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# Effector Role of Blood Monocytes in Experimental Visceral Leishmaniasis

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**In BALB/c mice, liver granulomas provoked by visceral infection with intracellular *Leishmania donovani* are rapidly populated by influxing blood monocytes. To determine the host defense effector role of these mononuclear phagocytes, we treated three populations of infected animals with 5C6, an anti-type 3 complement receptor monoclonal antibody (MAb), which inhibits monocyte recruitment into inflamed tissues. In naive BALB/c mice, injections of 5C6 impaired the initial acquisition of antileishmanial resistance and arrested the development of mature liver granulomas. In sensitized mice with established immunity, both resistance to rechallenge and accelerated granuloma formation were similarly inhibited by MAb administration. Finally, in naive mice, 5C6 MAb also abolished the antileishmanial activity induced by treatment with the macrophage-activating lymphokine gamma interferon. Together, these results suggest a key effector role for the influxing blood monocyte in both initial and established antileishmanial defense and granuloma assembly and in the infected liver as the mononuclear phagocyte target for the antimicrobial effects of gamma interferon.**

*Leishmania donovani*, the intracellular protozoan which causes visceral leishmaniasis, selectively parasitizes resident macrophages within the liver, the spleen, and the bone marrow. In experimental infections in BALB/c mice, visceral replication progresses rapidly until a T-cell-dependent response develops and induces host defense cell activation and the killing of intracellular amastigotes (15, 24). This mechanism involves the macrophage-activating lymphokine gamma interferon (IFN-γ) (23), and it is expressed in infected tissues by granuloma formation (9, 15, 23, 24).

In the liver, sinusoidal-lining resident macrophages (Kupffer cells) are the initial targets for *L. donovani* (15). Once fused, parasitized Kupffer cells serve as the central nidus for the recruitment of both T cells and influxing blood monocytes, which together form an encircling mantle to complete granuloma assembly (9, 15). The antileishmanial role of T cells in the granuloma probably involves the local secretion of activating lymphokines, including IFN-γ (23) and interleukin-2 (22); cytotoxicity directed at parasitized macrophages may represent an additional T-cell effect (20). However, little is known about the relative effector contributions of the two populations of mononuclear phagocytes present in the *L. donovani*-induced granuloma, Kupffer cells and monocytes.

Therefore, to determine the role of the influxing blood monocyte in antileishmanial defense in vivo, we exploited differential in situ expression of the type 3 complement receptor (CR3) by monocytes (present) and Kupffer cells (absent) (4, 6, 8, 9) and treated infected mice with an anti-CR3 monoclonal antibody (MAb). This MAb, 5C6, is specific for an epitope of the CR3 of mouse myelomonocytic cells and selectively blocks myelomonocytic cell tissue recruitment (18, 19).

## MATERIALS AND METHODS

**Visceral infection.** Female BALB/c mice (Charles River Laboratory, Wilmington, Mass.) (20 to 30 g) were injected via their tail veins with $10^7$ *L. donovani* amastigotes (1 Sudan strain) obtained from infected hamster spleen homogenates (15). The course of visceral infection was determined microscopically with stained liver tissue imprints (15), and liver parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of amastigotes per 1,000 hepatic cell nuclei times liver weight (in g) (15). Formalin-fixed liver sections stained with hematoxylin and eosin were used for histologic examination and granuloma scoring (9, 15, 23, 24). *L. donovani*-immune BALB/c mice (15), originally infected 10 to 12 months before, were rechallenged with $10^7$ amastigotes and examined 2 weeks later (14).

**Treatment with 5C6 MAb.** Naive and immune mice were challenged with *L. donovani* and then left undisturbed for 24 h. On day 1 and twice weekly thereafter, groups of three to four mice received intraperitoneal injections of saline containing 0.5 mg of purified 5C6 (19) or 0.5 mg of normal rat immunoglobulin G (IgG) (Sigma Chemical Co., St. Louis, Mo.). The preparation, specificity, and in vitro and in vivo activities of 5C6, a rat anti-mouse IgG2b MAb, have been reviewed in detail elsewhere (18). In vitro, anti-CR3 treatment does not appreciably alter *L. donovani* amastigote binding to mouse macrophages (1, 3). Since the calculation of liver parasite burden (in Leishman-Donovan units) is dependent upon the total number of hepatic cell nuclei, it was possible that Leishman-Donovan units in 5C6-treated mice might be increased by a reduction in cells influxing into the liver. However, as judged by low-power microscopy, overall cellularity in liver sections from infected controls at 4 weeks and that in those from 5C6-treated mice were similar (data not shown), and liver weights in control and treated mice were also not different (1.70 ± 0.3 versus 1.87 ± 0.3 g).

**Treatment with IFN-γ.** Control and 5C6-injected mice were also treated with *Escherichia coli*-derived, recombinant murine IFN-γ ($2 \times 10^7$ U/mg; Amgen, Thousand Oaks, Calif.) beginning 2 weeks after infection (13). IFN-γ ($2.4 \times 10^5$ U/day) was administered continuously for 7 days via a subcutaneously implanted osmotic pump (Alzet model 2002; Alza, Palo Alto, Calif.) (13).

**Statistical methods.** Differences in mean liver parasite

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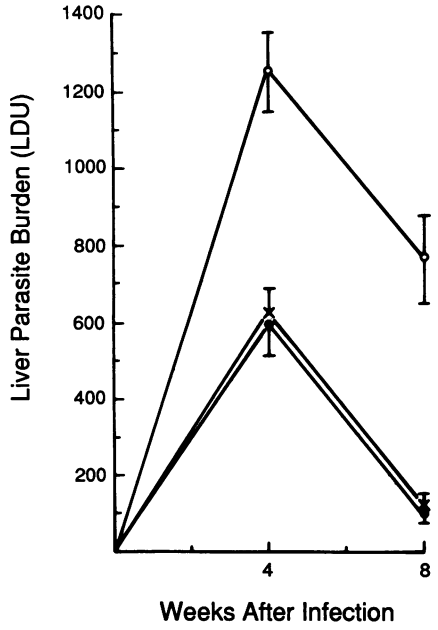


FIG. 1. Effects of 5C6 MAb treatment on *L. donovani* infection in mouse livers. One day after being challenged, mice received no further treatment (●) or twice-weekly injections of 0.5 mg of 5C6 (○) or rat IgG (×). Results (mean ± standard error of the mean values) are from six experiments at 4 weeks (18 mice total per group) and from two experiments at 8 weeks (6 mice total per group). Values for 5C6-treated mice at both 4 and 8 weeks are significantly different from those for rat IgG-treated animals ($P < 0.05$).

TABLE 1. Effects of 5C6 MAb treatment on granuloma development

| Mice, time of administration, and treatment[a] | Granuloma score (% of infected foci) with the following tissue reaction[b]: | | |
|---|---|---|---|
| | 1+ | 2+ | 3+ |
| Naive | | | |
| 4 wk | | | |
| None | 0 | 82 ± 2 | 18 ± 2 |
| IgG | 0 | 86 ± 4 | 14 ± 3 |
| 5C6 | 6 ± 1 | 94 ± 3 | 0 |
| 8 wk | | | |
| None | 0 | 66 ± 5 | 34 ± 5 |
| IgG | 0 | 68 ± 2 | 32 ± 2 |
| 5C6 | 0 | 91 ± 3 | 9 ± 3 |
| Immune (2 wk) | | | |
| None | 1 ± 1 | 41 ± 7 | 58 ± 8 |
| IgG | 0 | 39 ± 6 | 61 ± 7 |
| 5C6 | 8 ± 3 | 76 ± 3 | 16 ± 5 |

[a] Starting 1 day after infection of naive mice or rechallenge of immune mice, 0.5 mg of 5C6 or rat IgG was administered twice weekly for up to 8 weeks. At the indicated time points, liver sections from each of five to seven mice per group (two experiments) were used to identify 100 discrete infected foci in consecutive 63× microscopic fields.

[b] Tissue reactions; 1+, single or fused parasitized Kupffer cells with no cellular infiltrate (Fig. 2C and D); 2+, developing granulomas with fused Kupffer cells and some mononuclear cell influx; 3+, mature granulomas (Fig. 2A and B) with the Kupffer cell core surrounded by a well-organized mononuclear cell mantle (9, 15, 23, 24).

burdens of control mice and those of treated mice were analyzed by a two-tailed *t* test for independent samples (14).

## RESULTS AND DISCUSSION

**Effect of 5C6 MAb on initial control of visceral infection and granuloma formation in naive mice.** Twice-weekly injections of 5C6 inhibited the acquisition of resistance to *L. donovani* in naive mice and significantly increased liver parasite burdens 4 weeks after infection (Fig. 1). In parallel, granuloma development was also impaired and no mature granulomas were evident in the livers of 5C6-treated mice (Table 1). Treatment with 5C6 did not affect Kupffer cell fusion, the initial step in granuloma core formation (15), but it clearly inhibited the development of the organized mononuclear cell mantle which defines the mature granuloma in this model (Fig. 2) (9, 15, 23). Morphologically, the few residual cells surrounding fused Kupffer cells in 5C6-treated mice appeared to be predominantly lymphocytes. Since the encircling mantle in its intact state contains abundant $CD4^+$ and $CD8^+$ cells (9), the tissue effect of 5C6 treatment suggested that monocytes may help to direct the recruitment of both helper and cytotoxic T cells into the infected focus. At the same time, as judged by the minimal tissue reaction to *L. donovani* in nude BALB/c mice (15), T cells themselves also appear to be required for the optimal recruitment of monocytes into the infected liver (9, 15). Thus, granuloma assembly in response to *L. donovani* is probably governed by at least two cell populations, each of which may attract the other and amplify the tissue reaction.

**Effect of early treatment with 5C6 MAb.** To determine whether inhibition of monocyte influx during the early stage of visceral infection was sufficient to control subsequent events, mice were treated with 5C6 only during the first week after challenge. In mice injected with 1 mg of MAb on day 1 or days 1 and 4, however, there was no effect on either the development of antileishmanial resistance or granuloma formation 4 weeks after infection (two experiments; data not shown). Thus, the continuous presence of a blockade of CR3 appeared to be necessary to inhibit both responses.

This latter observation was also pertinent since a small number of neutrophils may migrate into hepatic foci in the first week after *L. donovani* infection in naive mice (9). However, neutrophils are seldom present in infected tissues at week 2 (unpublished data) and are essentially absent at week 4 (Fig. 2B), and 5C6 administration during week 1 of infection only had no apparent effect. At both 1 and 2 weeks after rechallenge with *L. donovani*, neutrophils are also scarce in the livers of immune mice (14). Natural killer (NK) cells can also express CR3 (25). While these cells have been implicated in defense against *L. donovani* in the spleen (7), they are not required for control of hepatic infections in either naive (7, 15, 21) or rechallenged immune (14) mice. In addition, granuloma formation in response to *L. donovani* is intact in both naive and immune NK-cell-deficient C57BL/6 beige mice as well as in naive and immune BALB/c mice treated with NK-cell-depleting anti-asialo GM1 antiserum (14; also unpublished data). Thus, while 5C6 MAb acts on other $CR3^+$ leukocytes, including neutrophils (2) and perhaps NK cells (25), we believe the results in Fig. 1 and 2 most likely reflect the well-established effect of 5C6 MAb on the tissue recruitment of monocytes (18).

**Effect of 5C6 MAb on resolution of infection.** After 8 weeks, visceral infection in untreated animals had largely resolved (Fig. 1). Liver parasite burdens also declined in 5C6-treated mice, but infection still remained well established at the late time point. Since antileishmanial activity (Fig. 1) and, to a

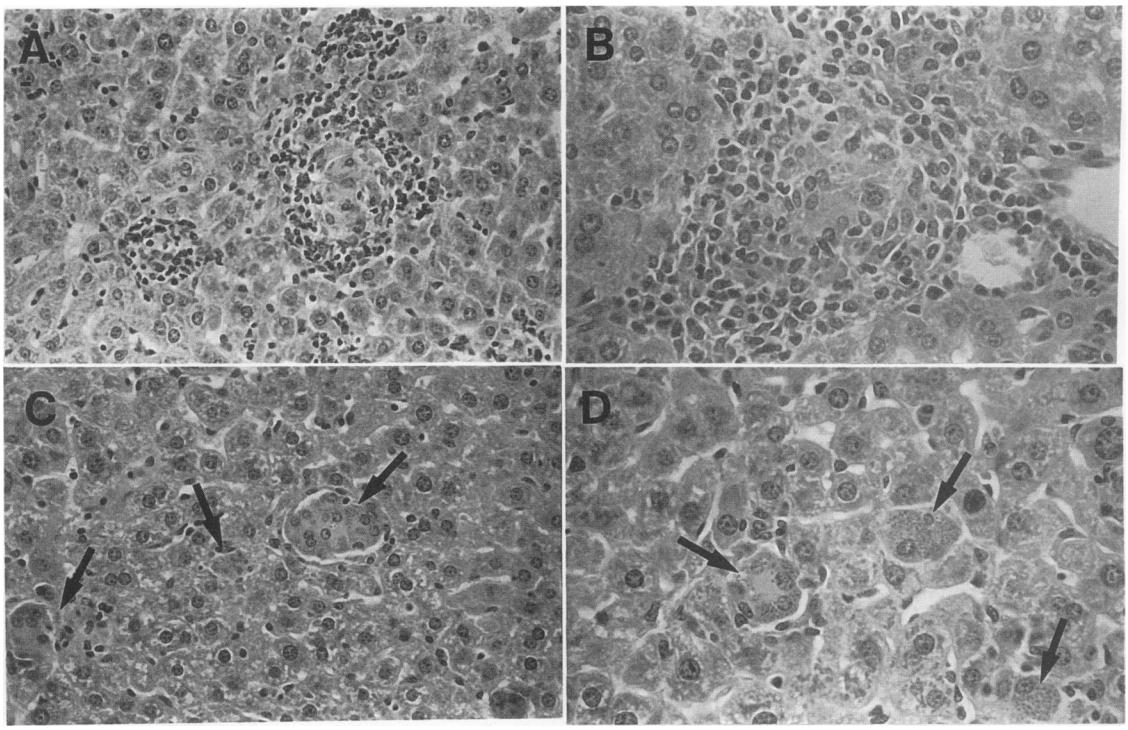


FIG. 2. Liver histologic responses to *L. donovani* in naive BALB/c mice 4 weeks after infection. In control mice (A and B), granulomas show a well-organized mononuclear cell mantle surrounding a core of fused Kupffer cells. In contrast, 5C6 treatment (C and D) has essentially abolished mononuclear cell influx into infected foci which consist of heavily parasitized fused as well as single Kupffer cells (arrows). Magnifications, ×200 (A and C) and ×320 (B and D).

lesser extent, granuloma formation (Table 1) had emerged despite 8 weeks of MAb treatment, we doubled the twice-weekly dose of 5C6 to 1 mg and repeated these experiments. At both 4 and 8 weeks after infection, however, the effects of high-dose 5C6 were no different from those illustrated in Fig. 1 and Table 1 (two experiments; data not shown).

Thus, the preceding results with naive mice suggested that (i) monocytes appeared to be required for the optimal, initial acquisition of resistance to *L. donovani* and for granuloma formation, (ii) these mononuclear phagocytes continue to enter liver foci and to act as effector cells beyond the acute stage of infection, and (iii) cells in addition to $CR3^+$ monocytes probably contribute to the eventual resolution of visceral infection in naive mice. It is also possible that a CR3-independent mechanism for monocyte recruitment (11) had emerged after week 4 of infection to explain why liver burdens declined despite continued injections of 5C6 MAb. A more likely explanation, however, relates to the kinetics of monocyte influx into the infected liver (9): the number of monocytes increases steadily and peaks at 4 weeks and then declines by >50% by 8 weeks (9).

**Effect of 5C6 MAb on established resistance in immune mice.** We next used 5C6 treatment to probe the host defense role of the blood monocyte in established antileishmanial resistance. Previous studies using *Listeria monocytogenes* have indicated that although the influxing monocyte is a key effector cell in primary infection (16, 19), it does not necessarily play a similar role in resistance to rechallenge (10) or in models of adoptive immunity (17). In our experiments, we employed chronically infected immune BALB/c mice which solidly resist rechallenge and show accelerated granuloma formation with rapid monocyte influx into the liver (9, 14, 15). Two weeks after the mice were rechallenged, liver parasite burdens in 5C6-treated immune mice (0.5 mg twice weekly starting on day 1) were 343 ± 21 LDU, a significant increase ($P < 0.05$) compared with those in either untreated (138 ± 24 LDU) or rat IgG-treated (131 ± 12 LDU) immune controls ($n$ = eight mice from two experiments). 5C6 injections also strongly inhibited granuloma formation in response to rechallenge (Table 1). Thus, the influxing monocyte appeared to be active in both initial as well as established resistance to *L. donovani*.

**Effect of 5C6 MAb on in vivo antileishmanial activity induced by exogenous IFN-γ.** Finally, we also utilized 5C6 administration to identify the target cell in the liver which responds to and which may mediate the antileishmanial effect of treatment with the macrophage-activating lymphokine IFN-γ (13, 15). In previous studies, Kupffer cells failed to respond to IFN-γ in vitro with enhanced activity against *L. donovani* (8), and they have also been previously shown to respond poorly to IFN-γ administered in vivo (5). Thus, the influxing monocyte, not the resident hepatic macrophage, seemed most likely to be the IFN-γ-responsive effector mononuclear phagocyte.

To test this hypothesis, IFN-γ was administered continuously by pump ($2.4 \times 10^5$ U/day) starting 2 weeks after infection to otherwise untreated mice or mice treated twice weekly since day 1 with 0.5 mg of 5C6 or normal rat IgG. Liver parasite burdens were determined after 7 days of IFN-γ treatment, during which time 5C6 and rat IgG injections were continued. In three experiments with nine mice per group, liver parasite burdens in mice treated with IFN-γ alone (524 ± 46 LDU) were significantly lower ($P < 0.05$) than those in untreated controls (1,007 ± 95 LDU). In rat

IgG-treated mice, IFN-γ also significantly ($P < 0.05$) decreased liver burdens (545 ± 23 LDU). In contrast, the effect of IFN-γ treatment was abolished in 5C6-treated mice, and their liver parasite burdens (950 ± 84 LDU) were not different from those of untreated control animals.

Together, these observations suggest a primary effector role for the blood monocyte in three key expressions of the host defense response to systemic intracellular infections caused by *L. donovani*: the acquisition of initial resistance, the maintenance of established immunity, and the assembly of granulomas during both of these distinct immunologic responses (14). In addition, the influxing monocyte also appears to be the likely mononuclear phagocyte target for IFN-γ, which in both its endogenous and its exogenous forms acts to control experimental *L. donovani* infection (13, 15, 23). The immigrant monocyte has also been well identified as a critical host defense cell in previous studies, particularly those employing *L. monocytogenes* (16, 19). However, work with other pathogens has also indicated either no role (18) or a potentially negative role (4, 12) for the monocyte as well. In the latter models (4, 12), influxing monocytes have been implicated as being preferentially parasitized by *Leishmania major* (cutaneous leishmaniasis) and therefore potentially capable of perpetuating intracellular infections. Our results suggest the opposite role for the blood monocyte in visceral infections caused by *L. donovani.*

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