Granulocyte Macrophage Colony-Stimulating Factor Expression by Both Renal Parenchymal and Immune Cells Mediates Murine Crescentic Glomerulonephritis

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GM-CSF has previously been demonstrated to be important in crescentic glomerulonephritis (GN). As both renal parenchymal cells and infiltrating inflammatory cells produce GM-CSF, their separate contributions to inflammatory renal injury were investigated by creation of two different types of GM-CSF chimeric mice: (1) GM-CSF–deficient (GM-CSF−/−)→wild-type (WT) chimeras with leukocytes that are unable to produce GM-CSF and (2) WT→GM-CSF−/− chimeras with deficient renal cell GM-CSF expression. Crescentic anti–glomerular basement membrane GN was induced in WT, GM-CSF recruitment, CD40 mice developed severe crescentic GN, whereas GM-CSF chimeras, WT3 significantly reduced in GM-CSF mice, indicating that either leukocyte or renal sources of GM-CSF are sufficient to drive these aspects of the inflammatory response. Restricted expression of GM-CSF revealed a major role for renal cell–derived GM-CSF but a minor role for leukocyte-derived GM-CSF in the formation of cellular crescents; glomerular MHC II expression; serum creatinine; and monocyte chemoattractant protein-1, vascular cellular adhesion molecule, and IL-1β expression. Glomerular macrophage accumulation, proteinuria, and interstitial infiltrate were equivalent in both chimeric groups but intermediate between WT and GM-CSF−/−, indicating that both sources are required for the full development of glomerular injury in crescentic GN.


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M-CSF is a key mediator of inflammation and autoimmunity. In murine models of inflammatory injury such as collagen-induced arthritis (1,2), experimental autoimmune encephalomyelitis (EAE) (3), LPS-induced lung inflammation (4,5), peritonitis (6), and glomerulonephritis (GN) (7) the importance of GM-CSF has been established. Mice with targeted disruption of GM-CSF or antibody (Ab) blockade of GM-CSF are protected from the development of severe disease and associated injury.

Originally recognized for the important role that it has in the generation of granulocytes and monocytes from hemopoietic precursors, GM-CSF is now regarded to act at several levels in the generation and propagation of immune responses (8,9). GM-CSF is made by T cells and macrophages and participates in enhancing phagocytosis and inducing MHC II, CD1a, CD80, and CD86 on the surface of dendritic cells, which augments their antigen presentation (10). GM-CSF has been demonstrated to be a chemoattractant for antigen-presenting cells (11) and is also required for antigen-specific T cell (12) and delayed-type hypersensitivity (DTH) responses.

Recently, we investigated the effect of GM-CSF deficiency in GN (7). A particularly severe form of GN, with a poor clinical outcome, crescentic GN is a T cell– and macrophage-dependent form of inflammatory renal injury. In mice, this disease can be induced by administration of heterologous anti–glomerular basement membrane (GBM) antibody, which selectively binds in glomeruli and acts as a planted antigen (Ag) target for a DTH-like autologous immune response. The presence of DTH effectors in the kidney has been well documented (13–18). Crescentic GN was studied in mice that were genetically deficient in GM-CSF (GM-CSF−/−), which do not exhibit myelopoietic deficiencies (19). These deficient mice were significantly protected from the development of severe crescentic injury, with significant reductions in glomerular crescent formation, T cell and macrophage glomerular influx, and preservation of renal function indicated by serum creatinine and proteinuria (7).

Elevated GM-CSF expression has been observed in human GN (20), and intrinsic renal cells, specifically podocytes, mesangial and tubular epithelial cells, have been demonstrated to produce GM-CSF in vitro (21–23). Reactive oxygen species is a major inducer of podocyte GM-CSF and is suggested to be one of the major pathways in which Reactive oxygen species me-
diates podocyte injury (22). Tubular cells are also potent producers of GM-CSF (23), and this production can be induced in a dose-dependent manner by low molecular weight proteins (24).

Substantial evidence exists for the participation of renal parenchymal cells in the nephritogenic immune response and subsequent inflammatory response. We have previously shown that intrinsic renal cells participate in the formation of glomerular crescents, renal leukocyte accumulation, and impairment of renal function via their production of IFN-γ, IL-12, and TNF and expression of MHC II, IL-1 receptor I (IL-1RI), and CD40 molecules (25–30). In view of the potential for both recruited leukocytes and renal parenchymal cells to contribute to GM-CSF production in crescentic GN, we sought to determine which cell population is responsible for the pathogenetic GM-CSF production. Our hypothesis is that in addition to the predicted important role of leukocyte GM-CSF, intrinsic renal cells contribute to the effector response in crescentic GN via GM-CSF production.

Materials and Methods

Initiation of GN and Experimental Design
Mice with a genetic deletion of GM-CSF on a C57BL/6 × 129Ola background and littermate controls C57BL/6 × 129Ola (WT) were bred and housed in a specific pathogen-free facility at Monash Medical Centre, Monash University (Clayton, Victoria, Australia). All animal procedures were approved by the Monash Medical Centre Animal Ethics Committee. Sheep anti-mouse GBM globulin was prepared as described previously (7). Nonaccelerated anti-GBM was induced in 13-wk-old WT and GM-CSF−/− mice by intravenous injection of 5.15 mg of anti-GBM globulin in two divided doses 3 h apart (total dose = 10.3 mg). For studies in chimeric animals, irradiation and bone marrow transplantation were performed when the mice were between 5 and 6 wk of age. GN was induced 8 wk after transplantation as described previously (7), and results are expressed as percent of kidney containing crescents. All mice that developed GN were challenged 24 h (day 20) before the end of the experiment by intradermal injection of 500 μg of sheep globulin in 50 μl of PBS into the right hind plantar surface of the footpad. The same dose of an irrelevant Ag (horse globulin) was injected into the contralateral footpad. Swelling was measured after 24 h using a micrometer (Mitutoyo, Kawasaki-Shi, Japan). DTH was assessed as the difference in swelling between the two footpads and is expressed as millimeters.

Assessment of Systemic Immune Responses

Humoral Immune Responses to Sheep Globulin. Mouse anti-sheep globulin antibody titers were measured by ELISA on serum collected at the end of each experiment. Assays were performed using microtiter plates that were coated with sheep globulin at a concentration of 10 μg/ml, and bound mouse Ig was detected using horseradish peroxidase–conjugated sheep anti-mouse Ig antibody (Amersham, Little Chalfont, UK), as described previously (31).

Induction of Dermal DTH to Sheep Globulin. All mice that developed GN were challenged 24 h (day 20) before the end of the experiment by intradermal injection of 500 μg of sheep globulin in 50 μl of PBS into the right hind plantar surface of the footpad. The same dose of an irrelevant Ag (horse globulin) was injected into the contralateral footpad. Swelling was measured after 24 h using a micrometer (Mitutoyo, Kawasaki-Shi, Japan). DTH was assessed as the difference in swelling between the two footpads and is expressed as millimeters.

Assessment of Renal Histology and Leukocyte Infiltration
For assessing glomerular crescent formation, kidney tissue was fixed in Bouin’s fixative and embedded in paraffin, and 4-μm sections were stained with periodic acid-Schiff (PAS) reagent. Glomeruli were considered to exhibit crescent formation when two or more layers of cells were observed in Bowman’s space. In a blinded protocol, a minimum of 50 glomeruli was assessed to determine the crescent score for each animal. The number of interstitial cells in periodic acid-Schiff–stained renal tissue sections was also assessed blindly. Five randomly selected cortical areas, which excluded glomeruli, were counted for each animal using a 10-mm2 graticule fitted in the eyepiece of the microscope. Each high-power field represented an area of 1 mm2. Data are expressed as cells/mm2 and represents the mean ± SEM for four animals in each group.

For determining the extent of inflammatory cell influx into glomeruli, kidney tissue and spleen were fixed in periodate/lysine/paraformaldehyde. Six-micrometer cryostat-cut sections were stained to demonstrate CD4+ T cells and macrophages with mAb GK1.5 and M1/70, respectively, using a three-layer immunoperoxidase technique (16). A minimum of 20 equatorially sectioned glomeruli were assessed blindly, and results are expressed as cells per glomerular cross section (c/gcs).

Renal Adhesion Molecule and Cytokine Expression

RNA Extraction. Total kidney RNA was extracted with TRIzol reagent (Life Technologies, Victoria, Australia) according to the manufacturer’s protocol. The final product was air dried and dissolved in DNase/RNase-free water (Life Technologies) and stored at −70°C. The concentration of RNA was determined by spectroscopy at 260 nm.

RNase Protection Assay. Total kidney RNA was assessed by the RiboQuant system (BD Bioscience/PharMingen, San Diego, CA) as previously reported (25). Image Gauge software (version 3.46; Fuji Photo Film, Tokyo, Japan) was used to evaluate the gel image. Adhesion molecule and cytokine expression were measured and normalized to the housekeeping gene L32. The results for E-selectin, intercellular adhesion molecule-1 (ICAM-1), IL-1β, migration inhibitory factor, vascular cellular adhesion molecule-1 (VCAM-1), IL-18, and IL-1RI were sufficient to allow quantitative analysis.

Reverse Transcription–PCR Assessment of Renal IFN-γ and TNF mRNA. Total kidney RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and the method outlined by the manufacturer. Concentration and purity were determined by measuring absorbance at 260 and 280 nm and by the A260/A280 ratio. One microgram of total RNA was treated with 1 unit of amplification-grade DNase I (Invitrogen) to remove any contaminating genomic DNA. DNase-treated total...
RNA was primed with 500 ng of Oligo(dT)\textsubscript{12,18} (Roche, Mannheim, Germany) and reverse transcribed with Super Script II (Invitrogen) according to the manufacturer’s specifications to produce cDNA. Gene-specific primers for mouse IFN-\(\gamma\) and mouse \(\beta\)-actin were designed using Vector NTI software (Invitrogen). IFN-\(\gamma\), TNF-\(\alpha\), and \(\beta\)-actin cDNA was amplified using the primers (IFN-\(\gamma\) F 5\'-GAAAGACAATCAGGCCCATCA-3\' and R 5\'-TTGCTGTGCTGAAGAAGGT-3\', TNF-\(\alpha\) F 5\'-GCCTCCTTCATTCTCCTGCT-3\' and R 5\'-CATTCTGGTGTTTTGCTACGA-3\', and (\(\beta\)-actin) F 5\'-AGGCTGCTGCTGCCCTGTAT-3\' and R 5\'-AAGGAAGGCTGAAAGACG-3\') to produce PCR products of 78 bp (IFN-\(\gamma\)), 203 bp (TNF-\(\alpha\)), and 388 bp (\(\beta\)-actin). Real-time PCR was performed on a Rotor Gene RG-3000 (Corbett Research, Sydney, Australia) using Faststart DNA master and Sybr Green I (Roche) according to the manufacturer’s specifications. Quantification of IFN-\(\gamma\), TNF-\(\alpha\), and \(\beta\)-actin mRNA was performed using serial dilutions of an exogenous standard. IFN-\(\gamma\) and TNF-\(\alpha\) levels were normalized to \(\beta\)-actin and expressed as a percentage. PCR products were confirmed by melt-curve analysis.

**Quantification of MHC Class II, GM-CSF, TNF, P-Selectin, CD40, and MCP-1 Expression.** A single-layer immunofluorescence protocol was performed for semiquantitative assessment of GM-CSF, TNF, MHC II, and MCP-1 protein expression. Briefly, cryostat-cut, snap-frozen kidney tissue sections (6 \(\mu\)m) were blocked with 10% normal rat serum in 5% BSA/PBS and then incubated for 60 min at room temperature with a directly conjugated Ab at a determined concentration: FITC-conjugated rat anti-mouse GM-CSF antibody (clone MP1–22E9; BD PharMingen) used at a 1:20 dilution, rat anti-mouse MHC II antibody (clone Y3P; a gift from Prof. K. Shortman, Walter and Eliza Hall Institute, Melbourne, Australia) was conjugated to an Alexa Flour dye 488 (Molecular Probes, Eugene OR) used at a final dilution of 1:50, and finally PE-conjugated hamster anti-rat/mouse MCP-1 (clone 2H5; BD Biosciences/PharMingen) was used at a dilution of 1:20. All sections were assessed in a blinded protocol; a minimum of 50 glomeruli per mouse were scored semiquantitatively (0 to 3\(^+\)), with 0 being equivalent to background and 3\(^+\) equal to the most intense fluorescence observed.

Renal P-selectin was identified using a two-layer immunofluorescence method with a rabbit anti-human P-selectin (a gift from Dr. M. Berndt, Monash University) Ab, as described previously (32). CD40 expression was detected by immunohistochemical ABC staining with rat anti-mouse CD40 (clone 3/23; BD Bioscience/PharMingen) as the primary Ab. Acetone-fixed kidney sections (6 \(\mu\)m) underwent citrate buffer Ag retrieval and endogenous biotin blocking (Dako, Botany Bay, Australia) before being incubated overnight at 4\(^\circ\)C with the primary Ab at a dilution of 1:50. The secondary Ab used was rabbit anti-rat biotin (BD Biosciences), and CD40 was detected by using an avidin-biotin complex detection system (Dako). Sections were counterstained with nuclear fast red.

**Functional Assessment of Glomerular Injury**

**Proteinuria.** Mice were housed individually in cages to collect urine before administration of anti-GBM globulin and during the final 24 h (day 20) of the experiment. Urinary protein concentrations were determined by a modified Bradford method. Before induction of GN, all groups of mice had 24-h urine protein excretion in the normal range (0.5 to 2.0 mg/24 h).

**Serum Creatinine.** Serum creatinine concentrations were measured by an enzymatic creatininase assay.

**Results**

**Immune Activation Is Intact in GM-CSF\(^--/\)- and Chimeric Mice**

Mice were sensitized by intravenous injection of sheep globulin; 20 d later, mice were challenged intradermally with sheep globulin and horse globulin (in the opposite footpad as a control Ag). Antigen-specific DTH was determined by the difference in resulting footpad swelling between Ag- and control-injected footpads, 24 h after challenge. WT mice developed significant Ag-specific swelling (0.50 ± 0.04 \(\Delta\)mm), whereas Ag-specific DTH was significantly reduced in GM-CSF\(^--/\)- mice (0.13 ± 0.02 \(\Delta\)mm; \(P < 0.05\)) as we have previously reported (7).

In both groups of chimeric mice, dermal DTH was not significantly reduced compared with WT mice (GM-CSF\(^--/\)-\(→\)WT 0.31 ± 0.05 \(\Delta\)mm; WT\(→\)GM-CSF\(^--/\)- 0.35 ± 0.09 \(\Delta\)mm), indicating that GM-CSF from either leukocytes or resident dermal cells is sufficient for this CD4\(^+\) T cell–directed cutaneous inflammatory response (Figure 1A).

Circulating titers of mouse anti-sheep globulin Ab were measured as an index of systemic humoral immune responses to sheep globulin. No differences were observed in the titers of sheep globulin–specific Ab in serum that was collected 21 d after immunization in either GM-CSF\(^--/\)- mice or chimeras (Figure 1B).

![Figure 1](image-url) - (A) Dermal delayed-type hypersensitivity (DTH) responses to sheep globulin in wild-type (WT) mice and GM-CSF\(^--/\)-\(→\)WT, WT\(→\)GM-CSF\(^--/\)- chimeric mice, and GM-CSF\(^--/\)- mice with glomerulonephritis (GN; 21 d after intravenous injection of sheep globulin), \(* P < 0.05\) compared with (c.f.) WT. (B) Serum titers of mouse anti-sheep globulin Ig in WT (■), GM-CSF\(^--/\)-\(→\)WT (○), WT\(→\)GM-CSF\(^--/\)- (□), and GM-CSF\(^--/\)- (○) mice developing crescentic GN. ▼, Ig titers observed in normal WT mice.
Effects of Restricted GM-CSF Expression on Glomerular Leukocyte Recruitment and Development of Crescentic GN

WT mice developed severe proliferative crescentic GN (Figure 2), with 33 ± 2.9% of glomeruli showing crescent formation (Figure 3A). The development of crescents in WT mice correlated with a substantial influx of inflammatory cells into the glomerulus (T cells 0.9 ± 0.08 c/gcs; macrophages 2.1 ± 0.23 c/gcs) and prominent glomerular MHC II expression (Figure 3B). GM-CSF was abundantly expressed in interstitial and intraglomerular areas as well as on tubular cells. Expression in glomeruli was associated with both leukocytes and intrinsic renal cells (Figure 4A).

Absent intrinsic renal cell GM-CSF production (WT→GM-CSF−/− chimeras) with intact leukocyte GM-CSF (demonstrated by confocal microscopy; Figure 4B) resulted in a significant attenuation of crescent formation compared with both WT and GM-CSF−/−→WT chimeras. Whereas glomerular T cell recruitment was unaffected with similar glomerular T cell numbers to WT mice (WT→GM-CSF−/− chimeras 0.80 ± 0.19 c/gcs; WT 0.9 ± 0.08 c/gcs), significant attenuation of glomerular macrophage recruitment was observed (WT→GM-CSF−/− chimeras 1.1 ± 0.18 c/gcs; WT 2.1 ± 0.23 c/gcs; P < 0.05). Furthermore, glomerular MHC II expression was significantly reduced (WT→GM-CSF−/− chimeras 0.40 ± 0.05; WT 0.87 ± 0.05; P < 0.05).

Chimeras with intact GM-CSF production by intrinsic renal cell but absent production by infiltrating leukocytes (Figure 4C) showed similar crescent formation (GM-CSF−/−→WT, 30 ± 2.7% glomeruli) and glomerular T cell recruitment (GM-CSF−/−→WT 0.87 ± 0.06 c/gcs) to WT mice. Despite significantly attenuated macrophage recruitment (GM-CSF−/−→WT 1.34 ± 0.28 c/gcs), glomerular MHC II expression was similar to WT levels, suggesting that renal-derived GM-CSF may significantly contribute to activation of leukocytes after their glomerular recruitment.

Effects of Restricted GM-CSF Production on Functional Renal Injury

WT mice developed renal impairment with elevated proteinuria (5.95 ± 0.5 mg/24 h; normal 0.98 ± 0.2 mg/24 h) and serum creatinine (27.12 ± 2.25 μmol/L; normal 9.8 ± 1.19 μmol/L). GM-CSF−/− and chimeric groups were moderately protected from renal impairment with a minimal rise in proteinuria and serum creatinine from normal range values. In comparison with WT mice, proteinuria was significantly attenuated in GM-CSF−/−→WT (4.4 ± 0.3 mg/24 h; P < 0.01 compared with (c.f.) WT) and WT→GM-CSF−/− (3.5 ± 0.3 mg/24 h; P < 0.001 c.f. WT) chimeras; however, serum creatinine was significantly reduced only in bone marrow–negative chimeric mice (GM-CSF−/−→WT 17.77 ± 1.42 μmol/L; P < 0.01 c.f. WT). Serum creatinine was slightly decreased in kidney-negative WT→GM-CSF−/− (24.46 ± 2.2 μmol/L) chimeras, but this was not statistically significant (Figure 5).

Both Intrinsic Renal GM-CSF and Immune GM-CSF Participate in Tubulointerstitial Inflammation

Tubulointerstitial inflammation is an important sequela of proliferative and crescentic forms of GN. WT developed a marked interstitial cellular infiltrate (172 ± 7 cells/mm²), whereas GM-CSF−/− mice displayed limited cell recruitment into the interstitium (108 ± 5 cells/mm²; P < 0.001 c.f. WT). The interstitial infiltrate was also significantly attenuated in both WT→GM-CSF−/− (134 ± 5 cells/mm²) and GM-CSF−/−→WT (128 ± 4 cells/mm²) chimeras (both P < 0.001 c.f. WT). This was accompanied by reductions in both macrophages and T cells in the interstitium of chimeric animals (data not shown). Thus, both cellular sources of GM-CSF contribute to interstitial inflammation (Figure 5).

The tubulointerstitial changes noted in WT mice were associated with impairment of renal function indicated by elevated proteinuria and serum creatinine. A reduction of tubulointerstitial inflammation and less functional renal injury was also observed in chimeric groups and GM-CSF−/− animals (Figure 5).

Renal GM-CSF Regulates VCAM-1 and MCP-1 Expression

Renal mRNA expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin was analyzed by an RNAse protection assay. WT, GM−/→WT, and WT→GM-CSF−/− mice with GN all had equivalent expression of ICAM-1 and E-selectin. However, VCAM-1 mRNA was significantly down-regulated in GM-CSF−/− mice and kidney-negative WT→GM-CSF−/− chimeras (P < 0.05) compared with elevated levels observed in WT and GM-CSF−/−→WT chimeras. Renal P-selectin and MCP-1 were assessed semiquantitatively by immunofluorescence. P-selectin expression was not different be-
between the groups; however, MCP-1 expression was significantly elevated in the WT and GM-CSF<sup>−/−</sup> → WT kidney-positive chimeric mice, suggesting that intrinsic renal cell GM-CSF augments MCP-1 expression. A significant decrease in the number of glomerular cells expressing CD40 was observed in the GM-CSF<sup>−/−</sup> mice, whereas in both chimeric groups and WT mice, the number of CD40-expressing cells was similar (Figure 6).

Renal GM-CSF Selectively Regulates Renal Cytokines

Expression of IL-1β and IL-RI mRNA was assessed by an RNAse protection assay, TNF and IFN-γ mRNA by reverse transcription-PCR, and TNF protein expression by immunofluorescence. IL-1β mRNA was significantly attenuated in GM-CSF<sup>−/−</sup> and WT → GM-CSF<sup>−/−</sup> chimeras (P < 0.05) compared with WT and GM-CSF<sup>−/−</sup> → WT chimeras, suggesting that GM-CSF from intrinsic renal cells has a role in amplifying IL-1β renal expression. IL-1RI mRNA levels remained unchanged in all groups. Either intrinsic renal cells or leukocytes were a sufficient source of GM-CSF for the induction of IFN-γ mRNA, as production was similarly intact in both types of chimeras and WT mice and significantly greater than in GM-CSF<sup>−/−</sup> mice. Likewise, TNF protein expression was similar in WT, GM-CSF<sup>−/−</sup> → WT, and WT → GM-CSF<sup>−/−</sup> chimeras and significantly attenuated in GM-CSF<sup>−/−</sup> mice. Unexpected, TNF mRNA was discordant with protein synthesis in GM-CSF<sup>−/−</sup> → WT kidney-positive chimeras with significantly raised in TNF mRNA compared with all other groups (Figure 7).

Discussion

Dysregulated expression of GM-CSF is associated with a range of pathologic conditions, including leukemias, and chronic inflammatory conditions such, as rheumatoid arthritis, EAE, and glomerulonephritis. As both leukocyte and many resident tissue cells produce GM-CSF, studies have attempted to determine the important cellular source of GM-CSF. A recent study has shown that EAE development was dependent on the local delivery of GM-CSF by retrovirally transduced Ag-specific T cells. Thus, overexpression of GM-CSF in the central nervous system drives the effector phase of EAE (33).

As both renal parenchymal cells and infiltrating immune
cells are capable of producing GM-CSF, it is important to determine whether GM-CSF produced by either or both cellular sources contribute to the exacerbation of GN. To address this issue, we studied the development of crescentic GN in chimeric mice: WT→GM-CSF−/− chimeric mice, which had restricted expression of GM-CSF to bone marrow–derived cells, and GM-CSF−/→WT chimeras, which had absent leukocyte GM-CSF but intact intrinsic renal cell ability to produce GM-CSF.

The effects of restricted GM-CSF expression on the development of crescentic GN indicated a major role for renal cell–derived GM-CSF in the formation of cellular crescents, glomerular MHC II expression, increased serum creatinine, MCP-1 production, and VCAM mRNA and IL-1β mRNA expression. These parameters were similar in GM-CSF−/−→WT kidney-positive chimeras and WT mice but significantly reduced in mice with total absence of GM-CSF and WT→GM-CSF−/− kidney-negative chimeric mice. In comparison, chimeras with GM-CSF–deficient bone marrow allowed the role of leukocyte–derived GM-CSF to be determined as relatively minor in the above-mentioned parameters. In the two chimeric groups, glomerular T cell recruitment, the number of CD40-positive cells in glomeruli, and renal IFN-γ mRNA and TNF protein expression were similar to elevated values measured in WT mice, indicating redundancy in the production of GM-CSF with either source being sufficient to drive these aspects of the inflammatory response.

Glomerular macrophages, proteinuria, and interstitial infiltrate were similar in both chimeras. These parameters of inflammation were significantly reduced from WT mice but also significantly increased compared with GM-CSF−/− mice, indicating that neither source of GM-CSF is capable of completely compensating for the other’s deficiency. Both sources are required for the full development of these aspects of glomerular injury in crescentic GN. Finally, IL-1RI, ICAM-1, P-selectin, and E-selectin expression was unaffected by the absence of either source of GM-CSF or its total absence, indicating that GM-CSF has no role in the regulation of these proinflammatory molecules.

As macrophage numbers were significantly reduced in both chimeric groups, we speculate that recruitment of monocytes into the inflamed kidney is particularly sensitive to GM-CSF levels. Several studies have demonstrated that GM-CSF promotes the infiltration of monocytes and macrophages after tissue injury (34,35). Furthermore, GM-CSF mRNA and protein synthesis occurs in both monocytes and endothelial cells as a result of direct cell-to-cell interaction (36). Therefore, GM-CSF produced locally (by both renal cells and bone marrow–derived cells) via monocyte–renal cell interactions may modulate adhesive interactions between intrinsic renal cells and monocytes and consequently the recruitment of macrophages into the glomerular lesion.

Despite glomerular macrophage numbers’ being significantly decreased in leukocyte-deficient GM-CSF chimeras, MHC II expression was unaffected. As MHC II is regarded as an activation marker for macrophages (37–39)/mesangial cells (40), this finding indicates that renal GM-CSF plays a dominant role in local activation of macrophages and/or mesangial cells. We demonstrated previously the dependence of cell-mediated immune renal injury in anti-GBM GN on intrinsic renal cell expression of MHC II (30). GM-CSF induces MHC II expression on the surface of bone marrow–derived monocytes (41), elevates MHC II expression on macrophages, and skew the cytokine response of T cells toward Th1 (42). Hence, renal cell–derived GM-CSF may be vital to sustain an elevated expression of MHC II on macrophages and thus helping to sustain the local Th1 biased response and exacerbate GN.

The correlation between glomerulopathies associated with proteinuria and progressive tubulointerstitial injury has been documented extensively in a clinical setting. One obvious explanation for this relationship is that the magnitude of proteinuria is a reflection of the severity of the underlying glomerular disease (43). Although some evidence suggests that proteinuria may contribute directly to renal injury (specifically tubular and interstitial changes), it remains unclear whether proteinuria is a cause or a consequence of progressive renal injury (24). In this study, GM-CSF−/−→WT chimeric mice developed severe glomerular crescent formation accompanied by glomerular MHC II expression but significantly attenuated proteinuria and tubulointerstitial inflammation. Our studies suggest that events in-
lower serum creatinine levels were demonstrated in WT and GM-CSF
activated serum creatinine demonstrated impaired renal function in
line indicates normal proteinuria in nonnephritic mice). (B) Ele-
significantly reduced proteinuria compared with WT mice (dotted
interstitial infiltrate values in nonnephritic mice).
Figure 5. Functional indices of injury and interstitial inflammation
in WT, GM-CSF chimeric, and GM-CSF/−/− mice with GN. (A)
WT mice with GN developed significant proteinuria, whereas
both groups of GM-CSF chimeras and GM-CSF/−/− mice had
significantly reduced proteinuria compared with WT mice (dotted
line indicates normal proteinuria in nonnephritic mice). (B) Elevated
serum creatinine demonstrated impaired renal function in
WT and GM-CSF/−/−→WT chimeric mice, whereas significantly
lower serum creatinine levels were demonstrated in WT→GM-
CSF/−/− chimeric and GM-CSF/−/− mice (dotted line indicates
normal serum creatinine in nonnephritic mice). (C) WT mice
displayed a marked increase in the number of inflammatory cells into
the tubulointerstitial areas, whereas the GM-CSF chimeric and
GM-CSF/−/− mice did not demonstrate augmented inflammatory
cell infiltrate into the interstitium (dotted line indicates normal inter-
stitial infiltrate values in nonnephritic mice). *P < 0.05 c.f. WT mice;
■P < 0.05 c.f. GM-CSF/−/−→WT kidney-negative chimeric mice.

dependent from the glomerular capillary circulation can con-
tribute to pathogenic proteinuria. Support for the pathogenic
role of interstitial mononuclear inflammatory cell infiltration
has come from studies in which reduced infiltrates have been
associated with attenuated renal injury and preserved renal
function (44–47).

As the direct adhesive interaction of monocytes and endo-
thelial cells is one proposed stage affected in both chimeric
mice, we explored the expression of adhesion molecules in-
volved in leukocyte emigration from vessels during inflamma-
tion: ICAM-1, VCAM-1, P-selectin, and E-selectin. We found
renal ICAM-1, P-selectin, and E-selectin expression to be equiv-
alent in WT, GM-CSF/−/→WT, and WT→GM-CSF/−/− mice
with GN. However, VCAM-1 expression was significantly down-
regulated in WT→GM-CSF/−/− chimeras (P < 0.05) compared
with elevated levels observed in WT and GM-CSF/−/−→WT chimeras. Substantial expression of renal VCAM-1 has been demonstrated
in proliferative and crescentic forms of nephritis both clinically and experimentally (48–50). In the first 7 d of a nephro-
toxic nephritis model, administration of anti–VCAM-1 mAb had
little effect on disease progression (49). However, a role for
VCAM-1 in the later development of renal injury has been dem-
strated. In a mercuric chloride model of nephritis, administra-
tion of anti–VCAM-1 mAb inhibited leukocyte recruitment into
the renal interstitium (51), whereas tubular expression of VCAM-1
seems to be associated with the degree of histologic damage (52).
Thus, GM-CSF produced by tubular cells may upregulate tubu-
lointerstitial VCAM-1 expression, which influences interstitial
inflammation and renal function.

The pathway of activation and interaction between intrinsic
renal cells and infiltrating immune cells invariably involves
proinflammatory cytokines. We previously demonstrated dif-
ferring contributions of IFN-γ, IL-12, TNF, IL-1, and IL-1RI to
this complex local inflammatory response. As GM-CSF has the
capacity to be involved in the upregulation of TNF, IL-1β,
MCP-1, and IL-6 (6), we explored possible mechanisms by
which renal- and leukocyte-derived GM-CSF may amplify in-
jury. Our studies demonstrate that GM-CSF does not influence
the expression of IL-1RI in the kidney and that either cellular
source of GM-CSF is capable of augmenting IFN-γ mRNA or
TNF protein expression. Finally, we demonstrated that GM-
CSF may influence the inflammatory phase of crescentic GN
and tubular injury via IL-1β and MCP-1. A significant reduction
of IL-1β mRNA and MCP-1 expression in WT→GM-CSF/−/−–
kidney-negative chimeras (P < 0.05) was observed, whereas
both were significantly increased in WT and GM-CSF/−/−→WT
kidney-positive chimeras. Therefore, we propose that GM-CSF
released by renal parenchymal cells increases the numbers of
activated macrophages that are capable of releasing further
inflammatory mediators (e.g., IL-1β), previously demonstrated
to be critical in the progression of GN (29). Mesangial cells
produce MCP-1 in response to IL-1β (53); furthermore, blocking
MCP-1 via Ab reduces glomerular injury and proteinuria
(54,55). Hence, renal GM-CSF may regulate glomerular and
renal injury through MCP-1 by influencing IL-1β or in an
autocrine feedback loop as renal cells have been demonstrated
to be the principal producers of MCP-1 (56).
These findings allow us to conclude that renal parenchymal GM-CSF production is essential for the development of crescents and the elevation of serum creatinine. Investigation of the underlying mechanistic pathways of GM-CSF–mediated injury highlighted the activation of monocytes/mesangial cells (MHC II expression) and the amplification of local inflammation by increasing MCP-1, IL-1β, and VCAM production to be regulated by renal-derived GM-CSF. Furthermore, GM-CSF derived from both infiltrating immune cells and renal parenchymal cells mediate tubulointerstitial inflammation, macrophage accumulation, and proteinuria in murine crescentic nephritis.

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Figure 6. Renal expression of vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin, CD40, and monocyte chemoattractant protein-1 (MCP-1) in WT, GM-CSF chimeric, and GM-CSF+/−/− mice. Renal VCAM-1, ICAM-1, and E-selectin expression relative to the housekeeping gene L32 assessed by RNAse protection assay and results are expressed in arbitrary units (AU). P-selectin and MCP-1 expression was assessed semiquantitatively by immunofluorescence, scored 0 to 3+. Glomerular CD40-positive cells were detected by immunohistochemical ABC staining. WT, GM-CSF+/−/− → WT chimeras, WT → GM-CSF+/−, and GM-CSF+/− mice had equivalent expression of ICAM-1, P-selectin, and E-selectin. A significant amplification of VCAM-1 mRNA and MCP-1 protein expression was noted in WT mice and GM-CSF+/−/− → WT chimeras compared with WT → GM-CSF+/− chimeras and GM-CSF+/− mice. The number of CD40-positive cells in the glomeruli of GM-CSF+/− mice was significantly less than in chimeric and WT mice. *P < 0.05 c.f. WT mice; **P < 0.05 c.f. GM-CSF+/−/− → WT kidney-negative chimeric mice.
References


Figure 7. Renal expression of cytokines IL-1β and IL-1RI mRNA assessed by RNAse protection assay, IFN-γ and TNF mRNA quantified by reverse transcription–PCR, and TNF protein expression identified by immunofluorescence in WT, GM-CSF chimeric, and GM-CSF−/− mice. Renal IL-1β and IL-1RI expression relative to the housekeeping gene L32 as assessed by RNAse protection assay. Results are expressed in arbitrary units (AU). TNF expression was assessed semiquantitatively by immunofluorescence, scored 0 to 3+. WT, GM-CSF−/−→WT chimeras, WT→GM-CSF−/−, and GM-CSF−/− mice had equivalent expression of IL-1RI mRNA. Total GM-CSF deficiency resulted in decreased production of IFN-γ mRNA and TNF protein; however, either cellular source was capable of promoting production of IFN-γ/TNF to WT levels. The absence of GM-CSF from the renal parenchymal compartment significantly reduced renal IL-1β expression to a level similar to that seen in GM-CSF−/− mice. Intact renal-derived GM-CSF (GM-CSF−/−→WT) restored IL-1β production to WT levels. *P < 0.05.


