

# Effect of Granulocyte-Macrophage Colony-Stimulating Factor in Experimental Visceral Leishmaniasis

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## Abstract

GM-CSF induces three effects potentially beneficial in visceral leishmaniasis: blood monocyte mobilization, macrophage activation, and amelioration of granulocytopenia. To determine the experimental role and effect of GM-CSF in this intracellular infection, livers from *Leishmania donovani*-infected BALB/c mice were tested for GM-CSF mRNA expression and mice were treated with anti-GM-CSF antiserum or GM-CSF. *L. donovani* infection upregulated hepatic GM-CSF mRNA expression by 10-fold, and anti-GM-CSF treatment exacerbated visceral infection and tripled liver parasite burdens 4 wk after challenge. In euthymic mice with established infection, treatment with 1–5  $\mu\text{g}/\text{d}$  murine GM-CSF induced three dose-related effects: peripheral blood leukocytosis, preferential accumulation of myelomonocytic cells at visceral foci of infection, and leishmanicidal activity comparable to that achieved by IFN- $\gamma$ . These effects were either largely or entirely T cell dependent. Treatment with human GM-CSF also induced antileishmanial activity but with little effect on peripheral leukocyte number or tissue myelomonocytic cell influx; human G-CSF stimulated marked peripheral granulocytosis and neutrophil tissue accumulation but induced little antileishmanial effect. These results identify a role for endogenous GM-CSF in the initial host defense response to *L. donovani*, reemphasize the influx of monocyte as an effector cell, and indicate that GM-CSF can be used as an antileishmanial treatment. (*J. Clin. Invest.* 1995. 95:1183–1192.) **Key words:** leishmaniasis • *Leishmania donovani* • granulocyte-macrophage colony-stimulating factor • granulocyte colony-stimulating factor • monocyte

## Introduction

In experimental visceral leishmaniasis in susceptible BALB/c mice, successful acquisition of resistance to intracellular *Leishmania donovani* is T cell dependent and mediated primarily by cytokine-activated mononuclear phagocytes (1–3). In the liver, this response is expressed by the formation of granulomas composed of a core of fused, parasitized resident macrophages (Kupffer cells) encircled by a mononuclear cell mantle con-

taining both T lymphocytes and influx of blood monocytes (1, 4). The latter cells appear to be critical in defense against *L. donovani* since both acquired resistance and granuloma assembly are impaired by inhibiting monocyte recruitment to infected tissue foci (5). In addition, the blood monocyte also appears to be the primary target cell for IFN- $\gamma$  (5), a mononuclear phagocyte-activating cytokine instrumental in host defense against *L. donovani* (6, 7).

To extend this analysis of the role of the monocyte, the present report examines the antileishmanial effect of GM-CSF, a cytokine capable of directly increasing the number of circulating monocytes (8, 9). We also selected GM-CSF for study because it (a) stimulates macrophages by itself or with IFN- $\gamma$  to exert in vitro and in vivo effects against a number of intracellular pathogens, including *L. donovani* (10–17); (b) is now in clinical use (8, 9); and (c) is currently being tested in human visceral leishmaniasis (18).

## Methods

**Visceral infection and treatment with cytokines.** Euthymic (*nu/nu*) and athymic (*nu/nu*) 20–30-g female BALB/c mice (Life Sciences, Hialeah, FL) were infected via the tail vein with  $10^7$  *L. donovani* amastigotes obtained from hamster spleen homogenates (1). 1 d before infection, some nude mice were reconstituted by intravenous injection of  $10^7$  *nu/nu* spleen cells. The spleen cells transferred either were unfractionated or were first depleted by > 90% of either CD4<sup>+</sup> or CD8<sup>+</sup> cells by treatment with anti-L3T4 mAb (GK 1.5, ATCC TIB 207) or anti-Lyt-2 mAb (53-6.72, ATCC TIB 105; American Type Culture Collection, Rockville, MD) followed by complement as previously described (2).

2 wk after infection (day 0), groups of three to five mice were treated continuously with recombinant murine GM-CSF (MuGM-CSF) ( $10^8$  U/mg; Amgen, Thousand Oaks, CA) and/or with MuIFN- $\gamma$  ( $2 \times 10^7$  U/mg; Amgen). Continuous treatment was given via subcutaneous osmotic infusion pumps (Alzet model 2001, Alza Corp., Palo Alto, CA) implanted on the back (6, 19). According to the manufacturer, these 0.2-ml pumps empty their contents 7 d after insertion. The calculated rates of release were 0.5–5.0  $\mu\text{g}/\text{d}$  for MuGM-CSF and  $2.4 \times 10^5$  U/d for MuIFN- $\gamma$  (6). Mice were also treated in the same fashion with 0.5–10  $\mu\text{g}/\text{d}$  pump-delivered recombinant human GM-CSF (HuGM-CSF) ( $10^8$  U/mg; Amgen) or granulocyte colony-stimulating factor (HuG-CSF)<sup>1</sup> ( $10^8$  U/mg; Amgen). Controls were left untreated or received pump-delivered saline containing 1 mg/ml BSA (6, 19).

All mice were killed after 7 d of treatment (day +7) except when noted. The course of visceral infection was determined by microscopic examination of Giemsa-stained liver imprints (1). Liver parasite burdens, expressed as Leishman-Donovan units (LDU) (1), were calculated as follows: number of amastigotes per 500 hepatocyte nuclei  $\times$  liver weight (grams) (19). Observations were made per hepatocyte (19) rather than per total cell nuclei since GM-CSF and G-CSF treatment increased the number of myelomonocytic cells in the liver. The

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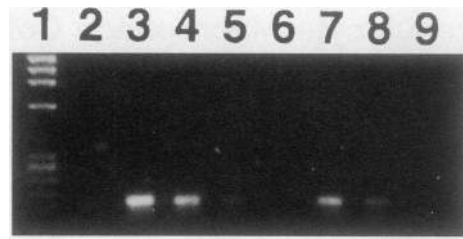
1. **Abbreviations used in this paper:** G-CSF, granulocyte colony-stimulating factor; LDU, Leishman-Donovan unit; RT-PCR, reverse transcriptase PCR; WBC, white blood cell.

histologic responses to infection and treatment were evaluated using formalin-fixed liver tissue stained with hematoxylin and eosin (1). Total white blood cell (WBC) and differential cell counts were performed using heparinized venous blood. Differences in liver parasite burdens and WBC counts between control and treated mice were analyzed by a two-tailed Student's *t* test (19).

**Anti-GM-CSF treatment.** Starting 1 d after *L. donovani* challenge and every 6 d thereafter for up to 8 wk, groups of 3–4 mice were injected intraperitoneally with 0.1 ml of normal rabbit serum or rabbit anti-MuGM-CSF antiserum. The dose and injection schedule was derived from *in vivo* titration experiments described in Results. To generate the antiserum, three New Zealand White adult rabbits were injected subcutaneously with 200  $\mu$ g of MuGM-CSF emulsified in Freund's complete adjuvant (20). On days 7, 21, 35, and 56, the rabbits were boosted subcutaneously with 200  $\mu$ g of MuGM-CSF in Freund's incomplete adjuvant. Rabbits were bled by ear vein on day 63, and serum was titered for antibody response to GM-CSF.

Reactivity to MuGM-CSF was first tested in a radioimmunoassay performed as follows. Using polystyrene wells (Immulon II, Dynatech Laboratories Inc., Alexandria, VA), GM-CSF was adsorbed to the surface at 0.5  $\mu$ g per well for 2 h at room temperature, and unreacted binding sites were blocked with 5% BSA. Dilutions of test and preimmune serum of  $\geq$  1:10 were then added to the wells for 2 h at room temperature. Wells were washed with an imidazole-buffered saline/Tween 20 wash solution (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD).  $^{125}$ I-labeled staphylococcal protein A (70–100  $\mu$ Ci/ $\mu$ g; Du Pont/New England Nuclear, Boston, MA) was then added to wells and incubated for 1 h at room temperature. After a final wash, wells were counted in a gamma counter, and bound radioactivity was graphed versus antibody dilution. Titer was defined as the serum dilution at which 50% of maximal specific counts bound was achieved. This titer was 1:3,000. Antiserum and preimmune serum were also titered against MuGM-CSF at 100 U/ml in a CFU-GM bioassay. Murine bone marrow cells from BDF<sub>1</sub> mice were depleted of adherent macrophages and T cells by plastic adherence on 100-mm dishes and treatment with anti-Thy 1.2 antibody followed by panning on plates coated with goat anti-mouse IgG, M, and A. The nonadherent T cell-depleted marrow cells were plated at  $4 \times 10^4$ /ml per plate in a single-layer 0.32% agarose system in IMDM media supplemented with 10% heat-inactivated FBS. Rabbit test and preimmune sera were heat inactivated at 56°C for 30 min before use. Sera at dilutions of 1:200, 1:400, and 1:800 were incubated for 1 h at room temperature with 100 U/ml GM-CSF in a total volume of 0.1 ml and then added to the agarose layer. Plates were incubated at 37°C in 5% CO<sub>2</sub>/95% air for 7–10 d, and colonies were counted using an inverted microscope. The antiserum showed complete neutralizing activity at 1:800, with no significant inhibition of colony formation by the preimmune serum.

**Expression of GM-CSF mRNA.** To determine relative levels of GM-CSF mRNA in uninfected versus infected liver samples, reverse transcriptase (RT-PCR) analysis for MuGM-CSF mRNA was performed using fully nested primers. Tissue preparation, cDNA synthesis, and initial PCR amplification were performed as described using 28–34 cycles of amplification (21). Each cDNA reaction contained 2  $\mu$ g of total RNA. Before the first round of GM-CSF amplification, cDNA samples were serially diluted by twofold and then amplified for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH) to show relative cDNA levels between samples; this amplification was in the log-linear phase. Adjusted amounts of cDNA (maximum adjustments were 1:1.3 or less between samples) were again amplified for GADPH using twofold serial dilutions to confirm that input cDNA was equal between samples. First-round GM-CSF PCR was then performed using the appropriate amounts of cDNA. Primers used for the first round of GM-CSF amplification (sense: TGTGGTCTACAGCCTCTCAGCAC; antisense: CAAAGGGGATATCAGTCACAAACC) flank three introns in the genomic sequence. cDNA from the initial GM-CSF amplification was serially diluted 10-fold and subjected to a second round of PCR amplification using 28 cycles and nested primers (sense: CCC-ACCCGCTACCCATCACTGTC; antisense: CCTGCTCGAATATC-



**Figure 1.** Serial dilution RT-PCR analysis of GM-CSF mRNA expression in homogenates of livers from uninfected and 2-wk infected BALB/c mice. Lane 1, size markers; lane 2, PCR control, no cDNA added. Lanes 3–5, infected liver; lanes 7–9, uninfected liver. Dilutions are as follows: 10-fold (lanes 3 and 7), 100-fold (lanes 4 and 8), and 1,000-fold (lanes 5 and 9). Results shown are representative of assays of livers from a total of 4 uninfected and 6 infected mice from two experiments.

TTACAGCGGG). The products of amplification were visualized on agarose gels. Each PCR was run with a negative control to exclude contamination of the reagents, and cDNA samples from different pairs of infected and uninfected liver samples were amplified at the same time.

***In vitro* activation of human macrophages.** Monocyte-derived human macrophages, first maintained for 5–7 d on glass coverslips (22), were treated for 3 d with HuGM-CSF (0.1–100 ng/ml), HuIFN- $\gamma$  (250 U/ml; Amgen) (22), or both cytokines in various combinations. These cultures were then challenged with  $5 \times 10^6$  *L. donovani* amastigotes. After 1 h, cultures were washed (time 0) and reincubated for 72 h in medium alone. Using duplicate coverslips, the number of amastigotes per 100 macrophages present microscopically at time 0 and 72 h was compared to determine the fold increase in intracellular replication (22).

## Results

**Effect of *L. donovani* infection on GM-CSF mRNA expression in the liver.** To determine whether the presence of *L. donovani* induced or upregulated GM-CSF mRNA expression *in vivo*, liver homogenates were tested by qualitative RT-PCR (21). GM-CSF mRNA expression was detected in livers of both uninfected and 2-wk infected mice. However, as shown in Fig. 1, semiquantitation (using serial 10-fold dilutions of input cDNA) suggested upregulation, with at least 10-fold more GM-CSF mRNA expressed in livers of infected mice.

**Role of endogenous GM-CSF.** In view of this upregulating effect and to determine the role of endogenous GM-CSF, BALB/c mice were injected every 6 d starting 1 d after *L. donovani* challenge with 0.1 ml of rabbit anti-GM-CSF antiserum. This dose and injection schedule was derived from initial experiments (Table I), which indicated that the enhancing effect of rMuGM-CSF treatment on the peripheral WBC count and tissue WBC influx (not shown; see the following discussion) could be entirely suppressed for 6 d by a single injection of  $\geq$  0.1 ml of antiserum.

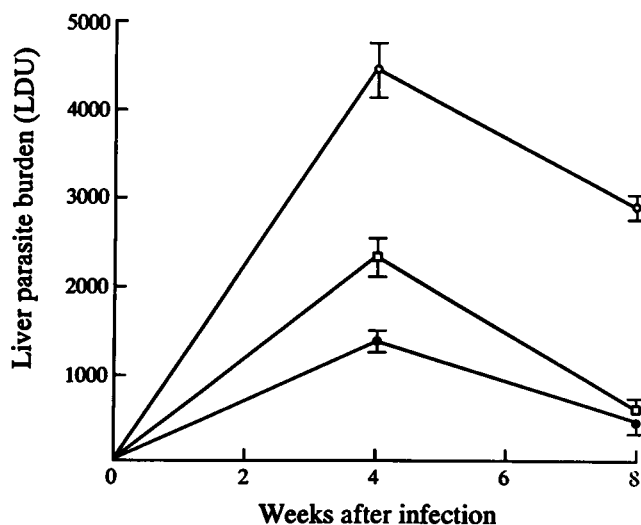
The results shown in Fig. 2 indicate that repeated injections of anti-GM-CSF antiserum exacerbated visceral infection, leading to a threefold increase in liver parasite burdens at week 4 ( $P < .05$  versus rabbit serum-injected controls). However, injections of rabbit serum alone also produced some increase in the level of infection at week 4, indicating a nonspecific component to this effect. After 8 wk of injections, liver burdens in animals treated with anti-GM-CSF antiserum were sevenfold higher than those in rabbit serum-treated mice; however, de-

**Table I. Dose-related Inhibition of GM-CSF-induced Leukocytosis by Anti-GM-CSF Antiserum**

Treatment	GM-CSF	WBC count	
		Day +6	Day +7
None	-	8.3±0.1	11.3±0.7
	+	38.1±4.0	67.2±7.4
Anti-GM-CSF 1.0 ml	+	6.7±0.8	9.5±0.8
	0.5 ml	10.5±0.6	12.7±0.4
	0.1 ml	10.2±0.5	28.9±1.4
Rabbit serum 1.0 ml	+	32.7±2.1	49.1±2.9

On day 0, mice received no treatment or a single intraperitoneal injection of 0.1–1.0 ml of anti-GM-CSF antiserum or 1.0 ml of normal rabbit serum. 2 h later, pumps delivering MuGM-CSF (2 µg/d) were inserted, and total peripheral blood WBC counts were determined after 6 or 7 d of GM-CSF treatment. Results are from one experiment representative of two performed and indicate mean±SEM values for 3 mice per group.

spite continued antiserum administration, visceral infection nevertheless came under control and liver parasite burdens began to decline (Fig. 2). This observation at week 8 suggested the emergence of a mechanism unrelated to endogenous GM-CSF during the resolution stage of visceral infection or perhaps suboptimal circulating levels of anti-GM-CSF activity after 8 wk of injections. The latter possibility seemed unlikely, however, in view of the following results. Infected mice were treated as in Fig. 2 every 6 d with 0.1-ml injections of anti-GM-CSF antiserum starting 1 d after challenge. After treatment for 7 wk (e.g., at the time of the ninth dose of antiserum), GM-CSF-



**Figure 2.** Effect of anti-GM-CSF treatment on visceral infection in BALB/c mice. Starting 1 d after infection and every 6 d thereafter, mice were injected with 0.1 ml of anti-GM-CSF antiserum (open circles) or normal rabbit serum (open squares); untreated mice (closed circles). Results are from two experiments and indicate mean±SEM values for 6–10 mice at each time point. *P* < 0.05 for differences between mice treated with anti-GM-CSF antiserum and rabbit serum-treated and untreated mice at weeks 4 and 8. Liver burdens in rabbit serum-treated versus untreated mice at weeks 4 and 8 were not significantly different.

**Table II. Circulating Anti-GM-CSF Activity in Antiserum-treated Mice**

Pretreatment of infected mice	GM-CSF	Peripheral WBC count on Day +56
None	-	5.9±0.3
	+	31.5±4.9*
Anti-GM-CSF	-	3.2±0.4
	+	6.4±0.9
Rabbit serum	-	7.2±0.9
	+	47.7±8.8*

Starting 1 d after infection and every 6 d thereafter for the following 7 wk, mice were treated with 0.1 ml of anti-GM-CSF antiserum or normal rabbit serum. On day +49, pumps delivering MuGM-CSF (2 µg/d) were inserted, and WBC counts ( $\times 10^3/\text{mm}^3$ ) were determined 1 wk later on day +56. Results indicate mean±SEM values for 6–9 mice from two experiments. See Fig. 4, *G* and *H*. \* Significantly different (*P* < 0.05) from untreated control value.

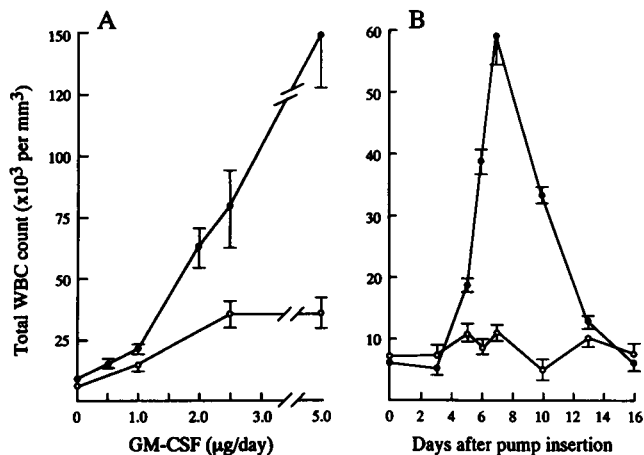
containing pumps (2.0 µg/d) were inserted, and WBC counts were measured 7 d later. These mice chronically treated with antiserum demonstrated sufficient circulating anti-GM-CSF activity to abolish both the peripheral leukocytosis-inducing effect of exogenous GM-CSF (Table II) and its capacity to enhance leukocyte influx into the liver (see Fig. 4, *G* and *H*). Controls treated identically with 0.1 ml of normal rabbit serum readily responded to GM-CSF with both leukocytosis (Table II) and myelomonocytic cell influx in the tissue (data not shown). We concluded from these findings that the capacity of mice treated for 8 wk with anti-GM-CSF antiserum to control and begin to reduce visceral infection was not likely to be the result of suboptimal levels of circulating antibody.

**Effect of exogenous GM-CSF treatment.** To determine whether GM-CSF in exogenous form could also induce anti-leishmanial activity, mice with established visceral infection were treated for 7 d with continuously administered cytokine beginning 2 wk after challenge (day 0). Whereas doses of  $\leq 1.0$  µg/d had little or no effect, treatment with 2.5 µg/d halted *L. donovani* replication, and 5.0 µg/d induced killing as judged by a 34% decrease in liver parasite burdens (day 0 LDU versus day +7 LDU) (Table III). The latter effect was comparable to

**Table III. Antileishmanial Effects of Treatment with MuGM-CSF**

Treatment of infected mice	Liver parasite burden	
	Day 0	Day +7
	LDU	
None (control)	1,560±82 (21)	2,480±181 (20)
BSA/saline		2,283±143 (12)
MuGM-CSF 1.0 µg/d		2,029±149 (10)
2.5		1,590±90 (16)*
5.0		1,034±44 (16)†
MuIFN-γ 2.5 × 10 <sup>5</sup> U/d		977±84 (12)†

2 wk after infection (day 0), the indicated treatments were started and given for 7 d. Results indicate mean±SEM values for (n) mice from three to five experiments. \* Significantly different (*P* < 0.05) from day +7 control value. † Significantly different (*P* < 0.05) from day 0 and day +7 control values.



**Figure 3.** Effect of MuGM-CSF treatment on total peripheral blood WBC count. (A) WBC counts in euthymic (closed circles) and nude (open circles) mice 7 d after insertion of pumps delivering the indicated doses of MuGM-CSF. Results (mean±SEM) are from two to four experiments with values for 9–13 euthymic and 6–12 nude mice at each of the indicated GM-CSF doses. (B) WBC counts in euthymic mice after insertion of pumps that delivered 2 µg/d MuGM-CSF (closed circles) or BSA/saline (open circles) during days 1–7. Results are from one experiment representative of two performed and indicate mean±SEM values for 3 mice at each time point.

that induced by treatment with pump-delivered IFN-γ (Table III), a previously identified antileishmanial cytokine (6). In separate experiments, two pumps, one containing GM-CSF and the other containing IFN-γ, were inserted on either side of the back. 7 d of simultaneous treatment with GM-CSF (5 µg/d) plus IFN-γ (2 × 10<sup>5</sup> U/d) (6), however, did not induce additional antileishmanial activity (two experiments; data not shown).

**Effect of GM-CSF on peripheral WBC count and tissue leukocyte influx.** Peripheral WBC counts in untreated mice infected for 2, 3, 4, or 8 wk (8.3±0.9, 9.2±0.8, 6.3±0.3, and 6.9±0.2 × 10<sup>3</sup>/mm<sup>3</sup>, respectively, *n* = 9–27 mice) were not appreciably different from those of uninfected controls (9.4±0.8 × 10<sup>3</sup>/mm<sup>3</sup>, *n* = 16). In contrast, in MuGM-CSF-treated mice, there were two prominent and linked leukocyte responses: a striking increase in peripheral WBC count and considerable influx of myelomonocytic cells into the liver. The data in Fig. 3 for euthymic mice summarize the dose-related increase in total WBC count and the kinetics of the peripheral blood leukocytosis induced by 7 d of GM-CSF treatment. WBC counts rapidly declined to baseline levels 5–6 d after the pumps had presumably run dry (Fig. 3 B). The effect of GM-CSF treatment on the differential percentages of circulating leukocytes is shown in Table IV.

Leukocytosis-inducing effects in the periphery were also accompanied by enhanced influx of cells into the liver (Fig. 4, A–D). Although MuGM-CSF stimulated similar increases in total peripheral blood WBC counts in both uninfected and 2-wk infected mice (data not shown), the distribution of influxing cells within the livers of these mice was quite different. In livers of uninfected mice treated with GM-CSF (Fig. 4, E and F), leukocyte infiltrates were less prominent and diffusely distributed, with few focal accumulations. In contrast, in infected livers (Fig. 4, C and D), influxing cells were much more plentiful

**Table IV.** Peripheral Blood WBC Differential Percentages

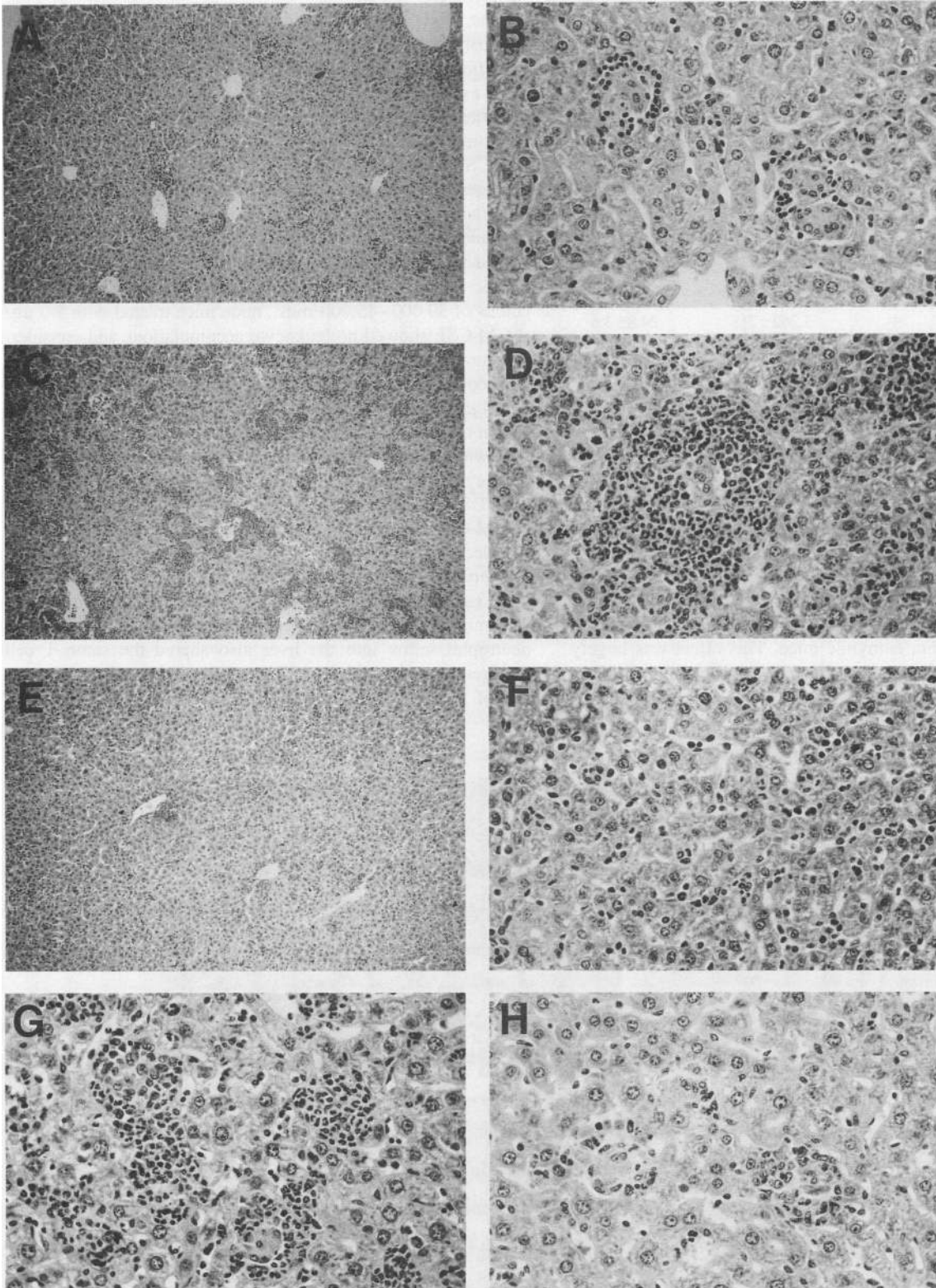
Mice and treatment	PMN*	Lymphocytes	Monocytes	Eosinophils
<b>Euthymic mice</b>				
Uninfected controls	11±2	86±3	2±1	< 1
Infected controls	9±1	83±2	8±2	< 1
+BSA/saline	12±2	80±2	8±1	< 1
+MuGM-CSF	55±7 <sup>†</sup>	18±3 <sup>†</sup>	18±3 <sup>†</sup>	3±1
+HuGM-CSF	36±5 <sup>†</sup>	51±5	13±1 <sup>†</sup>	< 1
+HuG-CSF	73±3 <sup>†</sup>	20±2 <sup>†</sup>	7±1	< 1
<b>Nude mice</b>				
Uninfected controls	35±2	38±5	6±2	11±1
Infected controls	35±4	57±4	7±1	1±1
+BSA/saline	29±2	64±5	5±2	2±1
+MuGM-CSF	56±7	22±5	17±4 <sup>†</sup>	5±1
+HuG-CSF	70±5 <sup>†</sup>	16±4 <sup>†</sup>	13±3	1±1

2 wk after infection, mice were left untreated (controls) or were treated for 7 d with saline, MuGM-CSF (5 µg/d), HuGM-CSF (2.5 µg/d), or HuG-CSF (10 µg/d). Results (given as percentages) are from two to four experiments and indicate mean±SEM values for 5–12 mice per group 7 d after treatment. \* Includes 0–5% band forms. <sup>†</sup> Significantly different (*P* < 0.05) from values for infected control mice.

and clearly accumulated in a focal fashion in areas of developing granulomas, and many infected foci were encased by layers of myelomonocytic cells (Fig. 4 D). In both uninfected and infected livers, neutrophils appeared to outnumber monocytes by approximately two- to threefold.

**Effect of anti-GM-CSF antiserum on WBC count and granuloma formation.** To complete this analysis, we also measured peripheral WBC counts and examined the histologic reaction in the infected mice treated with anti-GM-CSF antiserum in the experiments shown in Fig. 2. After 4 wk, mean WBC counts in mice treated with anti-GM-CSF antiserum (4.3±0.8) and rabbit serum (6.0±0.8) were similar to those of untreated controls (6.3±0.3) (× 10<sup>3</sup>/mm<sup>3</sup>, three experiments, 7–9 mice per group). After 8 wk, however, the WBC count had decreased by 56% in mice injected with anti-GM-CSF antiserum (Table II). At the same time, no differences were observed at either 4 or 8 wk between animals treated with anti-GM-CSF antiserum and control animals in their capacity to form hepatic granulomas (data not shown). Thus, although exogenous GM-CSF readily modified the tissue cellular response, endogenous GM-CSF was not apparently required for granuloma assembly or maintenance. Therefore, the capacity of anti-GM-CSF antiserum to impair initial acquired resistance and exacerbate visceral infection was not related to disruption of granuloma formation.

**Antileishmanial effect of GM-CSF in T cell-deficient mice.** We next treated *L. donovani*-infected nude mice with GM-CSF to determine whether its antileishmanial effect is direct or one that requires the presence of host T cells. Previous studies in this model have uniformly indicated such a requirement for the expression of the antileishmanial efficacy of IFN-γ, IL-2, and IL-12 (19, 23, 23a). In three experiments, pump treatment of 2-wk infected nude mice with 5 µg/d MuGM-CSF failed to induce any activity against *L. donovani*, and day +7 liver parasite burdens (LDU) in controls (3,640±252) and treated (5,029±305) mice were similar. As shown in Table V, however, a response to GM-CSF that was primarily leishmanistatic



**Figure 4.** Liver histologic reaction in euthymic BALB/c mice treated for 7 d with MuGM-CSF. (A and B) 3-wk infected controls not treated with GM-CSF show some focal cell accumulation with early granuloma formation. (C and D) Same mice treated with GM-CSF (5  $\mu\text{g}/\text{d}$ ) for the preceding 7 d demonstrate markedly enhanced leukocyte influx and large focal accumulations of myelomonocytic cells at areas of tissue infection and developing granulomas. (E and F) Normal uninfected mice treated with MuGM-CSF (5  $\mu\text{g}/\text{d}$ ) for 7 d show a diffusely distributed increase in leukocyte influx, with no focal cell accumulations. (G and H) Inhibition of GM-CSF-induced leukocyte influx by anti-GM-CSF antiserum pretreatment (see Table II). (G) 8-wk infected mice treated for the preceding 7 d with GM-CSF (2  $\mu\text{g}/\text{d}$ ). (H) Same 8-wk infected mice treated with 0.1 ml of anti-GM-CSF antiserum every 6 d for 7 wk before inserting a GM-CSF pump (2  $\mu\text{g}/\text{d}$ ) at the beginning of week 8.  $\times 50$  (A, C, and E);  $\times 200$  (B, D, and F-H).

Table V. Effect of MuGM-CSF Treatment in Reconstituted Nude Mice

Nude mice	GM-CSF	Day +7 liver burden	Day +7 WBC
		LDU	$\times 10^3/\text{mm}^3$
Control	-	3,943 $\pm$ 190	5.1 $\pm$ 0.6
	+	3,755 $\pm$ 133	22.7 $\pm$ 2.4
Reconstituted	-	4,096 $\pm$ 164	10.4 $\pm$ 0.9
	+	2,150 $\pm$ 181*	102.5 $\pm$ 12.0*
	+	1,758 $\pm$ 109*	40.0 $\pm$ 3.8*
	+	3,262 $\pm$ 218	24.8 $\pm$ 3.6

2 wk after infection (day 0), unreconstituted (control) nude mice and mice reconstituted with unfractionated (UNF), or CD8 cell- or CD4 cell-depleted *nu*+ spleen cells were left untreated or were treated for 7 d with pump-delivered MuGM-CSF (5  $\mu\text{g}/\text{d}$ ). Day 0 results for control nude mice ( $n = 6$ ) were 2,347 $\pm$ 191 LDU and WBC count of 4.6 $\pm$ 0.7  $\times 10^3/\text{mm}^3$ . Results indicate mean $\pm$ SEM from two experiments with 6 mice per group. \* Significantly different ( $P < 0.05$ ) from day +7 results in untreated and GM-CSF-treated control nude mice.

could be restored by reconstituting nude mice with unfractionated spleen cells from euthymic mice. This effect was largely inhibited by first depleting the transferred spleen cells of CD4<sup>+</sup> but not CD8<sup>+</sup> cells, suggesting that CD4<sup>+</sup> cells primarily medi-

ate the effect of or act in concert with GM-CSF to achieve antileishmanial activity.

**Effect of GM-CSF on WBC counts in nude mice.** These same experiments demonstrated that T cells can also regulate both the magnitude of the leukocytosis-inducing effect and the myelomonocytic cell tissue influx stimulated by exogenous GM-CSF. Although MuGM-CSF treatment increased the percentages of neutrophils and monocytes in the peripheral blood of both euthymic and nude mice (Table IV), total WBC counts plateaued in nude mice (Fig. 3 A), a finding that clearly contrasted with the effect of increasing doses of GM-CSF in euthymic animals. In addition, despite peripheral blood WBC counts of 30,000–45,000/ $\text{mm}^3$ , nude mice treated with 5.0  $\mu\text{g}/\text{d}$  GM-CSF showed no leukocyte accumulations and considerably fewer infiltrating cells in their livers than euthymic mice with comparable peripheral WBC counts induced by GM-CSF at 2.0  $\mu\text{g}/\text{d}$  (Fig. 5). Reconstitution with unfractionated *nu*+ spleen cells induced the capacity to respond to high dose GM-CSF with both peripheral leukocytosis (Table V) and enhanced leukocyte tissue influx (data not shown). Transfer of CD4<sup>+</sup> but not CD8<sup>+</sup> cells also augmented the leukocytosis-inducing effect of GM-CSF but not to the extent induced by unfractionated *nu*+ spleen cells.

Separate experiments in which rHuG-CSF was used as treatment (see the following section) provided the opportunity to determine whether optimal peripheral blood granulocytosis and neutrophil influx into the liver also shared the same T cell requirement. Although G-CSF treatment increased the percentage of neutrophils in peripheral blood of both euthymic and

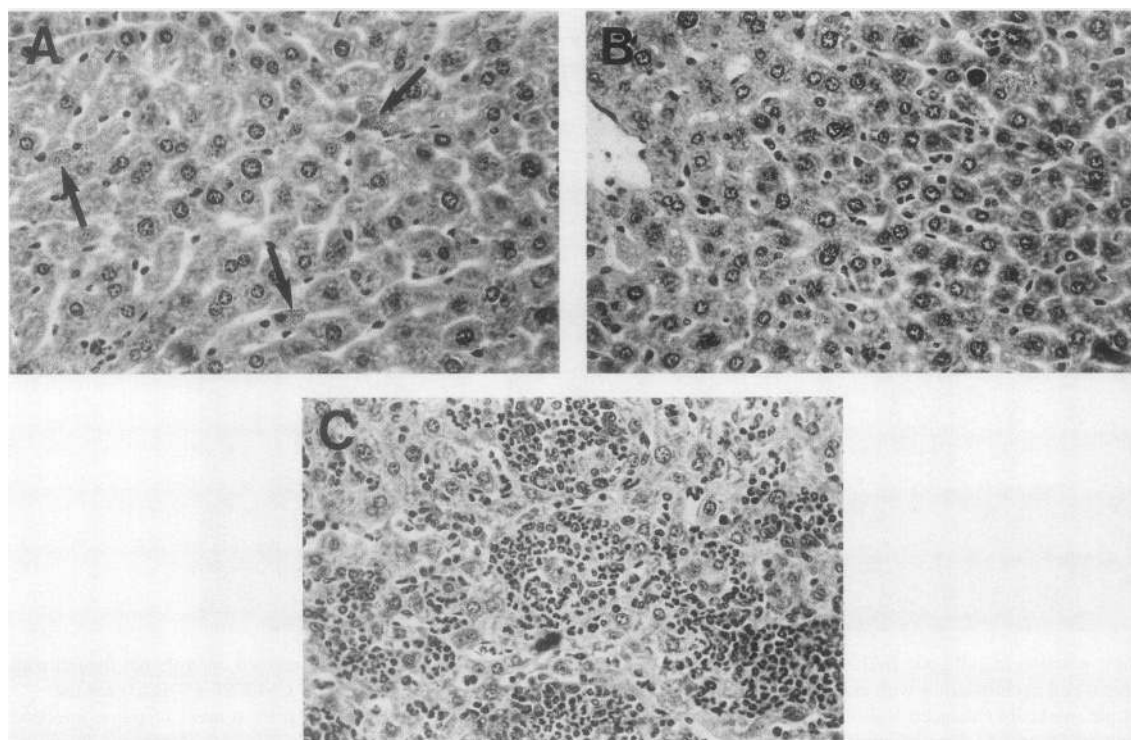


Figure 5. Liver histologic reaction in nude BALB/c mice treated with MuGM-CSF. (A) 3-wk infected nude mice not treated with GM-CSF show numerous infected foci (arrows) with no cellular response. (B) Treatment of the same mice with GM-CSF (5  $\mu\text{g}/\text{d}$ ) for the preceding 7 d induced little or no histologic response despite raising the mean peripheral blood total WBC count in the mice shown in B to 42,830/ $\text{mm}^3$ . (C) In contrast, the histologic reaction was strikingly altered in 3-wk infected euthymic mice, with a comparable mean peripheral WBC count (45,700/ $\text{mm}^3$ ) induced by 7 d of GM-CSF at 2  $\mu\text{g}/\text{d}$ .  $\times 200$ .



Table VI. Effect of Treatment with HuGM-CSF and HuG-CSF

Treatment for 7 d	Liver parasite burden	WBC count	
	Euthymic mice	Euthymic	Nude
None	2,975±106	8.0±0.6	4.5±0.6
BSA/saline	3,217±121	7.6±1.0	5.9±0.4
HuGM-CSF 0.5 µg/d	3,221±149	15.2±2.8	NT
1.0	2,294±160	17.9±2.1	4.2±0.6
2.5	1,407±92*	17.0±4.1	4.9±0.7
5.0	1,668±119*	13.1±1.3	NT
7.5	1,866±81	NT	NT
HuG-CSF 1.0 µg/d	3,076±194	34.5±8.7	9.9±1.6
2.5	2,456±143	80.5±7.5*	NT
5.0	2,658±106	85.4±6.3*	NT
10	2,401±172	83.5±9.9*	29.9±5.2*

2 wk after infection (day 0), just before beginning the indicated treatments, liver parasite burdens (given in LDU) were determined in euthymic mice (1,909±152), and peripheral blood WBC counts ( $\times 10^3/\text{mm}^3$ ) were determined in euthymic (6.8±0.4) and nude (4.0±0.4) mice. Data shown are results after 7 d of treatment, and indicate mean±SEM values from two to three experiments in euthymic mice ( $n = 6-13$  mice per group) and two experiments in nude mice ( $n = 6$  mice per group). NT, not tested. \* Significantly different ( $P < 0.05$ ) from untreated control day +7 value.

nude mice (Table IV), the data shown in Table VI and Fig. 6 for euthymic versus nude mice support the notion that T cells also participate in optimal peripheral and especially tissue neutrophil responses stimulated by exogenous G-CSF. Whereas treatment with G-CSF clearly modified the histologic reaction in euthymic mice, the same treatment had little or no effect on the largely absent tissue response to *L. donovani* in nude mice (Fig. 6).

**Effect of treatment with HuGM-CSF versus HuG-CSF.** In *in vitro* models using human cells, both monocytes (24) and neutrophils (25) can exert activity against *L. donovani*. Since MuGM-CSF treatment directed both types of phagocytes to infected tissue foci (Fig. 4 G), we attempted to distinguish the roles of monocytes plus neutrophils versus neutrophils alone by comparing the effects of treatment with GM-CSF versus G-CSF (Table VI). HuGM-CSF induced leishmanicidal effects but without a marked effect on either peripheral WBC count (Table VI) or histologic reaction (data not shown). In contrast, HuG-CSF treatment induced high WBC counts with granulocytosis and quite striking accumulation of influxing neutrophils and apparently immature myeloid cells discretely focused at sites of tissue infection (Table VI; Fig. 6). Although the latter effects were associated with modest inhibition of *L. donovani* visceral replication, G-CSF did not induce leishmanicidal activity (Table VI). Thus, these results with human cytokine preparations suggested that the capacity of MuGM-CSF to mobilize monocytes in addition to neutrophils may be important in inducing optimal antileishmanial activity *in vivo*. In addition, treatment with 2.5 µg/d HuGM-CSF reduced liver parasite burdens without appreciably increasing peripheral WBC count or promoting leukocyte influx into the liver. This observation suggested that a portion of the response to GM-CSF may reflect a direct activating effect on resident tissue macrophages and/or

on blood monocytes that are normally attracted to infected foci (4, 5).

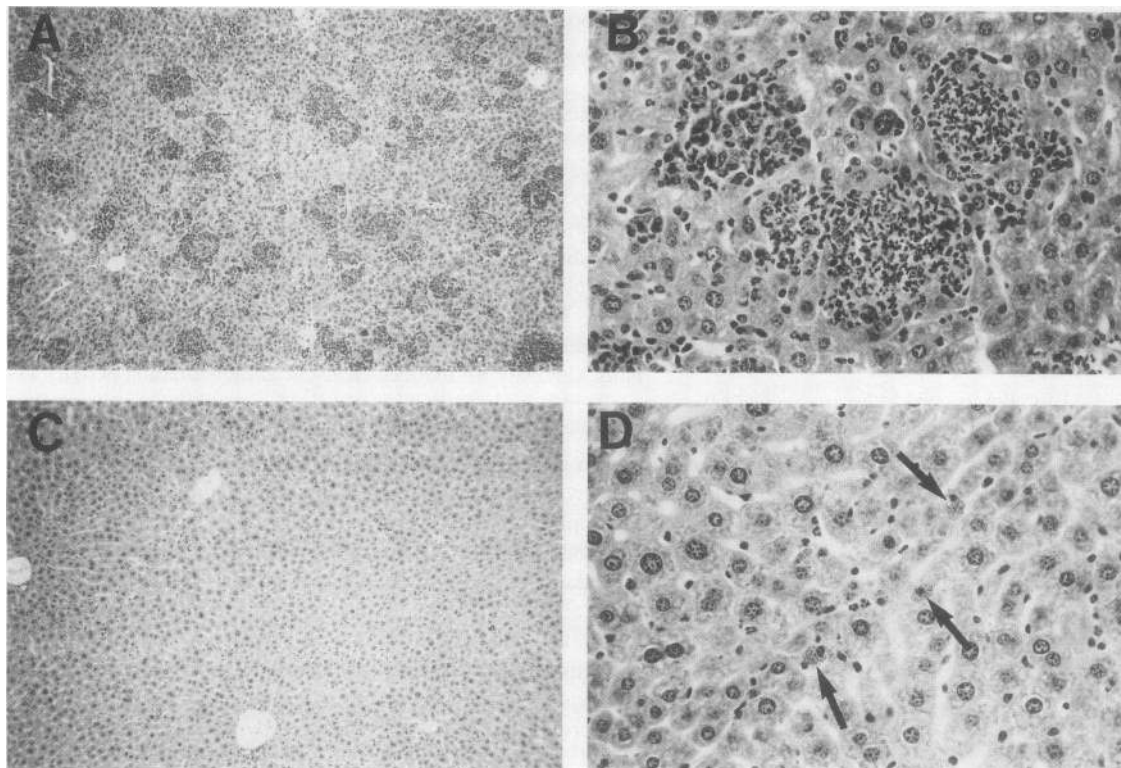
## Discussion

The results of this study suggest five conclusions related to the role and effect of GM-CSF in experimental visceral leishmaniasis. First, *L. donovani* infection upregulates the expression of GM-CSF mRNA in parasitized liver, and endogenous GM-CSF is involved in the initial response that leads to acquired resistance. This antileishmanial effect appears to be unrelated to inducing or maintaining granuloma assembly. Second, exogenous MuGM-CSF induces clear-cut antileishmanial activity associated with both mobilization of neutrophils and monocytes in the circulation and myelomonocytic cell influx and accumulation in infected tissues. Third, as judged by the results of treatment with HuGM-CSF and HuG-CSF, monocytes rather than neutrophils appear likely to be the more relevant GM-CSF-induced effector cells. Fourth, since HuGM-CSF induced antileishmanial activity in euthymic mice and MuGM-CSF stimulated activity in CD4<sup>+</sup> cell-reconstituted nude mice without high level leukocytosis, GM-CSF also may exert macrophage-activating effects *in vivo* as it does *in vitro* (10). The latter observation appeared important since, even though transient, the very high WBC counts induced by MuGM-CSF treatment would not be clinically feasible. Finally, fifth, host T cells are required not only for the induction of antileishmanial activity by GM-CSF, but also for maximal mobilization of myelomonocytic cells in the peripheral blood and optimal leukocyte entry into infected liver.

The effect of anti-GM-CSF treatment indicated that endogenous GM-CSF participates in the early response to *L. donovani*. This initial stage of the successful host defense response in this model is complex and multi-cytokine dependent, involving at least three other endogenous cytokines: IFN- $\gamma$ , IL-2, and TNF- $\alpha$  (7, 19, 26). Since chronic administration of anti-GM-CSF antiserum did not inhibit eventual reduction in liver parasite burdens, GM-CSF did not appear to be essential in the resolution stage of visceral infection. This differential role for a cytokine, active in initial but not late antileishmanial events, is similar to what we have observed for endogenous IFN- $\gamma$  and IL-2 (7, 19). In contrast, endogenous TNF- $\alpha$  is active in both the early response to *L. donovani* and the resolution of experimental visceral infection (26).

Anti-GM-CSF antiserum achieved its inhibitory effect on resistance without impairing the formation of granulomas that characterize the tissue response to intracellular *L. donovani* in the liver (1-5). In view of the role of blood monocytes in granuloma assembly (5) and the observed tissue effects of exogenous GM-CSF, it was surprising to find that granuloma development was undisturbed by anti-GM-CSF administration. However, whereas treatment of *L. donovani*-infected BALB/c mice with anti-IFN- $\gamma$  or anti-IL-2 antiserum simultaneously impairs acquired resistance and granuloma development (7, 19), anti-TNF- $\alpha$  injections also inhibit resistance without modifying the tissue reaction (26).

This antileishmanial treatment effect of exogenous GM-CSF was accompanied not only by the anticipated mobilization of neutrophils and monocytes in the peripheral circulation (8), but also by dose-related myelomonocytic cell influx into infected tissue. The histologic appearance of livers from GM-CSF-treated infected mice suggested that the presence of *L. donovani*



**Figure 6.** Liver histologic response in 3-wk infected euthymic and nude mice treated with HuG-CSF for the preceding 7 d. (A and B) Euthymic mice treated with G-CSF (2.5  $\mu\text{g}/\text{d}$ ) had a mean WBC count of 49,210/ $\text{mm}^3$  and show large neutrophil accumulations at tissue foci of infection. (C and D) In contrast, nude mice treated with G-CSF (10  $\mu\text{g}/\text{d}$ ) had a mean peripheral WBC count of 38,300/ $\text{mm}^3$  in this experiment but show no histologic reaction similar to untreated nude mice (Fig. 5 A). Arrows indicate parasitized Kupffer cells.  $\times 50$  (A and C);  $\times 200$  (B and D).

and/or the granulomatous response it provokes both enhanced overall leukocyte entry into the liver and preferentially attracted influxing cells to foci of established intracellular infection. Thus, from a host defense perspective, treatment with GM-CSF can be viewed as a method of augmenting focal delivery of neutrophils and monocytes to sites of tissue infection (Fig. 4). Treatment of infected euthymic mice with G-CSF induced the same type of focal accumulations of neutrophils (Fig. 6).

When used separately as treatment, both exogenous GM-CSF and IFN- $\gamma$  induced *in vivo* antileishmanial activity. However, we could not demonstrate an enhanced *in vivo* effect using both cytokines together despite the following potentially important interactions: (a) enhanced monocyte influx should have presented more effector cell targets (e.g., monocytes [5]) for activation by exogenous IFN- $\gamma$  (5, 6); (b) GM-CSF and IFN- $\gamma$  can induce each other's receptors (27–29) and increase monocyte production of the antileishmanial cytokine TNF- $\alpha$  (26, 30); and (c) neutrophils that can kill *L. donovani* (21) also respond to IFN- $\gamma$  with enhanced antimicrobial activity (31–33). Ideally, each of these activities should have had an opportunity to interact in the tissues of infected mice treated with both cytokines.

The lack of enhanced efficacy upon combining these two macrophage-activating (10, 22) cytokines *in vivo* contrasts with a previous *in vitro* report (10) and our own observations using human monocyte-derived macrophages (22) pretreated for 3 d with HuGM-CSF, HuIFN- $\gamma$ , or both cytokines together. In four such experiments, unstimulated cells supported a  $2.5 \pm 0.1$ -fold increase in the number of *L. donovani* amastigotes per 100

macrophages 72 h after challenge; cells pretreated with an optimal concentration of GM-CSF (0.5 ng/ml) did not kill *L. donovani* but completely inhibited its *in vitro* replication with only a  $1.1 \pm 0.2$ -fold increase in parasite number at 72 h. When 0.5 ng/ml GM-CSF was combined with suboptimal IFN- $\gamma$  (250 U/ml) (22), which by itself induced inhibition with a  $1.5 \pm 0.1$ -fold increase, leishmanistatic effects were converted to leishmanicidal effects with a  $0.4 \pm 0.1$ -fold increase (e.g., 60% killing) in parasite load.

Although our observations highlight a beneficial effect for GM-CSF in *L. donovani* infection, there are conflicting data concerning GM-CSF and the role of the monocyte in experimental infections caused by *L. tropica*, *L. major*, and *L. mexicana*. For example, *in vitro* and *in vivo* studies have suggested that blood monocytes or immature macrophages not only may not be active against these strains, but may be preferentially drawn into infected tissues and selectively parasitized (34–37). Such cells may be refractory to lymphokine stimulation (34) and thus paradoxically serve to promote intracellular infection (35). In experimental *L. donovani* infection, the opposite appears to be the case (5, 24, 37, 38). In addition, GM-CSF has been reported to enhance the growth of *L. mexicana* promastigotes (but not amastigotes) in a cell-free system (39), fail to induce antileishmanial effects *in vitro* in macrophages parasitized by *L. tropica* and *L. major* (40, 41), and exacerbate cutaneous *L. major* lesions when used as treatment *in vivo* (41). These results and the possibility that fresh monocytes mobilized by GM-CSF and attracted to infected tissues may provide additional “safe targets” for *L. major* (35) or *L. mexicana* (39) have raised concern



about using GM-CSF as treatment. However, other studies have indicated that (a) GM-CSF can activate macrophages in vitro to kill or inhibit these strains (42, 43); (b) infection is not promoted in GM-CSF transgenic mice (44); (c) anti-GM-CSF treatment does not alter *L. major* infection in mice (45); and (d) GM-CSF-treated, antigen-presenting macrophages can help to induce protective immunity to *L. major* in vivo (46).

Although GM-CSF has not been tested clinically in cutaneous leishmaniasis, in a pilot trial using GM-CSF plus conventional antimony therapy in leukopenic patients with visceral infection, combination treatment was safe, hastened resolution of leukopenia, may have reduced secondary infections, and had no adverse effect on antimony-induced parasite elimination (18). Treatment with G-CSF (9) would likely ameliorate granulocytopenia and its secondary complications just as well in such patients. However, the experimental results reported here clearly favor the use of GM-CSF in visceral infection in view of its capacity to mobilize and promote the tissue accumulation of an additional and critical effector phagocyte, the monocyte, and, at the same time, also stimulate mononuclear phagocyte anti-leishmanial mechanisms.

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