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Macrophage Progenitor Cell and Colony-Stimulating Factor Production during Granulomatous Schistosomiasis Mansoni in Mice

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***Schistosoma mansoni* worm eggs stimulate a T-cell-mediated granulomatous response in which macrophages play important inflammatory and regulatory roles. Although much has been learned about the functions of the schistosome granuloma macrophages, their origin and replicative ability are unknown. In the present sequential study, macrophage progenitor cells in the bone marrow (GM-CFC) and liver granulomas (M-CFC) were enumerated, and macrophage colony-stimulating factor (CSF-1) in the circulation and culture fluid of explanted granulomas of infected mice was assayed. During the acute phase of the infection, when the granulomatous response was vigorous (weeks 8 to 12) GM-CFC numbers were high in the bone marrow and M-CFC numbers were low within granulomas. Circulating CSF-1 levels were elevated, but the vigorous granuloma-secreted CSF-1 level was low. During the chronic phase of the infection, the number of GM-CFC within the bone marrow and levels of circulating CSF-1 returned to normal. Conversely, a sharp increase in the number of M-CFC occurred within the small immunomodulated granulomas that also secreted high levels of CSF-1. The frequency of M-CFC that proliferated under exogenously added CSF-1 within the immunomodulated granulomas was significantly higher than that of cells in the vigorous granulomas. Adherent macrophage-rich cells obtained after dispersal of the granulomas appeared to be one source of CSF-1 production. These data indicate that during the infection the macrophage supply to and replicative ability within the granulomas is influenced by systemically and/or locally produced CSF-1.**

Schistosomiasis mansoni is a major tropical disease in which eggs of adult worms are deposited in the liver and intestines. Macrophages, eosinophils, neutrophils, lymphocytes, and fibroblasts surround the eggs, and their inflammatory response results in hypersensitivity-type granuloma formation (18), which is considered to be an important factor in disease pathology (35). Over the years, various activities of the granuloma macrophages have been identified and quantitated. Studies included Fc (1); C3 receptor and Ia antigen expression (36); superoxide, anion, prostaglandin, and monohydroxyeicosatetraenoic acid production (7); antigen presentation (11, 24); tumoricidal activity (16); and regulatory cytokine production (12). Evidence indicates that macrophage functions appear to be regulated by T-effector, T-suppressor lymphocytes that influence granuloma size in schistosome-infected mice (5).

To date, very little is known about the origin and proliferation of the parasite egg-induced granuloma macrophage (26). The origin and proliferation of tissue macrophages under normal steady-state conditions and during inflammation has been studied for various systems (28). Controversy still exists whether tissue macrophages originate only from bone marrow-derived monocytes or whether they enlarge their numbers also by local proliferation.

The influence of growth factor(s) on the maturation of committed stem cells to monocytes within the bone marrow has been well established (19, 20). The supply of bone marrow-derived blood-borne monocytes to various tissues under steady-state or inflammatory conditions is also well documented (2, 3, 32–34). There is also a growing body of evidence for the proliferative ability of localized tissue macrophages (2, 3, 13, 15, 23, 29, 32, 33).

We present here data that suggest that granuloma macrophages are supplied by both bone marrow-derived and focally produced monocytes in *S. mansoni*-infected mice. The relative contributions of these two sources to the monocyte supply are subject to change as the vigorous granulomatous response undergoes immunologic downmodulation.

MATERIALS AND METHODS

Infection of mice. Female CBA/J mice (Jackson Laboratory, Bar Harbor, Maine) at 8 weeks of age were infected subcutaneously with 25 to 30 cercariae of the Puerto Rican strain of *S. mansoni*. Food and acidified water were provided ad libitum.

Bone marrow colony formation. Femurs of normal and infected mice, at the times of infection indicated, were removed from the animals and flushed with the alpha modification of minimum essential medium (α-MEM; GIBCO Laboratories, Grand Island, N.Y.). Cells were washed, counted, and adjusted to a final concentration of 25,000/ml per 35-mm tissue culture dish in semisolid agar (0.3% Noble agar; Difco Laboratories, Detroit, Mich.) containing α-MEM supplemented with 15% fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah) and 250 U of macrophage colony-stimulating factor (CSF-1) (prepared as described in reference 6). Cultures were incubated for 7 days at 37°C in the presence of 7.5% CO_2, at which time they were stained overnight by the addition of 1 mg of [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl] tetrazolium chloride per ml. The next day, aggregates containing 50 or more cells were counted as colonies.

Granuloma cell colony formation. The liver of each infected mouse was homogenized in a Waring blender, and the released granulomas were washed and dispersed into single-cell suspensions with collagenase as described previously (11). Cells were adjusted to a concentration of 1.5×10^6/ml

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in 10 ml of RPMI 1640 medium with 10% fetal calf serum in a gelatin-coated 25-cm^2 tissue culture flask (Beckton Dickinson Labware, Oxnard, Calif.) and incubated for 1 h at 37°C. Nonadherent cells were removed by two washes with α-MEM, and 10 ml of α-MEM supplemented with 10% fetal calf serum was added to the adherent cells. The cells were incubated overnight at 37°C in the presence of 7.5% CO_2. The next day, the supernatant was aspirated and saved for CSF-1 radioimmunoassay. Adherent cells were removed by incubation for 5 min at 37°C with 5 ml of 0.25% lidocaine in α-MEM and vigorous agitation. Cells were washed, counted, and adjusted to 1,000/ml per 35-mm dish in α-MEM supplemented with 15% fetal calf serum and 500 U of CSF-1. They were incubated at 37°C for 14 days in the presence of 7.5% CO_2 and stained with 0.5% crystal violet in 95% ethanol, and colonies, as defined above, were counted.

CSF-1 radioimmunoassay. Serum samples from infected mice were diluted to final concentrations of 1/2.5 and 1/25 in the presence of 25,000 cpm of ^{125}I-labeled CSF-1, rat anti-murine CSF-1, and normal rat serum (to reduce nonspecific interactions) in duplicate 5-ml tubes. This antiserum did not react with GM-CSF, gamma interferon, alpha/beta interferon, or interleukin-3 cytokines. The tubes were incubated at 4°C for 48 h, and aggregates were precipitated and washed with $(NH_4)_2SO_4$ solution. Pellets were counted for radioactivity, and the radioactivity level was converted to units of CSF-1 by a standard radioimmunoassay curve established in parallel with the samples. The assay was sensitive to 16 U of CSF-1; 1 U of CSF-1 is defined by the production of 2.5 to 3.0 colonies of 50 or more cells when 5×10^4 bone marrow cells are cultured in 1 ml of soft agar under optimal growth conditions (6).

CSF-1 radioimmunoassay in supernatants of granuloma explants and adherent granuloma macrophage cultures. At sacrifice, mice were bled by orbital bleeding and their livers were perfused with 8 to 10 ml of RPMI 1640 medium to remove blood from the organs. The livers were homogenized at low speed in a Waring blender, and the granulomas were collected in a conical flask and repeatedly rinsed with cold medium. Two hundred granulomas per ml of medium were counted under a stereomicroscope in a tissue culture dish (60 by 15 mm) (no. 3002; Beckton Dickinson Labware). The RPMI 1640 medium was supplemented with 2 mmol of glutamine per ml, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated fetal calf serum. For each mouse, two dishes containing 200 granulomas each were incubated for 24 h in a CO_2 incubator at 37°C, and then supernatant fluid was obtained and tested as described for serum.

RESULTS

Enumeration of GM-CFC in the bone marrow. At various times after infection, bone marrow cells of infected mice and age-matched, noninfected control animals were collected and cultured in semisolid agar in the presence of CSF-1. The number of macrophage progenitor cells per 2.5×10^4 bone marrow cells increased sharply during the acute phase of the infection (weeks 6 to 12) (Fig. 1). With the onset of immunologically mediated down-regulation of granuloma size (week 14 onward), GM-CFC numbers started to decline, and by the chronic phase of the infection (week 20), they reached preinfection values. The GM-CFC numbers at weeks 6 to 12 differed significantly ($P < 0.01$) from those observed in early- or late-infected mice. Values of a repeat experiment analyzing a different cycle of infection are as follows. At week 1 there were 62.2 ± 4.0 colonies per 2.5×10^4 cells (mean ± standard error [SEM] for three mice); at week 4 there were 58.7 ± 2.1 colonies per 2.5×10^4 cells (three mice); at week 6 there were 88.8 ± 5.2 colonies per 2.5×10^4 cells (four mice); at week 8 there were 82.3 ± 3.3 colonies per 2.5×10^4 cells (four mice); at week 12 there were 107.9 ± 8.1 colonies per 2.5×10^4 cells (four mice); and at week 20 there were 59.7 ± 3.6 colonies per 2.5×10^4 cells (four mice). P values between weeks 6 and 12 were significantly different ($P < 0.001$) from those at weeks 4 and 20.

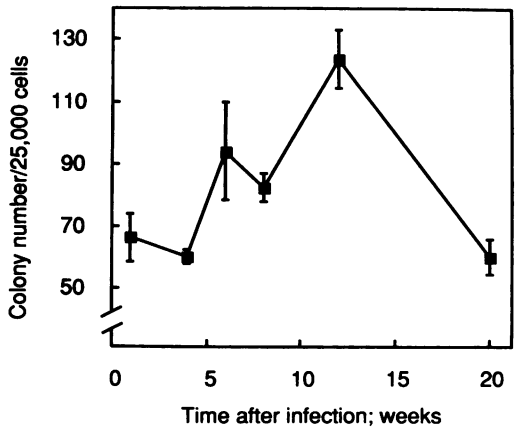

FIG. 1. Enumeration of GM-CFC in the bone marrow of infected mice. Bone marrow cells removed from sacrificed mice at various times after infection were cultured in soft agar at a concentration of 2.5×10^4 cells per dish in the presence of 250 U of CSF-1. After 7 days, aggregates of 50 or more cells were counted as colonies. Data represent the mean ± SEM of one representative experiment with four mice used for each point. Quadruplicate dishes were used for the samples from each mouse.

Enumeration of M-CFC in liver granulomas. Macrophage progenitor cells (M-CFC) are present in granulomas, since granuloma cells stimulated with CSF-1 produced colonies of typical macrophages (Fig. 2). The M-CFC count within granulomas, like that of bone marrow GM-CFC, also changed during the course of the infection. At the vigorous phase of the granulomatous response (weeks 8 to 12), the number of macrophage progenitor cells per 10^4 adherent granuloma cells was low. However, by the chronic stage of the disease (week 20), the number of progenitor cells doubled within the smaller, immunomodulated granulomas (Fig. 3). Values of a repeat experiment analyzing a different cycle of infection are as follows. At week 8 there were 18.5 ± 2.0 (mean ± SEM for four mice) colonies per 10^4 cells; at week 12 there were 18.8 ± 2.2 colonies per 10^4 cells (four mice); and at week 20 there were 29.9 ± 3.0 colonies per 10^4 cells (four mice). Controls contained 1 to 2 colonies per 10^4 cells. P values were <0.001 for the control versus weeks 8, 12, and 20 and <0.0026 for week 12 versus week 20. Occasionally, small, fibroblast-containing colonies were observed among the macrophage colonies. Such aggregates made up about 1% of the total number of colonies.

Enumeration of M-CFC in the vigorous and immunomodulated granulomas following stimulation with exogenous CSF-1. Cells from vigorous and immunomodulated granulomas were plated and cultured with graded doses of exogenous CSF-1 (0 to 250 U/ml). Increasing concentrations of added CSF-1 enhanced the number of colonies that developed from M-CFC (Fig. 4). At every dose of added CSF-1, cells of the immunomodulated granulomas produced significantly more colonies than did their counterparts derived from the vigor-

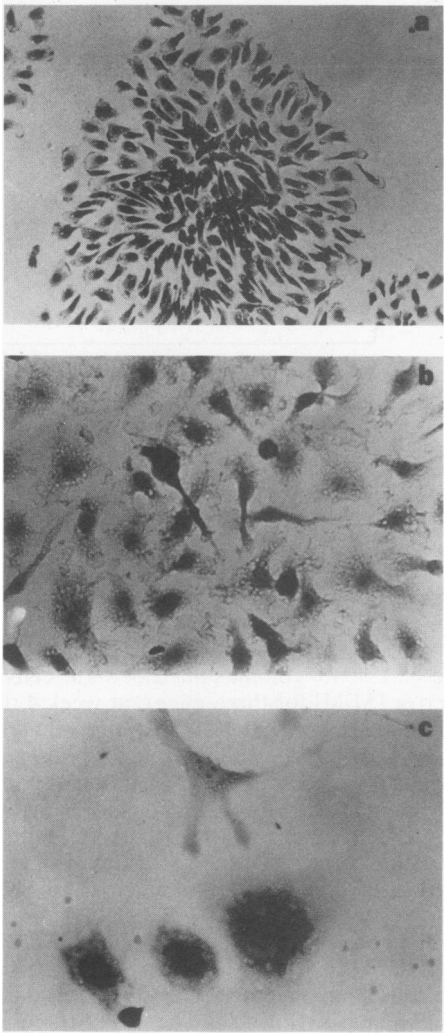

FIG. 2. Morphology of macrophages derived from M-CSF-stimulated granuloma cells. Adherent cells were cultured with 500 U of CSF-1 per ml for 14 days. Colonies were stained with 0.5% crystal violet and photographed at magnifications of ×100 (a), ×400 (b), and ×1,000 (c).

ous lesions. At saturating concentrations of CSF-1 (250 U), the number of colonies from the immunomodulated granulomas was double that seen in the vigorous lesions. Values of a repeat experiment analyzing a different cycle of infection are as follows. At week 8, the number of colonies (mean ± SEM for three mice) per 10^4 cells was 2.0 ± 0.5 (0 U of CSF-1), 5.3 ± 0.7 (15.6 U of CSF-1), 10.6 ± 0.9 (31.2 U of CSF-1), 15.3 ± 1.5 (62.5 U of CSF-1), 17.3 ± 1.9 (125 U of CSF-1), and 18.0 ± 2.0 (250 U of CSF-1). At week 20, the numbers were 67 ± 0.5 (0 U), 1.3 ± 0.7 (15.6 U), 2.3 ± 0.7 (31.2 U), 4.6 ± 0.5 (62.5 U), 7.3 ± 1.2 (125 U), and 7.7 ± 1.9 (250 U). P values at week 8 were highly significant at every point ($P < 0.001$) compared with values at week 20.

Measurement of circulating CSF-1 in serum. Serum samples collected from mice at various times after infection were measured by radioimmunoassay (Fig. 5). Circulating CSF-1 levels remained within the normal range during the first 4 weeks of infection. This period corresponds to the phase of larval migration and maturation. With the appearance and florid state of the granulomatous response (weeks 6 to 12),

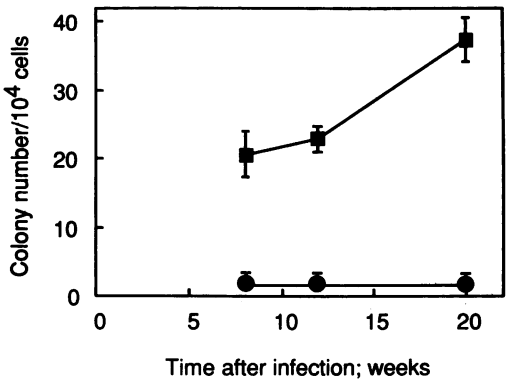

FIG. 3. Enumeration of M-CFC in liver granulomas of infected mice. The adherent cell fraction of granuloma cells was cultured in the presence (■) or absence (●) of 500 U of CSF-1 per ml for 7 days. The colony count per 10^4 cells plated is shown. Data represent the mean ± SEM of one representative experiment with four mice used for each point. Quadruplicate dishes were used for samples from each mouse.

CSF-1 levels increased significantly ($P < 0.01$) above control values. At the chronic phase of the infection (week 20), when granulomas were diminished in size, circulating CSF-1 levels returned to the normal range. Values of a repeat experiment analyzing a different cycle of infection are as follows. At week 1 the level of CSF-1 (mean ± SEM for three mice) was 59.3 ± 3.8 U/ml; at week 4 the level was 62.7 ± 2.8 U/ml (three mice); at week 6 the level was 68.0 ± 3.3 U/ml (four mice); at week 8 the level was 78.2 ± 2.0 U/ml (four mice); at week 12 the level was 52.0 ± 1.1 U/ml (four mice); and at week 20 the level was 67.7 ± 3.1 U/ml (three mice). Only at weeks 8 and 12 were significant differences found ($p < 0.001$) compared with week 1.

Measurement of CSF-1 in supernatants of granuloma explants and cultures of adherent granuloma cells. Table 1 presents the results of two experiments analyzing different cycles of infection. Supernatants of the larger, vigorous granuloma cultures of samples collected at weeks 6 to 8 contained undetectable levels (<16 U of growth factor per

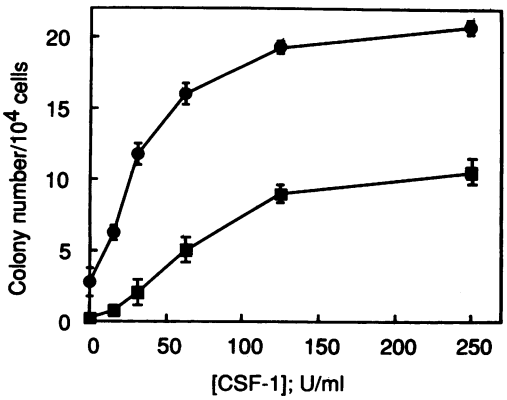

FIG. 4. Comparison of M-CFC counts in exogenous CSF-1-stimulated vigorous or immunomodulated granuloma cell cultures. The adherent-cell fraction of granuloma cells was cultured with graded doses of CSF-1 (0 to 250 U/ml). The colony count per 10^4 vigorous (●) or immunomodulated (■) granuloma cells plated is shown. Data represent the mean ± SEM of one representative experiment with four mice used for each point. Quadruplicate dishes were used for samples from each mouse.

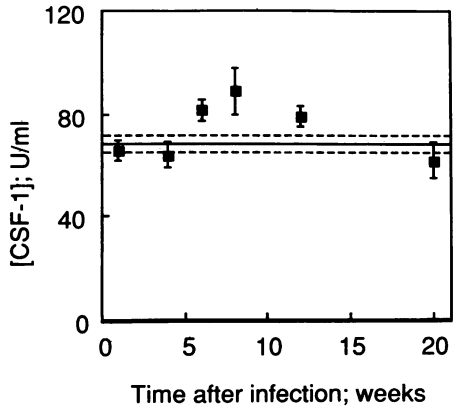

FIG. 5. Assay of circulating CSF-1 levels in sera of infected mice. Rat anti-CSF-1 antiserum was added to a 1:250 dilution of mouse serum containing 2.5×10^4 cpm of ^{125}I-labeled M-CSF. After incubation, aggregates were precipitated with $(NH_4)_2SO_4$ and counted. Data represent the mean ± SEM of one representative experiment with four mice used for each point. Duplicate determinations were used for samples from each mouse. ——, ---, mean and SEM range, respectively, for eight noninfected mice. At weeks 6, 8, and 12, $P < 0.01$ compared with the control.

ml). In contrast, the concentration tripled in the culture fluid of smaller, immunomodulated granulomas from chronically infected mice. After dispersal of the granulomas by collagenase, we separated the cell population into plastic-plate-adherent and nonadherent fractions by incubating cells for 2 h. The adherent fraction consisted of >85% esterase-positive macrophages. In addition, some macrophages were esterase negative. Occasionally, lymphocytes adhered to the plated macrophages. Their numbers in the monolayers made up 1 to 2% of the total cell population. Culture fluids of these cells contained no CSF-1 at week 6 but contained measurable amounts by week 8. This amount almost doubled in macrophage-rich cultures derived from immunomodulated granulomas. The nonadherent cell fraction contained 60 to 70% eosinophils, 10 to 15% lymphocytes, 8 to 10% nonadherent macrophagelike cells (not consistently staining with esterase stain), and some neutrophils. Because of the variable viability of the cells in culture, attempts to quantitate the level of CSF-1 were unsuccessful. However, supernatants of cultured cells contained no detectable levels of CSF-1 at week 8 but contained measurable amounts at week 20.

TABLE 1. CSF-1 activity in supernatants of cultured whole granulomas and adherent cells from dispersed granulomas

Duration of infection (weeks)	CSF-1 level[a] (mean ± SEM) (U) in:			
	Supernatant of whole granulomas		Supernatant of adherent cells from dispersed granulomas[b]	
	Expt 1	Expt 2	Expt 1	Expt 2
6	<16	<16	ND[c]	ND
8	<16	<16	50.7 ± 4.7[d]	43.8 ± 4.8[d]
20	50.0 ± 2.3	50.0 ± 2.3	113.0 ± 1.9	98.8 ± 4.3

[a] CSF-1 activity was determined by radioimmunoassay after 18 h of incubation. Each point represents the mean value of four mice.
[b] Mean CSF-1 levels (U) ± SEM per 10^7 adherent cells.
[c] ND, Not done, owing to the minimal number of cells obtained from young granulomas early in the infection.
[d] Comparison of values at weeks 8 and 20 shows significance: $p < 0.001$.

DISCUSSION

Cells of the mononuclear phagocytic system play a major role in host protection against invasive and destructive intracellular organisms (14). Protection is provided by bone-marrow-derived monocytes that organize into hypersensitivity-type granulomas (4, 8). Sequestration and killing of the microorganisms by the granuloma macrophages cause cell death, and hence a constant supply of fresh monocytes is required. The high cellular turnover of the granuloma macrophages is sustained by the increased output of monocytes by the bone marrow and the limited local proliferation of granuloma monocytes and macrophages (25). The schistosome egg-induced granuloma that surrounds the large parasite eggs is also dependent on the mobilization of macrophages and eosinophils (26). Data presented in this study show that during the acute phase of the infection (weeks 7 to 12), which encompasses the onset and florid expression of the granulomatous response, the bone marrow is highly responsive to the demand for fresh cell supply. This is seen in the elevated numbers of the progenitor GM-CFC present in the bone marrow. This response is likely to be regulated by the high levels of circulating CSF-1 found in serum samples of acutely infected mice. These observations are in accord with previous observations of elevated levels of circulating CSF-1 and progenitor M-CFC during the course of experimental infection with facultative intracellular pathogens (30, 37, 38), and experimental and clinical infections with *S. japonicum*, *S. mansoni*, and *S. haematobium* worms (17, 22). In all those instances, CSF-1 appears to fulfill an important role in the supply of monocytes and in the maintenance of the chronic inflammatory and protective response. In contrast, the vigorous granulomas contained low levels of macrophage progenitor cells and released low levels of CSF-active substance(s). Earlier studies with in vivo-labeled bone marrow precursor cells showed that labeled macrophages within the schistosome egg-induced liver granolomas (26), *Mycobacterium bovis* BCG-induced dermal granulomas (9, 31), and pulmonary interstitial inflammations (3) were recent arrivals from the bloodstream. The present observations indicate that both the bone marrow and the granulomas are potential sources of monocyte supply. Although it appears that the bone marrow plays an important role in the provision of monocytes, in vivo labeling studies are needed to identify it as the primary or predominant source of monocyte supply for the developing granulomas.

With the progression of infection to the chronic phase, the number of progenitor GM-CFC in the bone marrow and the levels of circulating CSF-1 decreased to the range seen in normal mice. Concurrently, the number of progenitor M-CFC within the immunomodulated granulomas rose sharply, and, on stimulation with exogenous CSF-1, the cells exhibited colony formation in a dose-dependent manner. Thus, with the onset of the immunologic down-modulation of the granulomatous response, the bone marrow becomes less active, probably because of the diminished levels of circulating CSF-1. The return of bone marrow activity to normal levels may be connected with the decreased overall demand for monocytes needed for the smaller lesions. In that regard, the rise in the numbers of monocyte precursors within the immunomodulated smaller granulomas is of great interest, because it points to the importance of the focal source of monocyte supply. Whether a major switch indeed occurred from systemic (bone marrow) to focal (granulomatous tissue) supply of the monocytes must be evaluated by labeling precursor cells. At present, the potential for focal

supply of monocytes is underscored by the enhanced CSF-1 release from the explanted modulated granulomas as well as cultured granuloma cells. The source of CSF-1 within these granulomas is as yet unidentified. Although over 85% of the adherent granuloma cells are esterase-positive macrophages, the presence of an undetermined low number of fibroblasts, makes the identification of the source of this cytokine difficult. Potentially, macrophages and fibroblasts may be considered sources of CSF-1 production (19–21, 27, 39). Within the context of the complex cellular interactions that prevail during the granulomatous response, it is conceivable that at different stages of the inflammatory response both sources may influence monocyte production.

Future studies should identify the major intragranulomatous source of CSF-1 production and the putative T-cell-mediated regulation of monocyte supply within the modulated granulomas. Moreover, CSF-1 is also considered to regulate the activation state of macrophages and their ability to secrete a variety of monokine mediators (20, 21, 39). Because some of these mediators participate in the modulation of the inflammatory and fibrotic host responses (10), the regulatory role of CSF-1 in monocyte supply, macrophage activation, and monokine secretion during the chronic granulomatous response warrants further investigation.

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