The Requirement for Granulocyte-Macrophage Colony-Stimulating Factor and Granulocyte Colony-Stimulating Factor in Leukocyte-Mediated Immune Glomerular Injury

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Abstract. Proliferative glomerulonephritis in humans is characterized by the presence of leukocytes in glomeruli. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) can potentially stimulate or affect T cell, macrophage, and neutrophil function. To define the roles of GM-CSF and G-CSF in leukocyte-mediated glomerulonephritis, glomerular injury was studied in mice genetically deficient in either GM-CSF (GM-CSF−/− mice) or G-CSF (G-CSF−/− mice). Two models of glomerulonephritis were studied: neutrophil-mediated heterologous-phase anti–glomerular basement membrane (GBM) glomerulonephritis and T cell/macrophage-mediated crescentic autologous-phase anti-GBM glomerulonephritis. Both GM-CSF−/− and G-CSF−/− mice were protected from heterologous-phase anti-GBM glomerulonephritis compared with genetically normal (CSF WT) mice, with reduced proteinuria and glomerular neutrophil numbers. However, only GM-CSF−/− mice were protected from crescentic glomerular injury in the autologous phase, whereas G-CSF−/− mice were not protected and in fact had increased numbers of T cells in glomeruli. Humoral responses to the nephritogenic antigen were unaltered by deficiency of either GM-CSF or G-CSF, but glomerular T cell and macrophage numbers, as well as dermal delayed-type hypersensitivity to the nephritogenic antigen, were reduced in GM-CSF−/− mice. These studies demonstrate that endogenous GM-CSF plays a role in experimental glomerulonephritis in both the autologous and heterologous phases of injury.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are growth factors and cytokines that are classically described as acting on hemopoietic cells, particularly granulocytes and monocytes. They are involved in the normal expression of innate and cognate immune responses and have been considered to be required for the normal development of hemopoietic progenitor cells (1,2). The generation of mice genetically deficient in either G-CSF (G-CSF−/− mice) or GM-CSF (GM-CSF−/− mice) have facilitated studies of their roles in hematoipoiesis and in immune and inflammatory injury. G-CSF−/− mice show abnormal numbers of bone marrow and circulating granulocytes (3) and deficient granulocyte defense to infective microorganisms (4). GM-CSF−/− mice have normal numbers of circulating leukocytes and bone marrow hemopoietic progenitors (5). GM-CSF has proinflammatory effects on granulocytes (1) and is required for normal function of at least some populations of dendritic cells (6,7), monocyte activation (1), T cell function (8), and, via its secretion by the T helper 1 (Th1) subset, the full expression of dermal delayed-type hypersensitivity (DTH) responses (9).

The most aggressive forms of glomerulonephritis (GN) show significant glomerular leukocytic infiltration (10). A number of studies in experimental models of GN have confirmed that infiltrating glomerular leukocytes are major effectors of glomerular injury in proliferative GN (11–13). Proliferative forms of human GN show a variety of immune effectors and histologic outcomes with variable involvement of leukocyte subsets (14). Underpinning many of these differences is the Th1 or T helper 2 predominance of the systemic nephritogenic immune response (14), with evidence emerging that Th1 responses are important in the development of severe proliferative and crescentic GN (14–17).

Neutrophils have been implicated in a number of forms of proliferative human GN, including anti-neutrophil cytoplasmic antibody–associated crescentic GN (18,19). The best-characterized experimental model of neutrophil-mediated immune glomerular injury is heterologous-phase anti–glomerular basement membrane (GBM) GN (or nephrotoxic serum nephritis). In this model, GN is induced by administering a heterologous globulin directed against the GBM. Glomerular injury is associated with neutrophil accumulation in glomeruli (11) and is induced predominantly by neutrophil degranulation with the release of proteolytic enzymes and reactive oxygen species (20,21). There is little known involvement for macrophages in
this form of acute glomerular injury in murine anti-GBM GN (11), although macrophages may have a role in the heterologous phase in rat models (22).

DTH responses have been shown to be important in experimental crescentic GN (12). The presence of DTH effectors in human crescentic GN, whether or not glomerular antibody deposition is present, implies that findings in experimental models are relevant to human disease (23,24). Th1-predominant nephritogenic immune responses result in strong glomerular DTH with T cell–directed macrophage recruitment and the development of crescentic GN (15,25). The experimental model most widely used to study these events is the autologous phase of experimental anti-GBM antibody–induced GN, in this model it is the host (autologous) immune response to the deposited heterologous Ig (the heterologous anti-GBM antibody) in glomeruli that induces immune injury (26).

In human glomerulonephritis, evidence that GM-CSF may play a functional role in inducing injury in GN comes from observations in humans in which GM-CSF has been found to be increased in human renal biopsies of patients with proliferative GN (27). In experimental GN, injection of tubular epithelial cells transfected with the gene for GM-CSF under the renal capsule of lupus-prone MRL/lpr mice induces inflammatory renal injury (28). G-CSF and GM-CSF have the potential to play important roles in the effector functions of inflammation induced by granulocytes, T cells, and monocytes. They are therefore potential therapeutic targets in inflammatory diseases. Because of evidence of participation of granulocytes and mononuclear leukocytes in several forms of proliferative GN, we sought to determine the roles of G-CSF and GM-CSF in models of leukocyte-induced glomerular injury in which either granulocytes or mononuclear cells are known to be the major effectors of injury.

Materials and Methods

Experimental Design
Anti-mouse GBM globulin was prepared from serum (adsorbed twice with mouse red blood cells then precipitated with ammonium sulfate) of a sheep immunized against homogenized and sonicated murine renal cortex in Freund’s complete adjuvant (Sigma Chemical Co., St. Louis, MO) and, later, Freund’s incomplete adjuvant. Mice with a genetic deletion of either GM-CSF or G-CSF on a C57BL/6J background were bred at Monash University (Melbourne, Victoria, Australia) in a specific pathogen-free facility. For studies in heterologous injury, male GM-CSF +/−, G-CSF +/−, and genetically normal C57BL/6 × 129Ola mice, 8 to 10 wk of age, were injected with 4 mg of sheep anti-mouse GBM globulin. In one experiment, glomerular neutrophil accumulation was studied at 1 h (n = 8 each group), the time of maximum influx in this model (29). In a further experiment, mice were placed in metabolic cages 8 h after injection, and urine was collected for 16 h for measurement of urinary protein excretion (n = 6 each group). For studies in autologous injury (n = 7 each group), mice were sensitized by subcutaneous injection of a total of 100 μg of sheep globulin in 50 μl of Freund’s complete adjuvant in divided doses in each flank 10 d before challenge with 2.5 mg of sheep anti-mouse GBM globulin. Renal injury was studied after a further 10 d. Results are expressed as the mean ± SEM. The significance of differences between groups was determined by ANOVA, followed by Tukey’s multiple comparison test for paired comparisons (GraphPad Prism, GraphPad Software Inc., San Diego, CA).

Assessment of Glomerular Crescent Formation
Kidney tissue was fixed in Bouin fixative and embedded in paraffin, and 3-μm tissue sections were cut and stained with periodic acid–Schiff. A glomerulus was considered to exhibit crescent formation if two or more layers of cells were observed in Bowman space. A minimum of 50 glomeruli was assessed to determine the crescent score for each animal.

Glomerular T Cell, Macrophage, and Neutrophil Accumulation
Kidney tissue was fixed in periodate lysine paraformaldehyde for 4 h, washed in 7% sucrose solution, and then frozen in liquid nitrogen. Tissue sections (6 μm) were stained to demonstrate CD3+ cells, neutrophils, and macrophages by use of a three-layer immunoperoxidase technique, as described elsewhere (12,30). The primary monoclonal antibodies were KT3 anti-mouse CD3 (American Type Culture Collection, Manassas, VA), the anti-mouse macrophage monoclonal FA/11 antibody (a gift of Dr. G.L. Koch, MRC Laboratory of Molecular Biology, Cambridge, UK) (31), and RB6–8C5 (anti-Gr-1, which recognizes neutrophils, a gift from DNAX Research Institute, Palo Alto, CA). A minimum of 20 equatorially sectioned glomeruli was assessed per animal, and results were expressed as cells per glomerular cross section (c/gcs).

Glomerular Deposition of Sheep Globulin, Mouse Ig, and C3
For detection of sheep globulin, immunofluorescence was performed on 4-μm cryostat-cut periodate lysine paraformaldehyde–fixed tissue, detected by use of FITC-rabbit-anti-sheep Ig (Silenus, Hawthorn, Victoria, Australia) at dilutions of 1:250 and/or 1:1000. For detection of autologous antibody and C3 in glomeruli, tissue was embedded in optimal Cutting Temperature Compound, frozen in liquid nitrogen, and stored at −70°C. Mouse Ig was detected by use of FITC-sheep anti-mouse Ig (Silenus, Hawthorn, Victoria, Australia) at dilutions of 1:100 and 1:10,000 and C3 by use of FITC-goat anti-mouse C3 (Cappel, Durham, NC) at dilutions of 1:100 and 1:5000. Fluorescence intensity was assessed semiquantitatively (0 to 3+). Sections in which only some glomeruli were positive were graded as 0.5 (i.e. +/−).

Proteinuria and Serum Creatinine
Urinary protein concentrations were determined by a modified Bradford method on timed urine collections. For studies in heterologous injury, mice were placed on metabolic cages 8 h after intravenous injection of sheep anti-mouse GBM globulin. Urine was collected for 16 h and results converted to provide a value for 24 h. For studies in autologous GN, urine was collected for 24 h from days 9 to 10 of GN. Serum creatinine concentrations at the completion of experiments in autologous phase GN (day 10) were measured by the alkaline picric acid method with the use of an autoanalyzer.

Skin DTH to Sheep Globulin
In the experimental groups where GN was induced and studied at day 10, mice were challenged 24 h before the end of the experiment by intradermal injection of sheep globulin (100 μg in 50 μl of phosphate-buffered saline) into the plantar surface of a hind foot. An
equivalent dose of an irrelevant antigen (horse globulin) in an equivalent volume of phosphate-buffered saline was injected in the opposite foot pad as a control. DTH was assessed 24 h later by measuring the difference between the sheep- and horse globulin-injected foot pads in each mouse by use of a micrometer (Mitutoyo Corporation, Japan).

**Titors of Serum Anti-Sheep Globulin Ig**

Titors of mouse anti-sheep globulin Ig were measured by enzyme-linked immunosorbent assay on serum collected at the end of experiments in autologous injury, as described elsewhere (16). Plates were coated with 10 μg/ml normal sheep globulin, washed, then blocked with 1% bovine serum albumin in phosphate-buffered saline. Plates were washed, then incubated with mouse serum at the dilutions of 1:100, 1:1000, and 1:10,000, and incubated for 1 h at 37°C. Bound mouse Ig was detected with horseradish peroxidase conjugated sheep anti-mouse Ig (Amersham, Little Chalfont, UK, 1:2000); 0.1 M 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (Boehringer Mannheim, Germany) substrate solution was added and the absorbance read at 405 nm. Sera from nonimmunized mice were tested to provide negative controls.

**Peripheral Blood White Blood Cell and Neutrophil Counts**

The effects of the administration of heterologous antigen on peripheral blood neutrophils were studied by use of mice 9 to 11 wk of age. Mice were bled at baseline or 1 h after intravenous injection of 4 mg sheep anti-mouse GBM, to determine total leukocyte count and the circulating neutrophil count. For baseline studies, six GM-CSF−/− mice, four G-CSF−/− mice, and six CSF wild type (WT) mice were used; for studies 1 h after administration of sheep anti-mouse GBM, eight GM-CSF−/− mice, five G-CSF−/− mice, and seven CSF WT mice were used. One hundred microliters of blood was obtained from each mouse and added to 50 μl of 3.3% tri-sodium citrate. A 100 μl volume of blood/tri-sodium citrate mix was transferred to a tube and red blood cells lysed (Q prep, Coulter Electronics, Sydney, Australia) and total white blood cell count determined via a hemocytometer. Subsequently, the percentage and absolute number of neutrophils present in the sample were determined by flow cytometry (Mo-fl cytometer, Fort Collins, CO), gating on the granulocyte population via forward and 90° scatter.

**Results**

**Both G-CSF−/− Mice and GM-CSF−/− Mice Are Protected from Neutrophil-Mediated, Heterologous-Phase GN**

CSF WT mice developed significant proteinuria 24 h after intravenous injection of 4 mg sheep anti-mouse GBM globulin (9.5 ± 0.7 mg/24 h; normal, 0.8 ± 0.2 mg/24 h, Figure 1B). Histologic examination of kidneys at the time of peak neutrophil accumulation in this model (1 h) (29) revealed exudative proliferative GN characterized by a significant neutrophil influx in CSF WT mice (2.5 ± 0.7 c/gcs; mice without GN, 0.1 ± 0.01 c/gcs; Figures 1A and 2A). Both G-CSF−/− and GM-CSF−/− mice were significantly protected from heterologous-phase glomerular injury (Figures 1 and 2, B and C). Proteinuria in G-CSF−/− mice was 3.5 ± 0.3 mg/24 h, and that in GM-CSF−/− mice was 3.7 ± 0.5 mg/24 h (both P < 0.01, Figure 1B). These reductions were associated with a significant and similar attenuation of glomerular neutrophil numbers (G-CSF−/−, 0.61 ± 0.12 c/gcs; GM-CSF−/−, 0.62 ± 0.08 c/gcs, both P < 0.05). There was no significant influx of T cells or macrophages into glomeruli at 1 h (CD3+ cells: CSF WT, 0.10 ± 0.03 c/gcs; G-CSF−/−, 0 ± 0 c/gcs; GM-CSF−/−, 0.10 ± 0.01 c/gcs and macrophages: CSF WT, 0.10 ± 0.04 c/gcs; G-CSF−/−, 0.05 ± 0.01 c/gcs; GM-CSF−/−, 0 ± 0 c/gcs) or at 24 h in this lesion (CD3+ cells: CSF WT, 0.17 ± 0.03 c/gcs; G-CSF−/−, 0.06 ± 0.03 c/gcs; GM-CSF−/−, 0.13 ± 0.03 c/gcs and macrophages: CSF WT, 0.14 ± 0.04 c/gcs; G-CSF−/−, 0.03 ± 0.02 c/gcs; GM-CSF−/−, 0.06 ± 0.03 c/gcs), consistent with an important role for neutrophils in this lesion in mice (11).

There was no difference in the deposition of sheep globulin in glomeruli among the three groups of mice (1:250 dilution average score 2+ [fluorescence intensity], 1:1000, + for all groups), which shows that the absence of either G-CSF or GM-CSF did not alter the deposition of the disease-initiating globulin. The potential for the disease to be affected by differences in circulating neutrophil numbers was assessed. The total white blood cell and circulating neutrophil count, both before and after injection of sheep anti-mouse GBM, was reduced in G-CSF−/− mice compared with CSF WT mice and GM-CSF−/− mice (Table 1). These findings are consistent with data published elsewhere on the phenotype of this strain of mouse (3). However, there was no difference in total white blood cell count or circulating neutrophil count between GM-CSF−/− mice and CSF WT mice, either before or after injection.
Table 1. Circulating total white blood cell (WBC) counts and neutrophil counts in mice before and 1 h after injection with sheep anti-mouse glomerular basement membrane globulin, showing leucopenia and neutropenia in G-CSF−/− mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>State</th>
<th>Total WBC (×10^3/ml)</th>
<th>Neutrophils (×10^3/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF WT</td>
<td>Preinjection</td>
<td>9.6 ± 2.8</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Postinjection</td>
<td>7.5 ± 2.6</td>
<td>2.7 ± 1.2</td>
</tr>
<tr>
<td>GM-CSF−/−</td>
<td>Preinjection</td>
<td>9.8 ± 1.8</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Postinjection</td>
<td>8.0 ± 2.8</td>
<td>2.8 ± 1.0</td>
</tr>
<tr>
<td>G-CSF−/−</td>
<td>Preinjection</td>
<td>1.9 ± 1.2</td>
<td>0.4 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Postinjection</td>
<td>2.2 ± 0.5</td>
<td>0.25 ± 0.07</td>
</tr>
</tbody>
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* G-CSF, granulocyte colony-stimulating factor; GM, granulocyte-macrophage; WT, wild-type; −/− mice, knockout mice. Results are expressed as the mean ± SEM.

**Only GM-CSF−/− Mice Are Protected from Crescentic Glomerulonephritis in the Autologous Phase of Injury**

Control animals develop a severe proliferative crescentic GN (51 ± 4% of glomeruli affected [Figures 2D and 3A]). This was associated with a significant influx of inflammatory cells, including CD3+ T cells, and macrophages and a minor degree of neutrophil accumulation (Figure 3, B through D). G-CSF−/− mice developed similar numbers of glomerular crescents and were not protected from autologous injury (Figures 2F and 3A), which demonstrates that G-CSF is not required for the development of crescentic GN (53 ± 4% of glomeruli affected). However, GM-CSF−/− mice were significantly protected from severe crescentic glomerular injury, glomerular crescents being present in only 13 ± 2% of glomeruli (Figure 2E and 3A). In this autologous form of injury, where injury is driven by an adaptive nephritogenic immune response directed against sheep globulin, there was no difference in the deposition of the nephritogenic antigen (sheep globulin) in glomeruli among the three groups of mice (1:1000 dilution average score + for all groups).

Reductions in crescentic injury observed in GM-CSF−/− mice with GN were paralleled by changes in the T cell and macrophage infiltrate in glomeruli. Although 1.4 ± 0.1 CD3+ cells per glomerular cross-section were observed in CSF WT mice with GN, GM-CSF−/− mice had one-third of this number in glomeruli (0.5 ± 0.10 c/gcs, Figure 3B) A similar reduction was observed in glomerular macrophage infiltrate (CSF WT, 3.3 ± 0.4 c/gcs and GM-CSF−/− 1.1 ± 0.05 c/gcs; Figures 2H and 3C). The modest increase in CD3+ cells in glomeruli of G-CSF−/− mice reached statistical significance (P < 0.05, Figure 3B), but other indices of injury were not significantly increased.

In contrast to the alterations observed in the cellular infiltrate in glomeruli, the deposition of humoral reactants (autologous antibody and C3) was not affected by deficiency of either G-CSF or GM-CSF. When examined at two dilutions, average semiquantitative fluorescence intensity was 3+ in each group at an antibody dilution of 1:100. Fluorescence intensity had fallen to +/− (i.e., only some glomeruli in each section were positive) in each group at a dilution of 1:10,000 for autologous antibody and 1:5000 for C3.

CSF WT mice developed renal impairment (serum creatinine 44 ± 5 μmol/L, Figure 4A) and heavy proteinuria (9.4 ± 1.2 mg/24 h, Figure 4B). G-CSF−/− mice with autologous, crescentic GN were not protected from functional renal injury. Their degree of proteinuria and renal impairment was similar to that of CSF WT mice (Figure 4). However, consistent with the significant reduction in glomerular crescent formation and glomerular T cell and macrophage infiltrate, GM-CSF−/− mice were significantly protected from the development of renal impairment (serum creatinine 20 ± 1 μmol/L, P < 0.01, Figure 4A). Proteinuria in GM-CSF−/− mice was reduced by ~40% (5.6 ± 0.4 mg/24 h, P < 0.01, Figure 4B).

**Systemic Immune Responses to Sheep Globulin**

In the final 24 h of experiments in autologous-phase (crescentic) injury, mice were challenged subcutaneously with sheep and horse globulin. The difference between the two sites of challenge served as a measure of dermal DTH. Control mice developed significant antigen-specific skin DTH (Figure 5). Although the degree of DTH was unchanged in G-CSF−/− mice, dermal DTH was significantly reduced in the absence of GM-CSF.

In the autologous phase of injury, all mice developed measurable titers of sheep globulin specific antibodies in serum drawn at day 10 of GN (Figure 6). However, there were no differences among CSF WT, G-CSF−/−, and GM-CSF−/− mice: all groups developed similar titers of antigen-specific circulating antibodies (Figure 6).

**Discussion**

These studies show that both neutrophil-associated glomerular injury and T cell/macrophage-mediated crescentic glomerular injury are mediated by endogenous GM-CSF−/−, whereas G-CSF is important in neutrophil-mediated injury (possibly by virtue of its effects on the development of neutrophils) and has no pathogenic role in experimental crescentic GN.

G-CSF is known to be important in the generation of granulocyte stem cell progenitors. Mice with a targeted disruption of the gene for this cytokine have a known predisposition to bacterial infection (4). However, this deficiency has variable effects on neutrophil recruitment and function in different models of inflammation. Zhan et al. (4) reported increased mortality to and exacerbated in vivo proliferation of *Listeria monocytogenes* in G-CSF−/− mice, and Metcalf et al. (32) observed that the intraperitoneal injection of casein (containing bacteria) into G-CSF−/− mice did not affect the subsequent neutrophil exudation observed. The model of heterologous anti-GBM antibody–induced GN used in those studies is associated with a significant glomerular neutrophil influx. The accumulating neutrophils have been shown to be responsible for the associated glomerular injury (11,21). In these studies, G-CSF−/− mice were found to be neutropenic, as reported...
elsewhere (3). These animals also showed a markedly reduced glomerular influx of neutrophils and, consequently, significant protection from the development of glomerular injury. However GM-CSF $-/-$ mice were also protected from heterologous injury, despite having normal circulating neutrophil numbers. Thus deficiency of either G-CSF or GM-CSF gave similar levels of protection in this model of acute antibody-induced neutrophil-mediated glomerular injury. Although there

Figure 2. Histologic features of injury in CST WT, GM-CSF $-/-$, and G-CSF $-/-$ mice with heterologous- and autologous-phase anti-GBM GN. (A through C) Heterologous injury: (A) genetically normal mice (CSF WT) developed proliferative GN with neutrophils in glomeruli (brown reaction product). Mice deficient in either GM-CSF (B) or G-CSF (C) had fewer neutrophils in their glomeruli. (D through F) Autologous-phase anti-GBM GN: (D) CSF WT mice developed severe proliferative and crescentic GN. GM-CSF $-/-$ mice had a lesser degree of injury (E), whereas renal injury in G-CSF $-/-$ mice was as severe as that of CSF WT mice (F), with substantial glomerular crescent formation. (G through I) Macrophage infiltration in autologous phase anti-GBM GN: CSF WT mice with GN had significant numbers of FA/11-positive macrophages (brown reaction product) within glomeruli (G). Numbers were significantly reduced in GM-CSF $-/-$ mice (H), but glomerular macrophage numbers (arrowheads) were unchanged in G-CSF $-/-$ mice compared with CSF WT mice with GN (I). (A through C and G through I) Immunoperoxidase with hematoxylin counterstain. (D through F) periodic acid–Schiff stain. Magnification: $\times200$. 

was only a minor degree of glomerular macrophage infiltrate at both 1 and 24 h in this murine model, it is difficult to exclude a role for GM-CSF in promoting macrophage-mediated proteinuria as measured from 8 to 24 h after injection of sheep anti-mouse GBM globulin.

The autologous model of anti-GBM injury involves quite different effector systems and is dependent on the active generation of DTH to the nephritogenic antigen (12,25). Although this model of injury is associated with development of both circulating antibodies and DTH to the inducing antigen, glomerular injury is dependent only on glomerular DTH effectors (25,33). Mice with agammaglobulinemia consequent to disruption of the \( \mu/H_9262 \) chain Ig gene developed equivalent injury to that observed in animals with an intact humoral response (33). Depletion of CD4\(^+\)/H\(_{11001}\) cells in the effector phase of injury is associated with attenuation of skin DTH to the nephritogenic antigen as well as prevention of the accumulation of glomerular DTH effectors and injury in this model (25,33). G-CSF \(-/-\) mice developed normal dermal DTH and titers of circulating antibody to the nephritogenic antigen. There was no deficiency in glomerular DTH, because significant numbers of CD3\(^+\)/H\(_{11001}\) cells and macrophages were observed in the glomeruli of injected animals. There was a statistically significant increase in CD3\(^+\) cells within glomeruli in G-CSF \(-/-\) mice, which suggests that endogenous G-CSF may in fact play a protective role in the development of this lesion. However, glomerular crescent formation, renal impairment, and proteinuria were not increased in the absence of G-CSF, which suggests that the increase in glomerular T cells, although statistically significant, was not biologically relevant. Glomerular

![Figure 3](image)

Figure 3. Glomerular injury in autologous (crescentic) anti-GBM GN, showing (A) reduced glomerular crescent formation, (B) CD3\(^+\) cells, and (C) macrophages in glomeruli of mice lacking endogenous GM-CSF but no reduction in glomerular crescent formation in G-CSF \(-/-\) mice and, in fact, an increase in intraglomerular T cells (\( * P < 0.05, ** P < 0.01 \) versus CSF WT mice for panels A through C). (D) The low numbers of neutrophils in glomeruli were not different in the absence of either G-CSF or GM-CSF.

![Figure 4](image)

Figure 4. Functional indices of injury in mice with autologous-phase anti-GBM GN. (A) Serum creatinine, showing impaired renal function in CSF WT mice with GN, and significantly lower serum creatinine in GM-CSF \(-/-\) mice (\( ** P < 0.01 \) versus CSF WT mice). (B) Proteinuria, showing a reduction in GM-CSF \(-/-\) mice compared with CSF WT mice (\( ** P < 0.01 \) versus CSF WT mice). G-CSF \(-/-\) mice were not protected from functional renal injury in this model. The horizontal dotted lines represent mean values for normal mice without GN.

![Figure 5](image)

Figure 5. Dermal delayed-type hypersensitivity responses (DTH) to sheep globulin (the nephritogenic antigen) at day 10 of the autologous-phase of injury, showing antigen specific swelling in CSF WT mice, unchanged responses in G-CSF \(-/-\), and reduced DTH responses in GM-CSF \(-/-\) mice (\( P < 0.05 \) versus CSF WT mice).
neutrophil accumulation is unlikely to be a major contributor to the development of crescentic GN in autologous-phase injury. The numbers of glomerular neutrophils were much lower in autologous-phase injury than those seen in the heterologous model. Injury in G-CSF −/− mice was similar to that seen in controls, despite their inability to fully develop a neutrophil-dependent (heterologous-phase) form of injury. In contrast to CSF WT and G-CSF −/− mice, GM-CSF −/− mice were unable to mount a normal dermal DTH response to the nephritogenic antigen. When autologous-phase anti-GBM GN was induced in these animals, they showed a reduced accumulation of CD3+ T cells and macrophages. There was significant protection from the development of crescent formation and impairment of renal function in GM-CSF −/− mice, and proteinuria was reduced but not to normal levels. The levels of circulating antibody to the immunizing antigen were unaffected by deficiency of GM-CSF, consistent with results elsewhere in studies of these mice (9).

GM-CSF-deficient animals are protected from collagen-induced arthritis, where similar alterations in the injurious immune response were observed (9). Levels of antibody, including titers of IgG subclasses, were unaffected, whereas DTH was reduced. Moreover, injection of GM-CSF systemically (in collagen arthritis) (9) and locally (by the inoculation of GM-CSF-transfected tubular epithelial cells under the capsule of the kidney and MRL/lpr-prone mice) (28) both exacerbated cell-mediated immune injury in the relevant organs. These observations are consistent with a role for GM-CSF in directing cell-mediated immune injury.

The protective effects of GM-CSF deficiency could be acting at several levels. It is known that antigen presentation is affected by GM-CSF deficiency (8); thus, systemic T cell responsiveness may be deficient in these animals. GM-CSF is produced by T cells to activate monocytes; therefore, deficient T cell production of GM-CSF is likely to be relevant to the diminution of injury observed. GM-CSF may be deficient in the target organ where its role as a proinflammatory cytokine may be important in the development of local injury. T cells are the predominant GM-CSF-producing leukocyte subpopulation. These cells are essential for the development of GN by their recruitment and activation of macrophages. The normal levels of antibody seen in GM-CSF −/− mice would suggest normal T cell help, at least for humoral responses. Thus the beneficial effects of GM-CSF deficiency in GN would appear to be predominantly via diminished T cell–induced macrophage activation.

The significant protection afforded to GM-CSF −/− mice with autologous GN is consistent with a role for GM-CSF as an important T cell–derived activator of macrophages. The incomplete protection observed in terms of crescent formation suggests partially redundant roles for several cytokines in the activation of macrophages, including interferon-γ (25,34) and macrophage migration inhibitory factor (35,36). Although autologous antibody has little role in glomerular crescent formation in this model (33), it has the capacity to induce complement-dependent proteinuric injury in the autologous phase of injury (25,37). GM-CSF plays little role in the generation of humoral responses in this model.

Augmented expression of GM-CSF has been observed in human proliferative GN in association with leukocyte infiltration (27). The demonstration that mesangial cells have the capacity to produce GM-CSF illustrates the potential for local production of GM-CSF to contribute to injury by activating macrophages (38). However, these studies do not address a potential role for GM-CSF in macrophage activation by intrinsic glomerular cells or in response to Fcγ receptor engagement.

These results suggest that both the G-CSF and GM-CSF are important in the generation of leukocyte-induced GN. G-CSF and GM-CSF are both required for normal neutrophil recruitment and glomerular localization, whereas G-CSF does not appear to be required for cell-mediated immune glomerular injury. In contrast, GM-CSF is required (potentially at several points in the immune response in the generation of DTH) for the development of crescentic GN. Targeting G-CSF as a therapeutic potential modality, although theoretically possible, would be of more limited therapeutic benefit and may only be effective in those forms of GN in which humoral immunity associated with neutrophil influx is a predominant immune effector mechanism. In addition, concerns exist about attenuating any potential anti-inflammatory effects of G-CSF, given our observations of increased glomerular T cell accumulation in G-CSF −/− mice, allied with in vitro studies that have shown that G-CSF reduces interferon-γ, tumor necrosis factor, and interleukin-1 production (39,40). The results of these studies suggest that targeting of GM-CSF could potentially be of benefit in all forms of leukocyte-associated GN.

Acknowledgments

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