzed by flow cytometry (MoFlo; DakoCytomation, Fort Collins, CO). Dead cells were excluded according to forward and side scatter properties, and BrdU incorporation was analyzed on CD4+ and CD8+ T cell populations. For *ex vivo* detection of apoptotic cells, freshly isolated lymph node cells were washed, then resuspended in 100 μl of Annexin-V-Flous labeling solution (Roche, Mannheim, Germany) that contained 10 μg of propidium iodide and incubated for 15 min (room temperature) before being analyzed by flow cytometry.

Results

Disease Expression in Genetically Normal C57BL/6 Mice (WT Mice)

Thirteen weeks after immunization, genetically normal C57BL/6 WT mice developed serum antibodies against α3-α5(IV)NC1 and linear deposition of IgG, IgG1, and IgG2a on the GBM and, to a lesser degree, the tubular basement membrane. Kidneys histologically showed injury with abnormalities present in 43 ± 5% of glomeruli (Figure 1, A and B) and a mild glomerular and interstitial leukocyte infiltrate. Crescent formation was observed in only 2% of glomeruli in three of eight mice. WT mice developed significant proteinuria, but serum creatinine values were within the normal range.

Histologic Injury in IFN-γ−/− and IL-12p40−/− Mice with EAG

IL-12p40−/− mice with EAG had only mild renal injury with few glomeruli affected (8 ± 2%; $P < 0.001$ *versus* WT; Figures 1, C and D, and 2A). However, renal disease in *IFN-γ−/−* mice was more severe than in *IL-12p40−/−* mice (Figure 1, E through H) with more glomeruli affected (69 ± 6%; $P < 0.01$ *versus* WT). Whereas no WT or *IL-12p40−/−* mouse developed severe GN, a significant number of *IFN-γ−/−* mice (4 of 10 *versus* 0 of 8 WT mice; $P = 0.042$ *versus* WT, χ^2) developed significant focal or diffuse glomerular and tubulointerstitial inflammation (Figure 1, G and H), with these severely affected mice showing glomerular crescent formation (8, 14, 36, and 38% of glomeruli affected). Analysis of glomerular cell numbers showed increased numbers of cell nuclei in *IFN-γ−/−* mice with GN that reached statistical significance when compared with *IL-12p40−/−* mice (Figure 2B).

Leukocytic Infiltrates in IFN-γ−/− and IL-12p40−/− Mice with EAG

WT mice developed a relatively sparse leukocytic infiltrate in glomeruli, and *IL-12p40−/−* mice showed trends to decreased leukocytes in glomeruli across all subsets (Figures 2, C through F). However, glomerular leukocyte numbers, except for CD8+ cells, were increased in the absence of IFN-γ when compared with *IL-12p40−/−* mice, although values did not reach statistical significance when compared with WT mice. In the cortical interstitium, overall trends were similar (Figures 3 and 4). However, whereas interstitial CD4+ and CD8+ cells were present in low numbers in both WT and *IL-12p40−/−* mice (Figure 3, A and B, and 4A, B, E, and F), results of quantitative analyses showed that there were fewer interstitial neutrophils present in *IL-12p40−/−* mice compared with WT mice (Figure 3D). A subset of *IFN-γ−/−* mice developed significant interstitial infiltrates that were predominantly CD4+ cells and macrophages, although CD8+ cells and neutrophils were also present. Figure 4, D, H, L, and P, shows

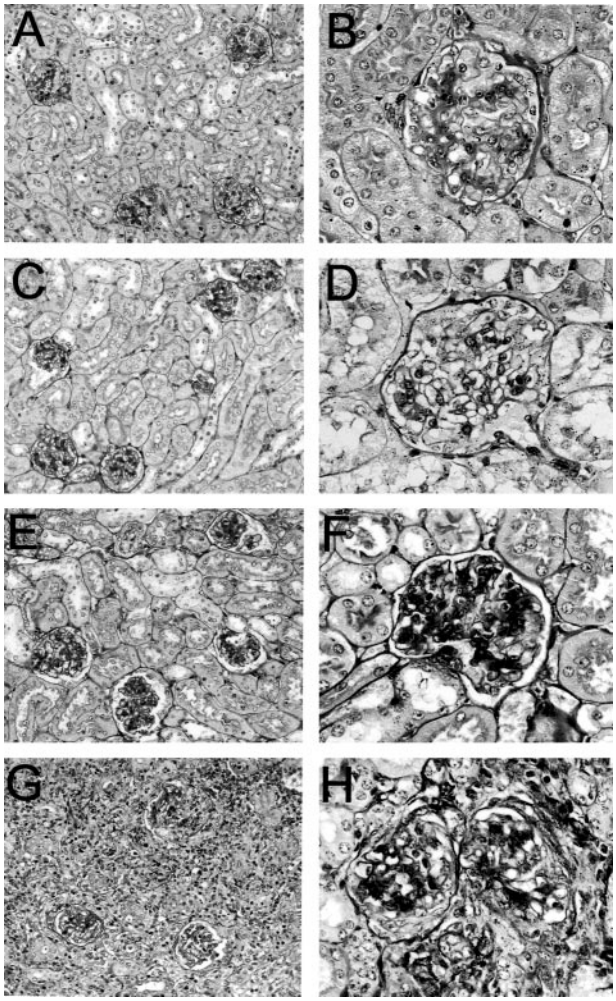

Figure 1. Representative photomicrographs of histologic features of renal injury in normal C57BL/6 (WT), *IL-12p40*−/−, and *IFN*-γ−/− mice with experimental autoimmune glomerulonephritis (EAG). Photomicrographs at low and high power demonstrate relatively mild renal injury in WT mice with EAG (A and B), less severe injury in *IL-12p40*−/− mice (C and D), more significant but relatively mild injury in the majority of *IFN*-γ−/− mice (E and F), and severe injury in some *IFN*-γ−/− mice with diffuse interstitial disease and more severe glomerular injury (G and H), including a significant degree of glomerular crescent formation (all periodic acid Schiff stain; A, C, E, and G low power; B, D, F, and H high power).

examples of significant infiltrates found in some *IFN*-γ−/− mice with GN. The monoclonal antibody M1/70 (anti–Mac-1) recognizes both macrophages and neutrophils. However, analysis of cells expressing Gr-1 demonstrated that most of the Mac-1+ cells recognized by M1/70 in glomeruli and in the interstitium were Gr-1 macrophages (Figure 3, C and D). No overall significant increases in interstitial leukocytes in *IFN*-γ−/− mice compared with WT mice was observed (ANOVA: CD4+ $P = 0.053$, M1/70 $P = 0.105$). The data may underestimate the true values for some *IFN*-γ−/− mice. In sections from one *IFN*-γ−/− mouse, the presence of diffuse dense infiltrates meant that individual CD4+, CD8+, M1/70+, or Gr-1+ cells could not be counted and therefore this mouse

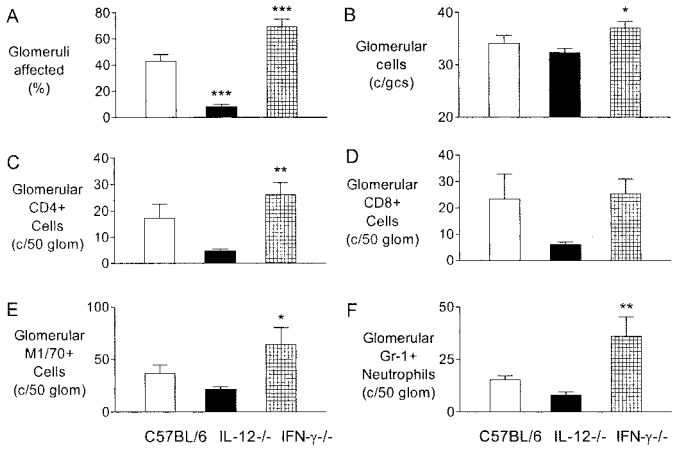

Figure 2. Glomerular pathology and leukocytes in EAG. (A and B) *IL-12p40*−/− mice have a lower proportion of glomeruli affected than WT mice and a trend to decreased glomerular cell numbers, whereas *IFN*-γ−/− mice have increased numbers of abnormal glomeruli compared with WT mice and increased cell nuclei compared with *IL-12p40*−/− mice. Analysis of leukocytes in glomeruli (C through F) show increased numbers of CD4+ cells, M1/70+ cells, and Gr-1+ leukocytes in *IFN*-γ−/− mice compared with *IL-12p40*−/− mice. *$P < 0.05$ *versus IL-12p40*−/− mice; **$P < 0.01$ *versus IL-12p40*−/− mice; ***$P < 0.01$ *versus* WT (ANOVA).

could not be included in these quantitative analyses. In three other *IFN*-γ−/− mice, areas of dense interstitial infiltrates (where individual cells were not discernible) were avoided, resulting in an underestimation of the degree of infiltrate.

Adhesion Molecules (P-Selectin and ICAM-1) in WT, IFN-γ−/−, and IL-12p40−/− Mice with EAG

Staining for P-selectin in both the glomerulus and the tubulointerstitium was evident in WT mice with EAG (Figure 5, A and B, and 6A) and was unaltered in the absence of IL-12p40 (Figure 6B). However, P-selectin expression was upregulated in the absence of IFN-γ (Figure 5, A and B) in *IFN*-γ−/− mice with severe disease (Figure 6D) and without dense leukocytic infiltrates (Figure 6C). Semiquantitative analysis of P-selectin expression showed significant increases in glomerular expression and a trend to increased interstitial P-selectin in *IFN*-γ−/− mice. Similar findings were present with respect to ICAM-1 expression (Figures 5, C and D, and 6, E through H), although significantly increased ICAM expression in *IFN*-γ−/− mice (compared with WT and *IL-12p40*−/− mice) was confined to the interstitial compartment.

Antibody Responses and IgG Subclasses in Mice with GN

Linear basement membrane staining was evident on the GBM of all strains and to a lesser degree the tubular basement membrane (Figure 7, A through C). Semiquantitative assessment of Ig deposition in glomeruli showed a similar degree of linear staining in WT, *IL-12p40*−/−, and *IFN*-γ−/− mice (Figure 7D). As anticipated, all mice made antibodies to α3-α5(IV)NC1, measured by ELISA on serum of mice collected at

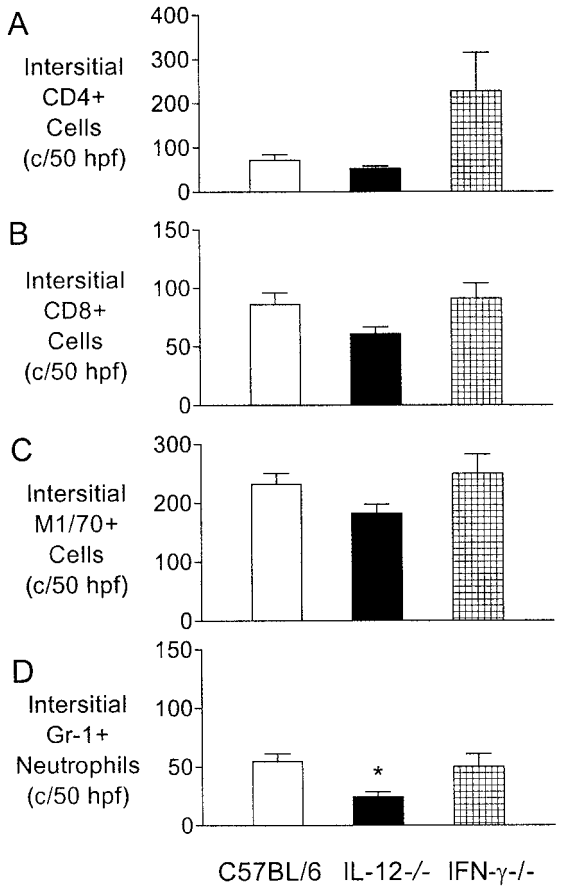

Figure 3. Analysis of interstitial leukocytic infiltrates in mice with EAG, showing fewer interstitial neutrophils in *IL-12p40*−/− mice and a trend to increased CD4+ cells in *IFN*-γ−/− mice. *$P < 0.05$ *versus* both C57BL/6 and *IFN*-γ−/− mice (ANOVA).

13 wk (Figure 7E). As IL-12 and IFN-γ can potentially affect IgG subclass switching in immune responses, IgG1 and IgG2a in serum and glomeruli were analyzed. Serum IgG1 was similar in all strains, but IgG1 deposition in glomeruli was decreased in *IFN*-γ−/− mice. Serum IgG2a was reduced in *IFN*-γ−/− but not *IL-12p40*−/− mice, and a trend to decreased glomerular IgG2a was observed in *IFN*-γ−/− mice (Table 1).

Clinical Evidence of Disease in WT, IFN-γ−/−, and IL-12p40−/− Mice with GN

Of 10 *IFN*-γ−/− mice immunized with α3-α5(IV)NC1, 2 mice developed oligoanuria and ascites at the end of 13 wk, and 1 mouse was killed humanely 1 d before the end of the experiment. No urine could be obtained from these clinically unwell mice, but other data derived from these mice were included in the results. Results of serum creatinine values and 24-h urinary protein excretion performed at the end of experiments are presented in Table 2. Two *IFN*-γ−/− mice with EAG developed elevated serum creatinine values (102.0 and 39.6 μmol/L).

Dermal DTH Responses in Mice Immunized with α3-α5(IV)NC1

Four weeks after immunization with α3-α5(IV)NC1 dimers, mice were challenged with collagenase solubilized murine RBM in the ear and α3-α5(IV)NC1 dimers in the footpad. WT mice demonstrated antigen-specific ear swelling (to murine RBM, measured at 48 h; Figure 8A) and footpad swelling (to α3-α5(IV)NC1, measured at 24 and 48 h; Figure 8B). DTH responses were increased in the absence of endogenous IFN-γ in *IFN*-γ−/− mice, the difference being most evident in ear swelling to murine RBM. Dermal DTH was reduced (to levels similar to or below that found in WT mice) in immunized *IFN*-γ−/− mice by the daily administration (from the time of sensitization) of low-dose rmIFN-γ, given to "reconstitute" *IFN*-γ−/− mice with IFN-γ.

Immune Responses in Secondary Lymphoid Organs in WT and IFN-γ−/− Mice

To explore whether IFN-γ exerts a protective effect during the early phases of the autoimmune process, we assessed CD4+ and CD8+ subsets, cellular proliferation, and apoptosis in lymph nodes draining the immunization sites by flow cytometric analysis of T cells in one group of WT mice ($n = 7$) and one group of *IFN*-γ−/− mice ($n = 7$) at 4 wk after a single immunization (Table 3). Draining lymph nodes from *IFN*-γ−/− mice had a higher proportion of their cells that were CD4+ compared with WT, whereas proportions of CD8+ cells were similar in both groups. Proliferation of CD4+ and CD8+ cells, assessed by the incorporation of BrdU in the 48 h before the end of the experiment, showed increased numbers of CD4+/BrdU+ cells in *IFN*-γ−/− mice. Proliferation in CD8+ cells was unchanged in the absence of IFN-γ. The degree of apoptosis, assessed as the proportion of cells that expressed annexin-V but that were negative for propidium iodide, were similar in both groups.

Discussion

These studies show a protective role for IFN-γ in EAG, together with a more predictable pathogenetic role for IL-12. In the absence of IFN-γ, there is an increased cellular infiltrate in both the glomerulus and the interstitium, with no net increase in antibody response and a decrease in the complement fixing and opsonizing (and therefore potentially more damaging) IgG2a subclass. A significantly higher proportion of *IFN*-γ−/− mice with EAG (4 of 10 mice; 40%) developed severe injury than did WT mice (0 of 8 mice). This is in contrast to the findings in *IL-12p40*−/− mice that when compared with WT and *IFN*-γ−/− mice developed less histologic injury and trends to a reduction in effector cell infiltrates. Some results did not reach statistical significance, for a variety of potential reasons, including the relative lack of severity of disease in WT mice. The development of clearly more significant histologic disease in a subset of *IFN*-γ−/− mice is somewhat analogous to some other models of autoimmune disease, including collagen-induced arthritis, whereby only some mice develop severe disease (41). Molecules involved in the adhesion of leukocytes in the kidney (P-selectin and ICAM-1) were upregulated in the

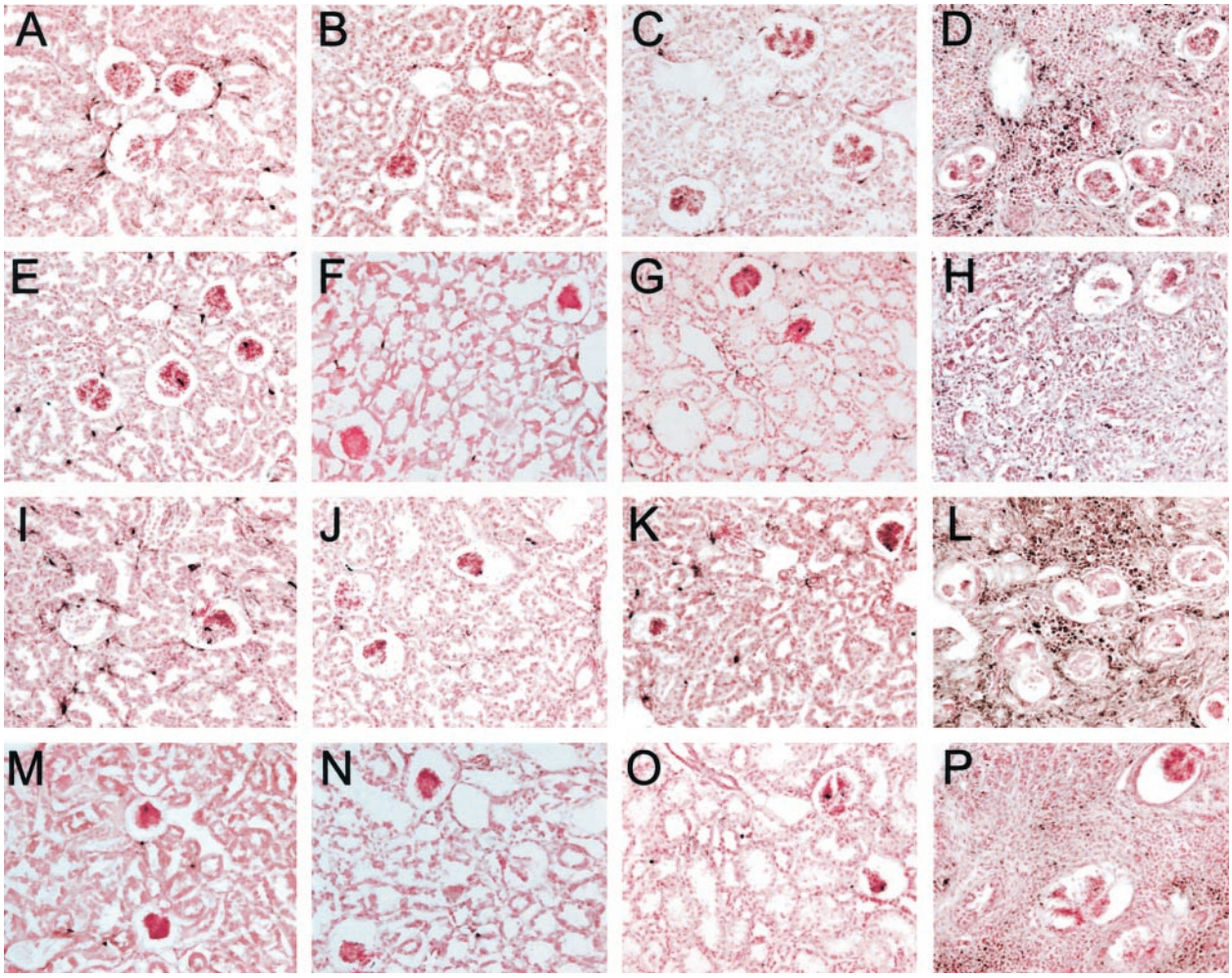

Figure 4. Immunohistochemical detection of leukocytes in EAG showing a predominant CD4+ cell and macrophage infiltrate in representative sections of more severely affected *IFN-γ−/−* mice. Positive cells are shown as a black reaction product. CD4+ cells in WT mice (A), *IL-12p40−/−* mice (B), and *IFN-γ−/−* mice (C). (D) A more significantly affected *IFN-γ−/−* mouse. CD8+ cells WT (E), *IL-12p40−/−* mice (F), *IFN-γ−/−* mice (G), and a more significantly affected *IFN-γ−/−* mouse (H). In general, CD8+ cells were less evident in *IFN-γ−/−* mice, including the dense infiltrates, than CD4+ cells. M1/70+ cells in WT mice (I), *IL-12p40−/−* mice (J), and *IFN-γ−/−* mice (K and L). Gr-1+ neutrophils in WT mice (M), *IL-12p40−/−* mice (N), and *IFN-γ−/−* mice (O and P). M1/70 detects both macrophages and neutrophils. Most of the M1/70+ cells were not Gr-1+, indicating a predominance of macrophages. Medium-power views using DAB (black reaction product) and nuclear fast red counterstain.

absence of IFN-γ, the differences being evident both in *IFN-γ−/−* mice with very severe disease and in *IFN-γ−/−* mice that did not have florid infiltrates. The phenotype of the model used in these studies differs in severity from that of Kalluri *et al.* (17), for reasons that are not clear. Although similarly prepared antigenic preparations were used, there may be differences in the potency of the antigen used for both sets of studies.

These studies demonstrate that IFN-γ is protective in renal organ–specific autoimmunity, and they highlight the complexity and potential differential roles of IFN-γ in immune renal disease. In the context of systemic autoimmunity, for example, severe murine lupus nephritis, IFN-γ's importance has been demonstrated by a number of studies, because of both its propensity to generate more damaging IgG2a and IgG3 autoantibodies (13) and its enhancing cell-mediated immunity (14). However, even within this system, IFN-γ has been shown to have some negative regulatory effects (42). In studies of the role of endogenous IFN-γ using planted antigen models of GN, conflicting results have emerged. In studies of severe crescentic injury in autologous-phase "anti-GBM GN" in C57BL/6 mice, endogenous IFN-γ was pathogenetic. These series of studies in autologous injury used both accelerated (antigen in adjuvant priming followed by intravenous antigen challenge) (7,8) and "nonaccelerated" (intravenous antigen and waiting for the development of the autologous phase of injury) (9) models and both anti–IFN-γ antibody treatment (7) and *IFN-γ−/−* mice (8,9). Results of these studies suggested that although IFN-γ was not essential for the generation of Th1 responses, it was important in effector phases of the nephritogenic immune responses, including IgG2a generation, macrophage activation, and T cell and macrophage recruitment.

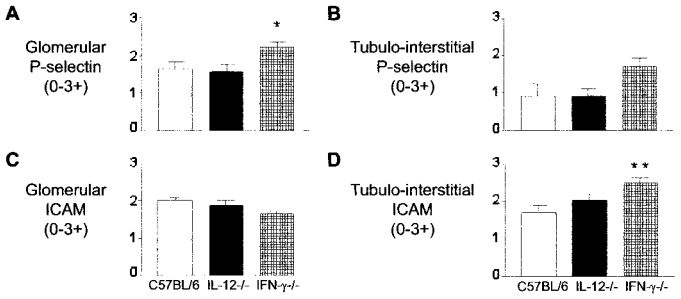

Figure 5. Semiquantitative analysis of adhesion molecule expression in kidneys of mice with EAG. (A and B) Upregulated P-selectin expression in the absence of IFN-γ, the difference reaching statistical significance (compared with both WT mice and *IL-12p40*−/− mice) in glomerular P-selectin. (C) No significant alteration in the expression of intracellular adhesion molecule 1 (ICAM-1) in glomeruli of the three groups of mice with EAG, but D shows significantly increased tubulointerstitial ICAM-1 expression in *IFN*-γ−/− mice compared with both WT and *IL-12p40*−/− mice. *$P < 0.05$ *versus* both WT and *IL-12p40*−/− mice; **$P < 0.05$ *versus IL-12p40*−/− mice and $P < 0.01$ *versus* WT mice (ANOVA).

Intrinsic renal cells were involved in amplification of this response (9). However, other studies using a mild model of injury showed that IFN-γ was protective (43), whereas further studies using *IFN*-γ*R*−/− mice have shown a pathogenetic effect that was modest (44) or even absent (45). Other roles of IFN-γ in the kidney include effects on mesangial cells, including an antiproliferative effect (46) and upregulation of mesangial cell FcR1 (47) as well potentially antifibrotic effects (48).

The mechanisms behind the protective effect of IFN-γ in this model and in other models of organ-specific autoimmunity are complex and remain incompletely understood. Factors implicated in IFN-γ's protective effect include suppression of autoreactive or alloreactive CD4+ or CD8+ cells by IFN-γ (27,49) and effects on M1/70+ cells (50). Our studies show that there is a significant T cell and macrophage infiltrate in some *IFN*-γ−/− mice. Relatively early (at least in this model) in the autoimmune process, dermal DTH responses to RBM collagens, including α3-α5(IV)NC1, are increased in *IFN*-γ−/− mice, and these responses can be suppressed by low-dose (10 units/d) rmIFN-γ, a dose chosen to approximate replacement therapy (51), rather than the higher "treatment" doses used in experimental models of infectious diseases (52) and in the treatment of chronic granulomatous disease in humans (53). Findings in the current studies of suppression of dermal DTH responses by IFN-γ in *IFN*-γ−/− mice are consistent with studies in collagen-induced arthritis, whereby systemic IFN-γ treatment inhibited disease (54). It is interesting that in collagen-induced arthritis, intralesional IFN-γ augments disease (55), suggesting differential local and systemic roles for this cytokine in organ-specific autoimmunity.

In addition to effects on dermal DTH, we found that *IFN*-γ−/− mice have increased proportions of CD4+ cells in draining lymph nodes. In this regard, our findings are congruent with studies that have shown increased T cell reactivity in the absence of IFN-γ in experimental autoimmune encephalitis (49,56). Although the hypothesis that IFN-γ is protective only in models in which FCA is used exists (50), in crescentic GN induced by planting foreign Ig that act as an antigen (autologous "anti-GBM" GN), we have found pathogenetic effects of IFN-γ in the both presence and the absence of FCA (7–9). We hypothesize that when glomerular injury does not require tolerance breaking and is severe, acute, and characterized by strong macrophage effector responses, IFN-γ is pathogenetic (7–9). When the immune response is organ specific, is autoimmune/allogeneic, and/or injury is less severe and more subtle, IFN-γ is protective (25,43,49). Last, in systemic autoimmunity, whereby tolerance is lost to ubiquitous self-antigens, IFN-γ is pathogenetic, partly, although not solely, because of the generation of pathogenetic autoantibodies (11–14).

In contrast to the complex role for endogenous IFN-γ in harmful immune responses, IL-12 has been shown to be patho-

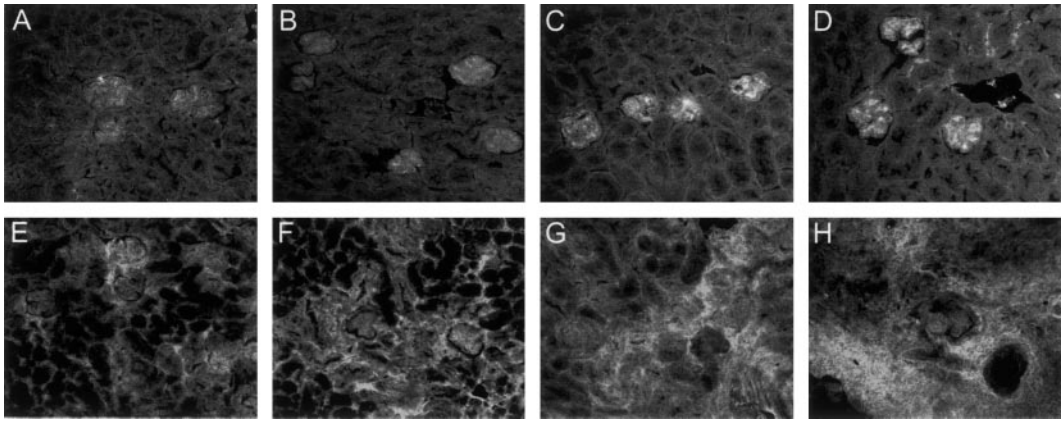

Figure 6. Immunofluorescent detection of adhesion molecule expression (P-selectin and ICAM-1) in EAG, showing in WT mice expression of P-selectin (A) and ICAM-1 (E) that was little different in the absence of IL-12p40 (P-selectin [B], ICAM-1 [F]). In *IFN*-γ−/− mice, increases in both P-selectin (C and D) and ICAM-1 (G and H; within the interstitium) were evident. The increased adhesion molecule expression observed was a feature of EAG in *IFN*-γ−/− mice regardless of the relative degree of injury (C and G show a less affected mouse; D and H show a more severely affected *IFN*-γ−/− mouse). Medium-power views using FITC (P-selectin) and PE (ICAM-1) as fluorochromes.

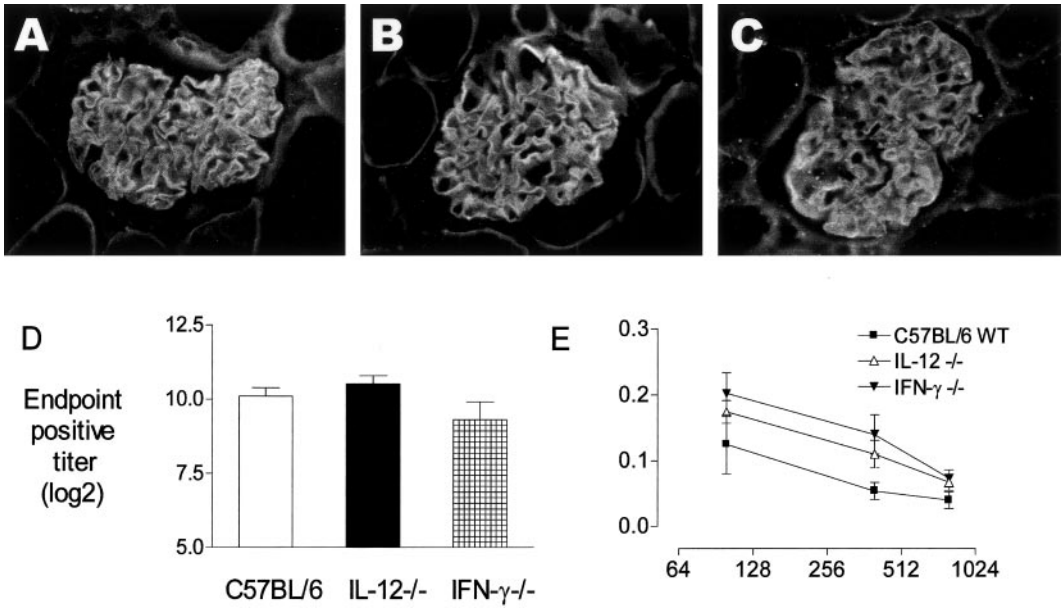

Figure 7. Autoantibodies in EAG. Linear staining of Ig was observed to a similar degree in C57BL/6 WT, *IL-12p40*−/−, and *IFN*-γ−/− mice (A through C); semiquantitative analysis in D. There was no significant difference in serum anti α3-α5(IV)NC1 antibody titers (E) between the three groups of mice.

Table 1. Serum and glomerular IgG subclasses in C57BL/6 WT, *IL-12p40*−/− and *IFN*-γ−/− mice with EAG[a]

	C57BL/6 WT	*IL-12p40*−/−	*IFN*-γ−/−
IgG1			
serum (OD_{405})	0.343 ± 0.081[b]	0.469 ± 0.092	0.503 ± 0.112
glomerular (0–3+)	2.2 ± 0.2	2.4 ± 0.2	1.5 ± 0.2[c]
IgG2a			
serum (OD_{405})	0.600 ± 0.183[b]	1.060 ± 0.030	0.221 ± 0.010[d]
glomerular (0–3+)	0.62 ± 0.12	0.61 ± 0.27	0.10 ± 0.10

[a] Sera were assessed by ELISA, and glomerular deposition was assessed by direct immunofluorescence (see Materials and Methods for details). Results are expressed as the OD_{405} mean ± SEM and the mean ± SEM of a semiquantitative score for each animal. WT, wild type mice; *IL-12p40*−/−, interleukin-12p40 deficient mice; *IFN*-γ−/−, IFN-γ deficient mice.
[b] Values for normal mouse sera were 0.098 ± 0.069 for IgG1 and 0.004 ± 0.002 for IgG2a.
[c] $P < 0.05$ *versus* C57BL/6.
[d] $P < 0.01$ *versus IL-12p40*−/− mice.

Table 2. Renal function and urinary protein excretion in C57BL/6 WT, *IL-12p40*−/−, and *IFN*-γ−/− mice with EAG[a]

	Serum Creatinine (μmol/L)	Urinary Protein (mg/24 h)
Normal mice no GN	10.9 ± 1.0	1.2 ± 0.3
C57BL/6 WT GN	12.9 ± 1.0	2.6 ± 0.3[b]
IL-12p40−/− GN	10.9 ± 1.2	1.4 ± 0.1
IFN-γ−/− GN	27.3 ± 11.2	1.4 ± 0.4[c]

[a] Results are expressed as the mean ± SEM. EAG, experimental autoimmune glomerulonephritis; GN, glomerulonephritis.
[b] $P < 0.01$ *versus* other three groups (ANOVA).
[c] Results in this group exclude two oligoanuric mice.

genetic in organ-specific autoimmunity, using similar models (21,22). Although in the current studies some of the results in *IL-12p40*−/− mice did not reach statistical significance, we believe because of the relatively mild nature of the injury in WT mice that overall IL-12 was certainly not protective and has a pathogenetic effect. These findings are consistent with studies of endogenous IL-12 in organ specific autoimmunity. The recent discovery of a new cytokine called IL-23, which

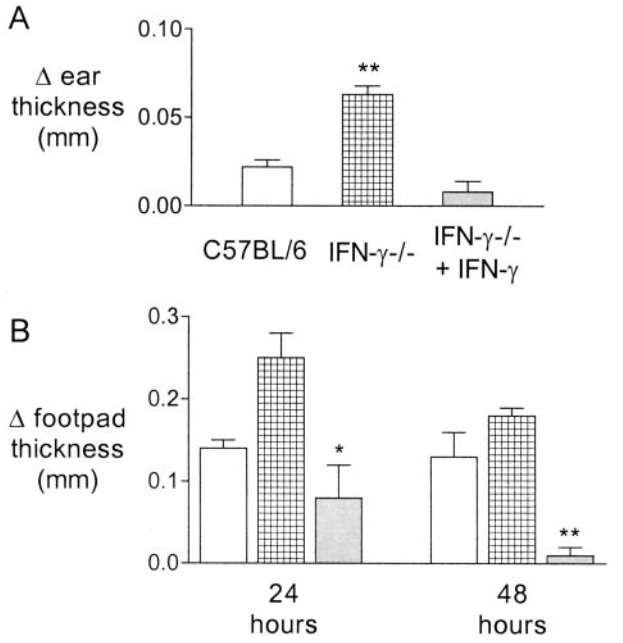

Figure 8. Dermal delayed-type hypersensitivity (DTH) responses in mice that were sensitized to α3-α5(IV)NC1. WT mice ($n = 4$) received an injection of vehicle alone (□), *IFN*-γ−/− mice ($n = 5$) received an injection of vehicle alone (▦), and *IFN*-γ−/− mice ($n = 5$) were given daily recombinant murine IFN-γ (rmIFN-γ; ■) from sensitization. Responses to collagenase solubilized murine renal basement membrane 48 h after challenge (A) showed an increase in *IFN*-γ−/− mice that was reversed by daily rmIFN-γ intraperitoneally. Responses to bovine α3-α5(IV)NC1 at both 24 and 48 h showed a similar pattern with suppression of DTH responses by rmIFN-γ, although results in *IFN*-γ−/− mice that were given vehicle alone did not reach statistical significance compared with WT mice. *$P < 0.05$ *versus IFN*-γ−/− mice; **$P < 0.001$ *versus* both other groups at the same time point (ANOVA).

Table 3. CD4+ cells, CD8+ cells, proliferation, and apoptosis in draining lymph nodes four weeks after immunization of WT and *IFN*-γ−/− mice[a]

	WT Mice	*IFN*-γ−/− Mice
CD4+ cells	20.8 ± 1.6	25.1 ± 0.8[b]
CD8+ cells	21.0 ± 1.4	21.0 ± 0.7
CD4+/BrdU+ cells	1.16 ± 0.06	1.53 ± 0.14[b]
CD8+/BrdU+ cells	0.43 ± 0.08	0.51 ± 0.16
Apoptotic cells	9.5 ± 0.7	10.2 ± 0.7

[a] Results are expressed in % of live cells, as the mean ± SEM.
[b] $P = 0.038$ *versus* WT (*t* test).

shares the IL-12p40 chain, means that the *IL-12p40*−/− mice used in the current studies are deficient in both IL-12 and IL-23 (4). Although the full range of IL-23's functions remains to be elucidated, it has stimulatory effects on memory T cells and on macrophages (4). Further studies will define the relationships between IL-12 and IL-23 in autoimmune disease. However, this new information raises the possibility that targeting the common chain of both IL-12 and IL-23 may inhibit the generation and maintenance of pathogenetic Th1 responses.

In summary, these studies demonstrate that in renal organ–specific autoimmunity, IFN-γ is protective, probably at the level of the generation of CD4+ cell–derived autoimmune process, whereas IL-12 is likely to be pathogenetic. They highlight the complex roles for IFN-γ in nephritogenic immune responses leading to GN.

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