IL-18 Has IL-12-Independent Effects in Delayed-Type Hypersensitivity: Studies in Cell-Mediated Crescentic Glomerulonephritis

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*J Immunol* 2000; 165:4649-4657;
doi: 10.4049/jimmunol.165.8.4649

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Interleukin-18 (formerly known as IFN-γ-inducing factor) was described and cloned in 1995 (1). Mononuclear cells and APC produce IL-18 during interactions with cognate T cells (2). There is growing evidence to support a role for IL-18 in the expression of Th1 responses (1, 3–6). IL-18 induces the production of IFN-γ and other proinflammatory cytokines. In an established Th1 clone, the levels of IFN-γ induced by IL-18 were, in fact, higher than those induced by IL-12, suggesting a potential role for IL-18 in the maintenance of Th1 responses (1). IL-18 acts in synergy with IL-12 to induce IFN-γ production in CD4+ cells (3), and both IL-12 and IL-18 are necessary for the full expression of Th1 responses. Further support for this synergistic effect comes from the observation that IL-12 and IL-18 use different signaling pathways in inducing IFN-γ production (7). IL-18−/− mice are deficient in IFN-γ production, examined by ex vivo IFN-γ production by CD4+ cells in response to established Th1-inducing stimuli (5). The extent of their deficiencies is comparable to that exhibited by IL-12−/− mice.

It is not clear whether IL-18 alone is capable of inducing Th1 responses (of which the delayed-type hypersensitivity (DTH) response is the classical example (8)). However, most work indicates that IL-18 cannot induce a Th1 response in the absence of IL-12. Culturing naive cells from IL-12−/− mice in the presence of IL-18 does not result in the development of IFN-γ-producing Th1 effector cells (5). This supports other in vitro data that implicate IL-18 as a cofactor rather than an initiator of Th1 development (2, 9).

The recruitment of leukocytes in DTH responses involves tethering and migration of cells into the target organ. Adhesion molecules and chemokines are important in the effector phase of DTH responses (10–13). IL-18 has the potential to induce the expression and production of adhesion molecules and chemokines, which could alter the expression and magnitude of the DTH response (14, 15). In particular, IL-18 has been shown to induce ICAM-1 expression on a human myelomonocytic line, an effect that is IFN-γ independent (14).

The potential for IL-12-independent actions of IL-18 in inducing and maintaining Th1-driven DTH-like responses in vivo was studied in experimental crescentic anti-glomerular basement membrane (GBM) glomerulonephritis (GN), a model of Th1-directed DTH-mediated immune injury. Crescentic GN is the most aggressive and damaging form of GN (16). In humans, crescent formation has features of DTH, with the consistent observation of T cells, macrophages, tissue factor, and fibrin in the glomerular lesion (17, 18). In the murine model used in the current studies, glomerular crescent formation is effector CD4+ (but not CD8+) dependent and is independent of the presence of autologous Ab

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IL-18 (formerly known as IFN-γ-inducing factor) enhances Th1 responses via effects that are thought to be dependent on and synergistic with IL-12. The potential for IL-18 to exert IL-12-independent effects in delayed-type hypersensitivity (DTH) responses was studied in a model of Th1-directed, DTH-mediated crescentic glomerulonephritis induced by planting an Ag in glomeruli of sensitized mice as well as in cutaneous DTH. Sensitized genetically normal (IL-12−/−) mice developed proteinuria and crescentic glomerulonephritis with a glomerular influx of DTH effectors (CD4+ T cells, macrophages, and fibrin deposition) in response to the planted glomerular Ag. IL-12p40-deficient (IL-12−/−) mice showed significant reductions in crescent formation, proteinuria, and glomerular DTH effectors. Administration of IL-18 to IL-12−/− mice restored the development of histological (including effectors of DTH) and functional glomerular injury in IL-12−/− mice to levels equivalent to those in IL-12+/+ mice. IL-18 administration to IL-12−/− mice increased glomerular ICAM-1 protein expression, but did not restore Ag-stimulated splenocyte IFN-γ, GM-CSF, IL-2, or TNF-α production. Sensitized IL-12−/− mice also developed cutaneous DTH following intradermal challenge with the nephritogenic Ag. Cutaneous DTH was inhibited in IL-12−/− mice, but was restored by administration of IL-18. IL-12−/− mice given IL-18 developed augmented injury, with enhanced glomerular and cutaneous DTH, demonstrating the synergistic effects of IL-18 and IL-12 in DTH responses. These studies demonstrate that even in the absence of IL-12, IL-18 can induce in vivo DTH responses and up-regulate ICAM-1 without inducing IFN-γ, GM-CSF, or TNF-α production. The Journal of Immunology, 2000, 165: 4649–4657.

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Received for publication November 24, 1999. Accepted for publication July 26, 2000.

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3 Abbreviations used in this paper: DTH, delayed-type hypersensitivity; c/ges, cells per glomerular cross-section; Ctrl, control group; GN, glomerulonephritis; GBM, glomerular basement membrane; PAS, periodic acid-Schiff’s reagent; PLP, periodate lysine paraformaldehyde.

This work was supported by grants from the National Health and Medical Research Council of Australia and the Australian Kidney Foundation. P.G.T. is a National Health and Medical Research Council Senior Research Fellow, and A.R.K. was supported by a National Health and Medical Research Council Medical Postgraduate Scholarship.
Materials and Methods

Induction of anti-GM glomerulonephritis and experimental design

Sheep anti-mouse GBM glubulin was prepared as previously described (30). Eight- to 10-week old male C57BL/6 mice (Monash University Animal Services, Melbourne, Australia) were assigned to groups that received IL-18 (IL-12 Ctrl; n = 9) or vehicle alone (IL-12 Ctrl; n = 9). Two mice that received IL-18 had to be killed on days 5 and 6 of GN because of poor condition with nephrotic syndrome: these mice had ascites and peripheral edema and are included in analyses of glomerular injury. IL-12 p40-deficient mice (31) (IL-12/-/- mice; obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at Monash University) received either IL-18 (IL-12 Ctrl; n = 9) or vehicle alone (IL-12 Ctrl; n = 6). Glomerulonephritis was induced by sensitizing mice by s.c. injection of a total of 100 μg of sheep glubulin in 100 μl of CFA in divided doses in each flank (day 0). Ten days later (day 0), GN was initiated with 2.5 mg of sheep anti-mouse GM. Glomerular injury and immune responses were assessed after a further 7 days (day 7). IL-18 was diluted in sterile, filtered 0.1% BSA in PBS and administered as follows: 1 μg in 200 μl of 0.1% BSA/PBS was injected i.p. on each of days −10, −9, and −8. A further 0.5 μg in 100 μl of vehicle was injected i.p. daily on days 1−5 of disease. Control mice received equivalent volumes of 0.1% BSA/PBS alone. An additional three groups of mice were studied to assess the effect of IL-18 on glomeruli of mice without GN: normal mice without GN (n = 6), mice sensitized with sheep glubulin but not challenged that received IL-18 in the same protocol as mice with GN (n = 4), and mice sensitized with sheep glubulin but not challenged that were injected with vehicle alone (n = 3). Results are expressed as the mean ± SEM. The statistical significance of differences between groups was determined by the Mann-Whitney U test.

Assessment of renal injury

Kidney tissue was fixed in Bouin’s fixative and embedded in paraffin, and 3-μm tissue sections were cut and stained with periodic acid-Schiff’s reagent (PAS). A glomerulus was considered to exhibit crescent formation when two or more layers of cells were observed in Bowman’s space. A minimum of 50 glomeruli were assessed to determine the crescent score for each animal. For deposition of PAS material in glomeruli, a 0–4+ scale was used as follows: 0, no accumulation of PAS material; 1, minor accumulation to cover up to 25% of the glomerular tuft; 2, PAS material in 25–50% of the tuft involved; and 4 ≤ 75% involved, with relative glomerular hypocellularity. Assessments were performed on coded slides. Urinary protein concentrations were determined by the method of Bradford (32) on 24-h urine collections from each mouse from days 1 and 2 of disease.

Immunohistochemistry for T cells, macrophages, NK cells, and ICAM-1

Kidney or footpad tissue was fixed in paraformaldehyde (PLP) for 4 h, washed in 7% sucrose solution, then frozen in liquid nitrogen. Tissue sections (6 μm) were stained to demonstrate CD4+ cells, CD8+ cells, and macrophages using a three-layer immunoperoxidase technique, as previously described (33–35). The primary mAbs were GK1.5 (anti-mouse CD4, American Type Culture Collection, Manassas, VA), 53-6.7 (anti-mouse CD8, American Type Culture Collection), and M1/70 (anti-mouse Mac-1, American Type Culture Collection). For the detection of NK cells (CD11c+) tissues were fixed with 10% formalin in 0.1 M sodium phosphate/BSA/PBS. A rabbit anti-mouse/ rat asialo-GM1 Ab (Cedarlane, Hornby, Canada) (15) was applied for 1 h (dilution, 1/200), followed by a swine anti-rabbit HRP-conjugated Ab (Dako, Carpinteria, CA; 1/100). To quantify glomerular CD4+ T cells, macrophages, and NK cells, a minimum of 20 glomeruli was assessed per animal, and results were expressed as cells per glomerular cross-sectional area (μm2), using the Leica QCapture Pro software (Leica Microsystems, Wetzlar, Germany). A minimum of 20 glomeruli were scored in each mouse, using a scale of 0–4+ on the basis of the intensity of staining, with 0 being an intensity similar to that seen in normal mice and 4 being heavy staining over the entire glomerular tuft.

Glomerular deposition of fibrin, sheep globulin, and mouse Ig

Tissue was embedded in Optimal Cutting Temperature Compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at −70°C. Immunofluorescence was performed on 4-μm cryostat cut tissue. Glomerular fibrin deposition was detected on a minimum of 30 glomeruli/mouse using an FITC-anti-mouse fibrin/fibrinogen polyclonal Ab (Nordic Immunological Laboratories, Berks, U.K.) at a dilution of 1/50. Fibrin deposition was scored semiquantitatively (0–3+) as follows: 0, no fibrin deposition; 1, fibrin occupying up to one-third of the glomerular cross-sectional area; 2, fibrin occupying one-third to two-thirds of the glomerulus; and 3, greater than two-thirds of the glomerulus. Deposition of sheep globulin was evaluated using FITC-rabbit anti-sheep Ig (Nordic Immunological Laboratories) at a dilution of 1/1,000, and sections were scored 0–3+ on the basis of fluorescence intensity. There were no differences in the deposition of the nephritogenic Ag, sheep globulin, in the glomeruli of IL-12 Ctrl mice (IL-12 Ctrl, 1.7 ± 0.2; IL-12 Ctrl + IL-18, 1.9 ± 0.3) or IL-12/-/- mice (IL-12 Ctrl, 1.8 ± 0.2; IL-12 Ctrl + IL-18, 2.2 ± 0.3). Two methods were used for assessment of mouse Ig (FTTC-anti-sheep anti-Ig: Silenus, Hawthorn, Australia) in glomeruli. Firstly, fluorescence intensity was assessed semiquantitatively (0–3+) using a dilution of 1/150 for mouse Ig. Secondly, serial dilutions of these Abs were made to determine the end-point positive titer in each animal, and results were expressed as the log2 of this end-point titer.

Cutaneous DTH to sheep globulin

To assess the cutaneous DTH response to sheep globulin, mice were challenged 24 h before the end of the experiment by intradermal injection of sheep globulin (500 μg in 50 μl of PBS) into the plantar surface of a hindfoot. Four mice from each of the IL-12 Ctrl group, and all six mice from each of the IL-12 Ctrl groups were challenged with sheep globulin. The same dose and volume of an irrelevant Ag (horse globulin) were injected into the opposite footpad as a control. DTH was assessed 24 h later in a blinded fashion by measuring the difference in thickness between sheep globulin and horse globulin-injected foot pads in each mouse using a micrometer. Footpads were dissected and fixed in PLP as described above.

Measurement of cytokine production by Ag-stimulated splenocytes

Spleens from diseased mice (IL-12 Ctrl, n = 9; IL-12 Ctrl, n = 6; IL-12 Ctrl + IL-18, n = 6; IL-12 Ctrl + IL-18, n = 6) were removed aseptically on day 7 and placed in DMEM/5% FCS medium. Single-cell suspensions were prepared by gently teasing tissue apart. Erythrocytes were lysed by incubation in Boyle’s solution (0.17 M Tris/0.16 M ammonium chloride) for 1 min at 37°C. Cell suspensions were washed in DMEM/5% FCS. Splenocytes (4 × 106 cells/ml, in DMEM/10% FCS) were incubated
for 72 h at 37°C in 5% CO₂ in 48-well tissue culture plates (1 ml/well) with protein G-purified normal sheep IgG at a concentration of 10 μg/ml. IFN-γ and IL-4 in culture supernatants were measured by ELISA as previously described (23). The mAbs used were rat anti-mouse IFN-γ (R46A2 [PharMingen, San Diego, CA] and biotinylated XMG1.2 [PharMingen]) for the IFN-γ ELISA and rat anti-mouse IL-4 (1 B11; American Type Culture Collection) and biotinylated BVD6 (DNAX Research Institute, Palo Alto, CA) for the IL-4 ELISA. IL-2 was measured using a similar protocol, with the mAbs being rat anti-mouse IL-2 (JES6-1A12 [DNAX] and biotinylated JES6-5H4 [DNAX]). TNF-α and GM-CSF were measured using commercially available immunoassays (for TNF-α: DuoSet, Genzyme, Cambridge, MA; for GM-CSF: Quantikine M, R&D Systems, Minneapolis, MN). The sensitivities of the assays were as follows: IFN-γ, 48 pg/ml; IL-4, 31 pg/ml; IL-2, 15 pg/ml; TNF-α, 28 pg/ml; and GM-CSF, 1 pg/ml.

Circulating mouse anti-sheep globulin Ig, IgG1, and IgG2a

Titers of total mouse anti-sheep globulin Ab and anti-human globulin IgG1 and IgG2a were measured by ELISA in serum collected from each mouse at the end of each experiment as previously described (30). Samples were incubated at dilutions of 1/500 for total Ig and IgG1 and 1/50 for IgG2a. Bound mouse Ig was detected with HRP-conjugated Abs: sheep anti-mouse Ig (Amersham, Little Chalfont, U.K.; 1/2000) for total Ig and HRP-conjugated goat anti-mouse IgG1 or IgG2a Ab (Southern Biotechnology Associates, Birmingham, AL; 1/4000). Sera from six nonimmunized mice were tested to provide normal controls.

Results

IL-18 restores DTH responses in glomeruli of IL-12−/− mice and enhances responses in IL-12+/− mice

Vehicle-treated C57BL/6 IL-12−/− mice with GN showed an influx of the effectors of DTH (CD4+ T cells, macrophages, and fibrin deposition in glomeruli; Fig. 1). Vehicle-treated IL-12−/− mice with GN developed a lesser degree of injury, with reduced glomerular CD4+ T cell and macrophage accumulation and reduced fibrin deposition (Fig. 1; CD4+ cells, p < 0.05; macrophages and fibrin, p < 0.01). The administration of IL-18 to IL-12−/− mice restored the numbers of infiltrating CD4+ T cells and macrophages and the extent of glomerular fibrin deposition to values comparable with those of IL-12-intact animals. IL-12+/− mice with GN given IL-18 developed increased accumulation of the effectors of DTH (CD4+ T cells, macrophages, and fibrin) in glomeruli compared with vehicle-treated IL-12−/− mice (Fig. 1; p < 0.01), suggesting that IL-18 is synergistic with IL-12 in enhancing glomerular injury. CD8+ cells were present in glomeruli of IL-12+/− mice with GN (0.31 ± 0.05 c/gcs) and were reduced in the absence of IL-12 (IL-12−/− Ctrl, 0.16 ± 0.06 c/gcs; p = 0.04). However, IL-18 administration did not increase glomerular CD8+ ~

Table 1: Glomerular findings in genetically normal (IL-12+/−) mice without GN

<table>
<thead>
<tr>
<th>Total Cells</th>
<th>CD4+ Cells</th>
<th>Macrophages</th>
<th>CD8+ Cells</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse</td>
<td>35 ± 1</td>
<td>0.02 ± 0.01</td>
<td>0.21 ± 0.04</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>No GN + vehicle</td>
<td>36 ± 1</td>
<td>0.02 ± 0.02</td>
<td>0.17 ± 0.06</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>No GN + IL-18</td>
<td>33 ± 2</td>
<td>0.02 ± 0.01</td>
<td>0.11 ± 0.03</td>
<td>0.08 ± 0.03</td>
</tr>
</tbody>
</table>

* Injection of vehicle alone or IL-18 into sensitized IL-12+/− mice that were sensitized but not challenged did not result in glomerular injury. Values are expressed as the mean ± SEM of cells per glomerular cross-section, apart from ICAM-1 expression that was scored 0–4+ as described in the Materials and Methods. At least 20 glomeruli from each animal were assessed. No NK cells were detected in glomeruli of normal mice.
cell number in either IL-12-intact or IL-12-deficient mice (IL-12<sup>-/-</sup> + IL-18, 0.18 ± 0.04 c/gcs; IL-12<sup>+/+</sup> + IL-18, 0.31 ± 0.06 c/gcs). Small numbers of NK cells were present in glomeruli of IL-12<sup>-/-</sup> mice with GN (0.11 ± 0.03 c/gcs). Genetic deficiency of IL-12 or the administration of IL-18 to either IL-12<sup>+/+</sup> or IL-12<sup>-/-</sup> mice did not significantly alter the degree of NK cell infiltrate (IL-12<sup>-/-</sup> Ctrl, 0.12 ± 0.02 c/gcs; IL-12<sup>-/-</sup> + IL-18, 0.17 ± 0.05 c/gcs; IL-12<sup>+/+</sup> + IL-18, 0.14 ± 0.04 c/gcs).

Changes in histological indexes of glomerular injury and proteinuria paralleled the changes in DTH effectors seen in the three groups. Compared with IL-12<sup>+/+</sup> mice without GN (Figs. 2 and 3, A and B), control treated IL-12<sup>-/-</sup> mice had developed proliferative GN with accumulation of PAS<sup>+</sup> material and crescent formation on day 7 of GN and significant proteinuria (Figs. 2 and 3, D and E). Deficiency of IL-12 (in vehicle-treated IL-12<sup>-/-</sup> mice) significantly attenuated glomerular injury and proteinuria (Figs. 2 and 3, G and H).

FIGURE 3. Histological features of glomerular injury and ICAM-1 expression in glomeruli. A and B, Low and high power views of kidneys of IL-12<sup>+/+</sup> mice without GN. Vehicle-treated IL-12<sup>+/+</sup> (IL-12<sup>+/+</sup> Ctrl) mice developed proliferative GN with accumulation of PAS<sup>+</sup> material in glomeruli (D and E) on day 7 of disease. Light ICAM-1 staining was observed in glomeruli of normal mice without GN (C). ICAM-1 expression was increased in IL-12<sup>+/+</sup> mice with GN, seen segmentally in glomeruli, in some proximal tubules, and in periglomerular regions (F). Control treated IL-12<sup>-/-</sup> mice (IL-12<sup>-/-</sup> Ctrl) developed mild GN with less proliferation and accumulation of PAS<sup>+</sup> material (G and H). ICAM-1 expression was not significantly altered by IL-12 deficiency (I). Administration of IL-18 to IL-12<sup>-/-</sup> mice resulted in restoration of the glomerular injury to levels comparable with those in IL-12<sup>-/-</sup> Ctrl mice (J and K). ICAM-1 expression was increased compared with that in IL-12<sup>-/-</sup> Ctrl mice (L). IL-12<sup>+/+</sup> mice given IL-18 (IL-12<sup>+/+</sup> + IL-18) developed a more severe lesion than vehicle-treated IL-12<sup>+/+</sup> mice (M and N), with significantly increased deposition of PAS<sup>+</sup> material, increased glomerular crescent formation (arrows), and an increased interstitial infiltrate. ICAM-1 expression was increased in both glomeruli and interstitium (O). For A, B, D, E, G, H, J, K, M, and N, paraffin sections (3 μm) were stained with PAS and counterstained with hematoxylin (A, D, G, J, and M, low power; B, E, H, K, and N, high power). For C, F, I, L, and O, medium power views of 6-μm frozen sections were stained for ICAM-1 using an mAb-based immunoperoxidase technique and were counterstained with hematoxylin.
and IL-18 has been shown to exacerbate renal injury in mice with glomerulonephritis (GN) (Fig. 1 and 3, J and K). However, administration of IL-18 to IL-12−/− animals resulted in an increase in glomerular injury such that glomerular crescent formation, the deposition of PAS+ material in glomeruli, and proteinuria were similar to those observed in vehicle-treated IL-12−/− mice (Figs. 2 and 3, G and H). Consistent with these findings of increased cellular effectors in glomeruli of genetically normal mice given IL-18, renal injury was increased in IL-12−/− mice given IL-18 (Fig. 2). Glomerular crescent formation and deposition of PAS+ material in glomeruli were significantly increased, and there was a trend toward increased proteinuria (p = 0.08). In the absence of GN, IL-18 administration or the injection of vehicle alone did not alter glomerular histology, total glomerular cell number, or the number of CD4+ cells or macrophages in glomeruli. NK cells were not detected in glomeruli of mice without GN (Table I).

**ICAM-1 expression in glomeruli is increased in IL-12−/− mice by rmIL-18 administration**

Immunostaining for ICAM-1 in IL-12−/− mice with GN revealed moderate staining within glomeruli, in some proximal tubules and periglomerular regions (Fig. 3F), and in the endothelium of interstitial blood vessels compared with light staining in some glomeruli and tubules in IL-12+/+ mice without GN (Fig. 3C). ICAM-1 expression in vehicle-treated IL-12−/− mice was not statistically significantly diminished on semiquantitative assessment using a score of 0–4+ for at least 20 glomeruli/mouse (IL-12+/+ Ctrl, 1.1 ± 0.1; IL-12−/− Ctrl, 0.8 ± 0.1; Fig. 3J). Administration of rmIL-18 to mice deficient in IL-12 enhanced the expression of ICAM-1 protein in glomeruli of IL-12−/− mice (IL-12−/− Ctrl, 0.8 ± 0.1; IL-12−/− + IL-18, 1.4 ± 0.1; p < 0.005; p, p, p, 0.05 compared with IL-12+/+ Ctrl mice.

**FIGURE 4.** Ag-specific dermal DTH in mice with GN. Vehicle-treated IL-12+/+ mice (IL-12+/+ Ctrl) developed cutaneous DTH to sheep globulin, measured 24 h after challenge. DTH was decreased in IL-12−/− Ctrl mice and was restored by IL-18 administration to IL-12−/− mice. IL-12++ mice given rmIL-18 developed increased DTH compared with IL-12++ Ctrl mice. *, p < 0.05 compared with IL-12++ Ctrl mice.

**FIGURE 5.** Immunohistological feature of cutaneous DTH. Genetically normal, control treated mice sensitized to sheep globulin (IL-12+/+ Ctrl) developed a CD4+ cell and macrophage infiltrate in footpads when challenged with sheep globulin (A, CD4+ cells; B, macrophages). In the absence of IL-12, this infiltrate was reduced (D, CD4+ cells; E, macrophages). Administration of rmIL-18 to IL-12−/− mice restored the degree of CD4+ and macrophage influx (G, CD4+ cells; H, macrophages). Administering rmIL-18 to IL-12−/− mice enhanced CD4+ and macrophage infiltration (J, CD4+ cells; K, macrophages). A relatively minor NK cell infiltrate was detected that did not significantly vary in groups of mice (C, F, I, and L). Photomicrographs are of low power views of PLP-fixed sections were stained by three-layer immunoperoxidase and counterstained with hematoxylin.
Fig. 3L), demonstrating that IL-18 can increase ICAM-1 in the absence of IL-12. Administering IL-18 to IL-12−/− mice with GN further increased glomerular ICAM-1 staining (IL-12−/− Ctrl, 1.1 ± 0.1; IL-12−/− + IL-18, 1.6 ± 0.1; p < 0.005; Fig. 3O). Administering IL-18 or vehicle alone to mice without GN did not increase glomerular ICAM-1 expression above that seen in normal, untreated mice without GN (normal mice, 0.12 ± 0.04; no GN + vehicle, 0.10 ± 0.05; no GN + IL-18, 0.14 ± 0.05).

IL-18 restores dermal DTH responses in IL-12−/− mice and enhances responses in IL-12+/+ mice

IL-12+/+ mice treated with vehicle alone showed Ag-specific dermal DTH (Fig. 4), with a prominent influx of CD4+ T cells and macrophages (Figs. 5, A and B). In vehicle-treated IL-12−/− mice, DTH was significantly reduced, and a lesser influx of T cells and macrophages was observed (Figs. 4 and 5, D and E). In IL-12−/− mice given IL-18, Ag-specific skin swelling was present to the same extent as in vehicle-treated IL-12−/− animals, with a similar leukocytic cell influx as that in genetically normal mice (Figs. 4 and 5, G and H). Dermal DTH responses in IL-12−/− mice were increased by administration of rmIL-18 (Fig. 4), with increases in CD4+ T cells and macrophages (Fig. 5, J and K). There were no differences between any groups in the relatively minor NK cell infiltrates observed in the footpads of mice (Fig. 5, C, F, I, and L).

Humoral immune responses in mice developing GN

All mice with GN developed Ag-specific Ab responses that were measured in serum drawn at the end of experiments (Table II). Total serum Ag-specific Ig and IgG2a titers were unchanged in IL-12−/− mice, although IgG1 titers were significantly increased. IL-12−/− mice given IL-18 had higher titers of total Ig and both IgG subclasses than vehicle-treated IL-12−/− mice. IgG2a was increased in IL-12−/− mice by the administration of IL-18 (p = 0.04). There were no significant changes in serum Ag-specific Ig, IgG1, or IgG2a titers in IL-12+/+ mice given IL-18. Glomerular deposition of autologous Ab (measured either by end-point positive titer measurements or semiquantitative scoring of fluorescence intensity) was not affected by either IL-12p40 gene deletion or IL-18 administration, suggesting that the changes in renal injury observed were not due to alterations in Ab deposition (Table III).

Cytokine production by Ag-stimulated splenocytes from mice with GN

IFN-γ, GM-CSF, TNF-α, and IL-2 were detected in the supernatants of splenocytes isolated from IL-12−/− mice with GN (Fig. 6). The production of these Th1 proinflammatory cytokines was reduced in IL-12−/− mice. Treatment with IL-18 did not restore Ag-stimulated splenocyte production of IFN-γ, GM-CSF, TNF-α, or IL-2 in IL-12−/− mice. IL-4 was detected in all groups of mice studied. There were no significant alterations in IL-4 production in either vehicle- or IL-18-treated IL-12−/− mice. IL-4 was not measurable (<31 pg/ml) in three of nine vehicle-treated IL-12−/− mice, three of six vehicle-treated IL-12+/+ mice, four of six IL-12−/− IL-18-treated mice, and two of six IL-12−/− mice given IL-18. However, Ag-stimulated splenocyte cytokine production was modified by IL-18 administration. As expected, the production of GM-CSF was increased, and there was a trend toward increased IFN-γ production (p = 0.11) after 72 h of culture (Fig. 6). TNF-α production was unchanged, and IL-2 levels fell. It is possible that the IL-2 results may relate to increased expression of the IL-2R, as is the case with mice injected with IL-12 (22, 31). IL-4 levels were not affected, consistent with previous data on the actions of IL-18 (2).

Discussion

The role of IL-18 is being increasingly recognized in the initiation and maintenance of Th1 responses, of which DTH is a classical example. It is clear that IL-18 serves as a cofactor in effector Th1 responses and in the induction of IFN-γ production by Th1 CD4+ T cells. The effects of IL-18 are synergistic with those of IL-12. IL-12 has been considered to be pivotal to the initiation of Th1 responses, and most in vitro work suggests that IL-12 is required to establish a Th1-predominant response. Moreover, IL-12 does induce Th1 cells in the absence of IL-18 (5). The ability of IL-18 to facilitate Th1 responses in vivo in the absence of IL-12 is less well defined and has not been studied at the tissue level in models of Th1-dependent injury. While some conflicting data exist, overall consideration of in vitro data suggests that IL-18 by itself is insufficient to induce effector Th1 responses when assessed by cytokine production by CD4+ cells (2, 5, 9).

Although the actions of IL-18 have been extensively studied in vitro and to some degree in immune responses where noncognate mechanisms play a major role, there have been few studies in Th1-dependent cognate immune responses. In particular, it is not known whether IL-18 can increase DTH or Th1 responses in vivo independent of IL-12. To address this question, IL-18 was administered to IL-12−/− C57BL/6 mice in two models of DTH: Ag-

### Table II. Ag-specific serum Ig, IgG1, and IgG2a titers in mice with GN

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Ig</th>
<th>IgG1</th>
<th>IgG2a</th>
</tr>
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<tbody>
<tr>
<td>Normal mouse</td>
<td>0.017 ± 0.003</td>
<td>0.004 ± 0.002</td>
<td>0.041 ± 0.016</td>
</tr>
<tr>
<td>IL-12−/− Ctrl</td>
<td>1.250 ± 0.114</td>
<td>0.583 ± 0.057</td>
<td>0.167 ± 0.045</td>
</tr>
<tr>
<td>IL-12−/− Ctrl</td>
<td>1.385 ± 0.092</td>
<td>0.805 ± 0.062b</td>
<td>0.159 ± 0.040</td>
</tr>
<tr>
<td>IL-12−/− + IL-18</td>
<td>1.768 ± 0.143b</td>
<td>0.913 ± 0.046b</td>
<td>0.368 ± 0.072b</td>
</tr>
<tr>
<td>IL-12−/− + IL-18</td>
<td>1.551 ± 0.143</td>
<td>0.724 ± 0.045</td>
<td>0.309 ± 0.081</td>
</tr>
</tbody>
</table>

a Serum sheep globulin-specific total Ig, IgG1, and IgG2a were measured by ELISA on serum drawn at the end of experiments (day 7 of GN) in IL-12-intact (IL-12−/−) and -deficient (IL-12−/−) mice treated either with vehicle alone (Ctrl) or rmIL-18. Sera from six nonimmunized mice were tested as negative controls. Results are expressed as the mean OD405 ± SEM at a dilution of 1/5000 for Ig and IgG1 and 1/500 for IgG2a.

b p < 0.05 compared with IL-12−/− Ctrl mice.

### Table III. Deposition of autologous Ab in glomeruli of mice developing GN

| Condition       | End Point Positive Titer (log2) | Fluorescence Intensity (0–3+)
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>IL-12−/− Ctrl</td>
<td>10.7 ± 0.4</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>IL-12−/− Ctrl</td>
<td>10.6 ± 0.2</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>IL-12−/− + IL-18</td>
<td>11.0 ± 0.4</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>IL-12−/− + IL-18</td>
<td>10.9 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Deposition of autologous Ab in glomeruli of mice was unaltered by IL-18 administration or IL-12 deficiency 7 days after initiation of GN. Results are expressed as the mean ± SEM of the log2 of the end point positive titer and as the mean ± SEM of the semiquantitative score of immunofluorescence (Ab dilution 1/150) for each animal.
FIGURE 6. Cytokine production by Ag-stimulated splenocytes from mice developing GN. Cytokine production was measured in culture supernatants (4 x 10^6 cells/ml) at 72 h by ELISA. IFN-γ, GM-CSF, TNF-α, and IL-2 levels were lower in the absence of IL-12. Administration of rIL-18 to IL-12−/− mice (in vivo) did not restore cytokine production. Administration of rIL-18 to IL-12−/+ mice increased GM-CSF levels and resulted in a trend toward increased IFN-γ production (p = 0.11). IL-4 production was not significantly altered in any group, although there was a trend to reduced IL-4 levels in supernatants from IL-12−/− mice in both the control and IL-18-treated groups. *, p = 0.02; **, p < 0.005 (compared with IL-12−/+ Ctrl mice).

Specific dermal DTH in sensitized mice and experimental proliferative and crescentic GN (accelerated anti-GBM GN). Dermal DTH is considered to be a classical Th1 response. Recent studies have characterized experimental crescentic anti-GBM GN as akin to DTH in the glomerulus and directed by the Th1 subset. Crescent formation and the influx of CD4+ cells and macrophages are effector CD4+ and IL-12- and IFN-γ-dependent (19, 22, 30), while crescentic GN can be attenuated by IL-4 and/or IL-10 (23, 24).

Consistent with earlier published work on the role of endogenous IL-12 in this model (22), mice genetically deficient in IL-12 developed a lesser degree of renal injury and DTH effectors in glomeruli compared with genetically normal mice. While clearly diminished in the absence of IL-12, renal injury was still present, as evidenced by glomerular cellular proliferation, abnormal numbers of T cells and macrophages, autologous Ab deposition, and abnormal proteinuria. Furthermore, skin DTH was reduced in IL-12−/− mice, with reduced footpad swelling and a diminution in the degree of CD4+ and macrophage infiltration. In this system the absence of IL-12 did not result in a default Th2 response, at least as measured by Ag-stimulated IL-4 production, although IgG1 levels were increased in IL-12−/− mice. Administering IL-18 to IL-12−/− mice restored renal injury and glomerular and skin DTH to levels seen in IL-12−/+ mice injected with vehicle alone. There were increases in effectors of DTH: CD4+ cells, macrophages, and (in the glomerulus) fibrin. Other potential mechanisms of glomerular injury (CD8+ cells, NK cells, and Ab) were assessed. CD8+ cells were present in glomeruli and were decreased in the absence of IL-12, but were not affected by IL-18 administration. These findings together with the observation that injury in this model is not CD8 dependent (21) do not support a role for CD8+ cells in this study. NK cells were not present in greater numbers in either the footpad or glomeruli of IL-18-treated mice. However, as IL-18 stimulates NK cell IFN-γ production (that would not necessarily have been measurable in Ag-stimulated splenocyte cultures), it is possible that increased IFN-γ production by NK cells present in the lesion may have contributed to the increased injury observed. There was no increase in the deposition of autologous Ab in glomeruli, confirming that humoral mediators were not responsible for the increased injury observed with IL-18 administration.

These results demonstrate that IL-18 can induce DTH responses in vivo in the absence of IL-12, and that the effectors present in the lesions are those classically associated with the DTH response. IL-18 is capable of inducing a number of proinflammatory cytokines that are relevant to Th1 responses, including IFN-γ, IL-2 (2), GM-CSF (36), and TNF-α (37). In this study, although there were increases in DTH effectors in both the glomerulus and the skin, splenocyte production of any of these proinflammatory cytokines was not induced by IL-18 in the absence of IL-12. It remains possible that other proinflammatory cytokines, such as IL-1 or lymphotoxin-α, may have been induced by IL-18.

To effect a cell-mediated immune response cells are required to localize within the target tissue, a process requiring both adhesion molecules and chemokines. The relevance of a number of adhesion molecules and chemokines has been demonstrated in crescentic glomerulonephritis (27–29) and dermal DTH (11–13). In the absence of any demonstrable effect by IL-18 in IL-12−/− on proinflammatory cytokines such as IFN-γ, GM-CSF, and TNF-α, the expression of the adhesion molecule ICAM-1 was studied within glomeruli. IL-18 has been shown to up-regulate ICAM-1 expression in an IFN-γ-independent manner (14), suggesting the potential for alterations in ICAM-1 (and indeed other adhesion molecules, such as VCAM-1, that has been shown to act in synergy with ICAM-1 in contact hypersensitivity (10)) to be relevant to the current studies. ICAM-1 is functionally important in the development of proliferative and crescentic glomerulonephritis (27, 28). Administering IL-18 to IL-12−/− mice resulted in increased glomerular ICAM-1 expression compared with that in IL-12−/− control mice with GN. Mice without GN that received IL-18 did not exhibit increased glomerular ICAM-1 expression, implying that in the absence of a local stimulus IL-18 does not up-regulate ICAM-1 expression. These findings show that despite reductions in Th1 proinflammatory cytokines found in control IL-12−/− mice and the failure of IL-18 to restore these deficits, at a tissue level Th1 responses (in this case the DTH response) can be restored by IL-18 via the up-regulation of chemoattractants or adhesion molecules such as ICAM-1. Furthermore, they imply that the increase in ICAM-1 expression observed in IL-12−/− mice given IL-18 is a direct effect of IL-18, although the mechanism by which ICAM-1 expression is increased by IL-18 could be indirect, involving other inflammatory mediators themselves affected by IL-18. Increased glomerular ICAM-1 expression in IL-12−/− mice given IL-18 demonstrates the principle that adhesion molecules can be up-regulated by IL-18 in an IL-12-independent manner in DTH responses. These studies do not address whether IL-18 induces T cell or macrophage integrin expression, or chemokine and chemokine receptor expression, both of which have the potential to contribute to injury in DTH. While these studies have demonstrated IL-12-independent effects of IL-18 in target tissues and have not shown
effects on the systemic immune response (via cytokines and Ab production), IL-18 may play an important IL-12-independent role in the initiation and generation of the immune response (for instance, at Ag presentation and during T cell proliferation and differentiation).

Genetically normal mice that were given IL-18 showed enhanced DTH responses, renal injury, and skin swelling compared with those in vehicle-treated IL-12+/− mice. These results demonstrate that although IL-12 can facilitate DTH responses in the absence of IL-12, when IL-18 is administered to IL-12-intact mice, a synergistic enhancement of DTH responses and injury occurs, with increased DTH effectors within the lesion. This increased injury observed in normal C57BL/6 mice given IL-18 was associated with increased splenocyte production of GM-CSF together with a trend toward increased IFN-γ. Glomerular ICAM-1 expression was also increased. Therefore, the enhancement of Th1 response by IL-18 in normal animals is likely to be mediated both by increases in the generation of Th1 effector cytokines and by induction of adhesion molecules.

The effects of IL-18 on Ag-specific Ab responses and on IgG subclasses in vivo have not, to our knowledge, previously been studied. In vitro studies have demonstrated that the combination of IL-12 and IL-18 suppress IgG1 and enhance IgG2a levels (38). However, studies of murine graft-vs-host disease suggest that IL-18 lacks this discriminatory effect and, in fact, suppresses total Ig, IgG1, and IgG2a levels in vitro (39). While IL-18 would be expected to induce IgG2a production in vivo (given its role in Th2 differentiation), there were no significant changes in Ag-specific total Ig, IgG1, or IgG2a titers when IL-18 was administered to IL-12−/− mice.

In summary, these studies demonstrate that IL-18 is capable of substituting for IL-12 in the generation of in vivo cell-mediated DTH-like responses, in this case experimental crescentic glomerulonephritis and dural DTH. These effects were not demonstrated to be due to alterations in patterns of cytokine synthesis and secretion. However, IL-12-independent effects of IL-18 on adhesion molecules were demonstrated, suggesting direct effects of IL-18 in enhancing cellular recruitment. In animals with an intact IL-12/IL-12R system, IL-18 enhances DTH-like responses via CD4+ and macrophage-mediated cellular mechanisms. In the presence of IL-12, the effects of IL-18 are likely to be multifactorial and include effects on proinflammatory cytokines and adhesion molecules. The current studies show that IL-18 is capable of inducing effector Th1 responses in the absence of IL-12 and point toward an important role for IL-18 in DTH and DTH-like inflammatory responses.

Acknowledgments
We thank A. Turner, J. Sharkey, and A. Wright for technical assistance. We thank The DNAX Research Institute (Palo Alto, CA) for the hybridomas used in the IL-4 and IL-2 assays.

References

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