

## THE DYNAMICS OF GRANULOMA FORMATION IN EXPERIMENTAL VISCERAL LEISHMANIASIS

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*Leishmania donovani* is an intracellular parasite that infects parenchymal macrophages of susceptible animals (1). Unlike infection with *L. major* (2) or *L. mexicana* (3), which usually leads to death, intravenous challenge with *L. donovani* results in acute visceral infection in the BALB/c mouse that eventually resolves (4). The athymic (*nu/nu*) BALB/c mouse infected with *L. donovani* develops progressive, noncuring, visceral disease. Despite different outcomes of infection in euthymic and athymic BALB/c mice, both form hepatic granulomas, defined as inflammatory foci containing infected cells surrounded by mononuclear cells and granulocytes.

We examined the dynamics and composition of hepatic granuloma formation in experimental *L. donovani* infection of euthymic and athymic BALB/c mice using immunocytochemistry. We studied the evolution of the granulomatous response during longitudinal infection and after rechallenge. We found an association between the size and number of granulomas formed, the cellular composition of the granulomas, and the eventual recovery from infection.

### Materials and Methods

*Mice.* BALB/c mice (+/+) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Congenitally athymic nude (*nu/nu*) and euthymic (*nu/+*) littermates on a BALB/c background were received from Life Sciences (St. Petersburg, FL). All mice were female, weighed 20–25 g when infected, and were housed together.

*Visceral Infection.* Mice were injected intravenously with  $10^7$  amastigotes (1 Sudan strain) freshly isolated from spleen homogenates of infected Syrian golden hamsters (4). The animals were killed at 2, 4, 8, and 20 wk after primary infection. Hepatic parasite burden was quantitated as Leishman-Donovan units (5) from liver weights and Giemsa-stained liver imprints. Groups of euthymic mice were rechallenged similarly with  $10^7$  amastigotes after ~18 wk of primary infection. These animals were killed 2 wk after rechallenge.

Kupffer cells (KC)<sup>1</sup> were labeled with carbon in designated animals by i.v. injection of 0.2 ml of sterile filtered 5% India Ink in PBS, pH 7.4, within 4 h before infection. Groups of mice were killed 1, 4, 8, 10, and 14 d after infection.

*Tissue Processing.* The livers were removed, cut into small pieces, fixed 3 h in periodate-lysine-paraformaldehyde (6), and suspended in graded sucrose solutions for immunocyto-

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<sup>1</sup> Abbreviation used in this paper: KC, Kupffer cells.

TABLE I  
*Rat mAbs for Mouse Leukocytes*

Clone (American Type Culture Collection Number)	Ig subclass	Specificity	Reference
M1/70	IgG2b	Macrophages, polymorphonuclear leukocytes (C3Bi receptor)	7
F4/80	IgG2b	Macrophages	8
B21.2	IgG2b	I-A <sup>b,d</sup>	9
GK1.5, L3T4 (TIB 207)	IgG2b	Mouse T4	10
RA-3A1/6.1 (TIB 146)	IgM	220-kD glycoprotein on B lymphocytes	11
53-6.72 (TIB 105)	IgG2a	Ly-2	12

chemical studies as previously described (3). Frozen sections were incubated for 1 h with 1:5 dilutions of culture supernatants from growing hybridomas containing rat anti-mouse mAbs, specified in Table I. Sections were incubated with biotinylated rabbit anti-rat IgG, and were mouse adsorbed (Vector Laboratories, Inc., Burlingame, CA) for 45 min, with avidin-biotin-peroxidase complex (Vector Laboratories, Inc.) for 30 min and finally with 0.4 mg/ml 3,3-diaminobenzidine tetrahydrochloride in 0.02 M Tris buffer, pH 7.6, containing 0.01% hydrogen peroxide. Sections were washed between each incubation with PBS. Nonspecific staining was evaluated by substituting PBS for reactant at each step. One of two slides from each animal was counterstained with Gill's hematoxylin (Formulation No. 1; Fisher Scientific Co., Fairlawn, NJ). The tissue sections were dehydrated with 100% ethanol and xylene, and coverslips were attached to the slides with Permount (Fisher Scientific Co.).

Portions of liver were fixed in 10% buffered formalin, paraffin embedded, mounted onto glass slides, and stained with hematoxylin-eosin.

*Evaluation of Hepatic Infection.* A Nikon Microphot FX microscope was used for viewing stained tissue and photography. Positive staining cells were enumerated from 20 random fields under a (60 ×) oil immersion objective. Granuloma size was determined under (40 ×) magnification using a 12.5 μm<sup>2</sup> grid. Student's *t* test was used to analyze the significance of differences between means.

*Euthymic mice.* As previously reported (13), the course of infection and granulomatous response in the heterozygous (*nu/+*) and homozygous (*+/+*) BALB/c mice were similar. Data from the two groups were pooled and the group, referred to as euthymic mice, was compared with the athymic (*nu/nu*) mice.

## Results

In the following sections, we will describe the generation and maintenance of the hepatic granuloma, its influence on the fate of intracellular *Leishmania* amastigotes, its cellular composition throughout infection and rechallenge, and the influence of thymus-derived lymphocytes upon these processes.

*Generation of the Hepatic Granuloma.* The intravenous injection of *L. donovani* amastigotes led to their ingestion by hepatic KC, transfer into parasitophorous vacuoles, and intracellular replication in these permissive host cells. Infected KC were characterized by their sinusoidal localization, uptake of colloidal carbon, staining of their limiting membranes with the macrophage marker F4/80 mAb, and with a reagent for MHC class II determinants (B21.2). The initially parasitized cells failed to stain for the C3bi receptor with M1/70 mAb and could therefore be differentiated from

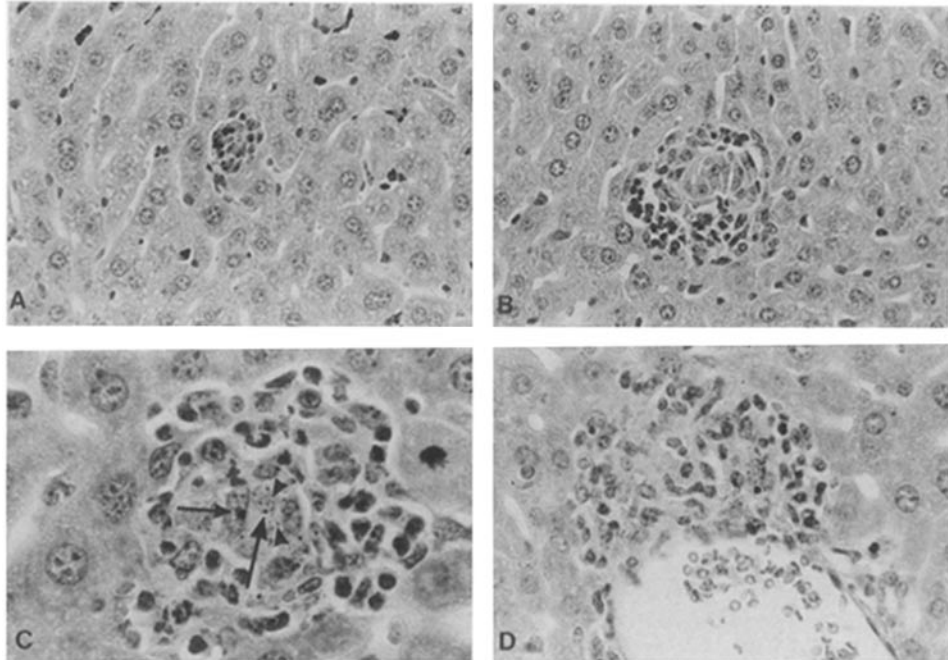


FIGURE 1. Photomicrographs of granuloma formation in hematoxylin-eosin-stained livers of euthymic mice infected with *L. donovani*. (A) The first evidence of granuloma formation was observed at day 8 of infection, with small clusters of granulocytes surrounding KC ( $\times 72$ ). (B) By day 15, the granuloma has enlarged and mononuclear cells have migrated to the edge of the granuloma ( $\times 72$ ). (C) KC labeled with carbon (arrows) are seen in the center of the granuloma at 4 wk of infection. Intracellular leishmania amastigotes are also visible within the KC (arrowheads) ( $\times 108$ ). (D) Moderate to large granulomas form alongside hepatic vessels by 4 wk of infection ( $\times 108$ ).

newly immigrated blood monocytes. During the first 2–3 wk it appeared that both infected and noninfected KC aggregated on the surface of sinusoids, leaving other areas relatively devoid of these cells.

The initial accumulation of KC served as the nidus for the developing granuloma. With time these cells impinged on the underlying hepatocytes, gradually became surrounded with newly immigrated cells from the vasculature, and soon lost their physical association with the sinusoid. Some of the earliest cells that appeared around the infected KC were granulocytes and monocytes. As shown in Fig. 1, A and B, peroxidase-positive granulocytes were closely apposed to the KC. Both neutrophils and eosinophils were present. By 2 wk, significant numbers of lymphocytes appeared as a rim around the infected KC (Fig. 1 B and C). Later, this stratification was less apparent (Fig. 1 D), and greater mixing of all cell types occurred. L3T4<sup>+</sup> and Ly-2<sup>+</sup> T cells were intermixed, with L3T4<sup>+</sup> cells slightly predominant at all early stages of infection (Figs. 2, C and D, and 3, A and B). Small numbers of B lymphocytes were seen throughout the infection both within and on the periphery of granulomas.

Both the number and size of granulomas increased rapidly during the first 4 wk of infection (Fig. 4, A and B). Presumably, parasites were taken up rapidly after intravenous challenge, but infectious foci only became apparent as intracellular repli-

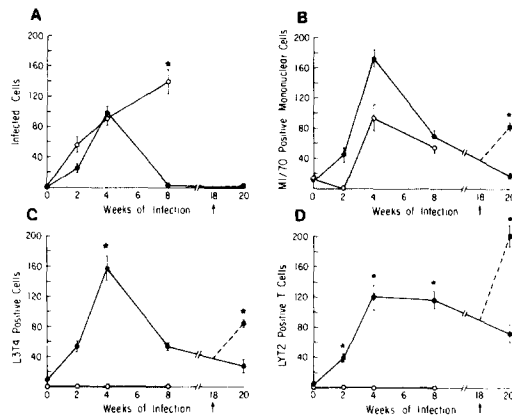


FIGURE 2. Course of *L. donovani* infection in euthymic (●), euthymic rechallenged (■), and athymic (○) BALB/c mice. The arrow indicates the time of reinfection. Infected cells (A) or cells staining positive with specific mAbs (B–D) by the immunoperoxidase method were enumerated from 20 random fields under  $60\times$  oil immersion. Livers from at least four animals per group were examined at each time period. (A) Infected KC were evaluated from slides in which specific mAbs were omitted. (B) M1/70<sup>+</sup> staining cells were primarily immigrant monocytes. Granulocytes with endogenous peroxidase were omitted in these counts. (C and D) Counts were taken from specimens without hematoxylin counterstain to better differentiate membrane staining of L3T4<sup>+</sup> (C) and Ly-2<sup>+</sup> (D) T cells from granulocytes with endogenous peroxidase. (\*) Significantly different,  $p \leq 0.01$ . Error bars indicate SE.

cation ensued and leukocyte migration progressed. In euthymic mice, numbers of L3T4<sup>+</sup> and Ly-2<sup>+</sup> T cells, monocytes, granuloma size, and number, all peaked at 4 wk of infection (Figs. 2–4). Similarly, the number of infected cells (Fig. 2 A) reached its highest level at the same time. Thereafter, a precipitous fall in intracellular parasites and Leishmania-infected cells took place, and by 8 wk, only a rare KC or monocyte could be found with organisms (Figs. 2 A and 5 A). The clearing of parasites during the 4–8-wk period was associated with a gradual reduction in all the above parameters. It was of interest that the number of L3T4<sup>+</sup> T cells fell more rapidly and reached lower levels than the Ly-2<sup>+</sup> phenotype (Figs. 2, C and D, and 5, B and C). This “helper” population was maintained at higher levels throughout the first 4 wk of infection with the L3T4<sup>+</sup>/Ly-2<sup>+</sup> T cell ratio of 1:3. In contrast, levels of Ly-2<sup>+</sup> T cells were maintained in the granuloma almost without change until 18 wk. At the 8-wk period, the L3T4<sup>+</sup>/Ly-2<sup>+</sup> T cell ratio had been reversed and was  $\sim 0.03$  (Fig. 3, A and B). Granuloma diameter and number were maintained during the 4–8-wk period (Fig. 4), in the face of striking losses of L3T4<sup>+</sup> cells (Fig. 2 C). This suggested ei-

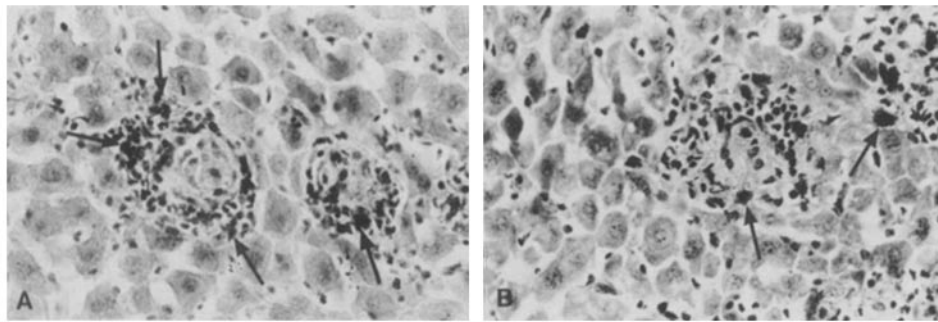


FIGURE 3. Photomicrographs of the T cell response (arrows) defined by immunoperoxidase staining after 4 wk of infection in the *nu/+* BALB/c liver. Hematoxylin counterstain was used ( $\times 72$ ). (A) L3T4<sup>+</sup> T cells. (B) Ly-2<sup>+</sup> T cells.

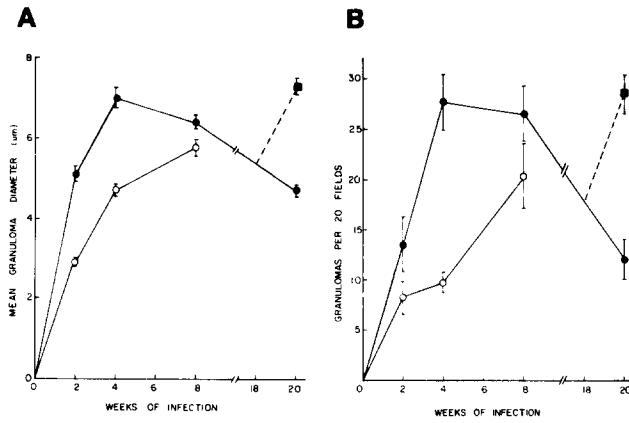


FIGURE 4. Evaluation of mean granuloma size and number formed during *L. donovani* infection in euthymic (●), euthymic rechallenged (■), and athymic (○) BALB/c mice. (A) Granulomas were aligned within a 12.5- $\mu\text{m}^2$  eyepiece grid ( $\times 40$ ). The diameter represents the mean of two perpendicular measurements within the grid. 20 granulomas were measured per liver, and four livers were examined per group at each time point. (B) Granulomas were enumerated in 20 random fields per liver ( $\times 60$ ). Four livers were examined per group at each time point. Error bars indicate SE.

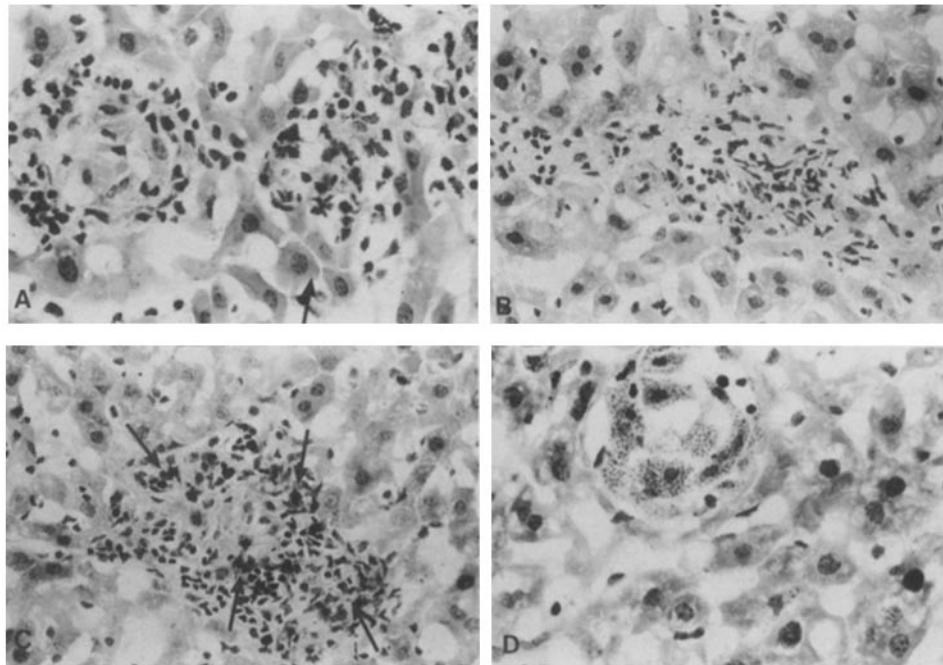


FIGURE 5. Photomicrographs of granuloma formation at 8 wk of infection using the immunoperoxidase method with hematoxylin counterstain ( $\times 72$ ). (A) Euthymic (*nu/+*) liver, with multiple large, well-circumscribed granulomas. Numerous inflammatory cells, including endogenous peroxidase-positive granulocytes, surround the central core, which is devoid of *Leishmania* amastigotes. The  $\text{L3T4}^+$  T cell response (B) was diminished by 8 wk, whereas the  $\text{Ly-2}^+$  T cells (arrows) persisted within the granulomas (C). (D) Athymic (*nu/nu*) liver with ill-defined granuloma consisting of several large heavily infected KC and a few surrounding inflammatory cells.

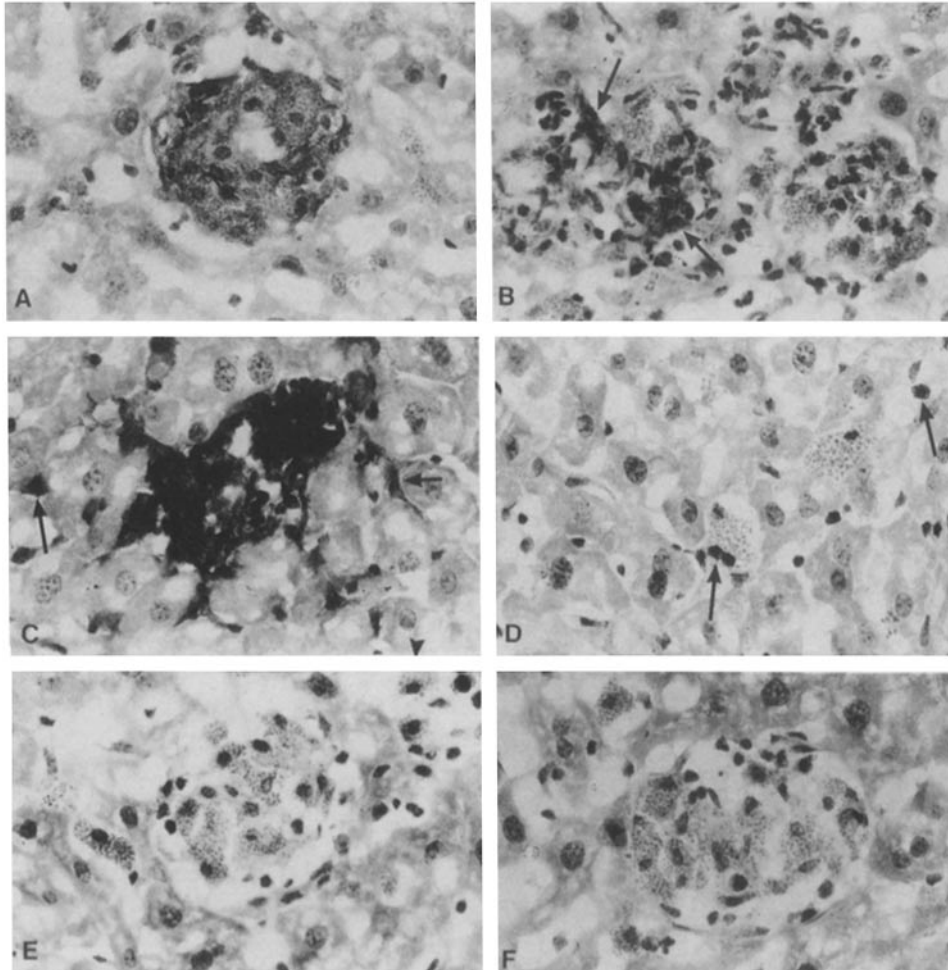


FIGURE 6. Photomicrographs of immunoperoxidase staining with hematoxylin counterstain of the athymic (*nu/nu*) hepatic granuloma after 4 wk of *L. donovani* infection ( $\times 108$ ). (A) Infected KC are stained with F4/80. (B) Inflammatory monocytes staining with M1/70 (arrows) and granulocytes are surrounding KC. (C) Staining of all cells in the granuloma and single KC (arrows) with Class II MHC antigen (B21.2). (D) B lymphocytes (arrows) staining with antibody to 220-kD glycoprotein are found alongside infected KC. L3T4<sup>+</sup> T cells (E) and Ly-2<sup>+</sup> T cells (F) are absent.

ther the persistent influx or the local proliferation of Ly-2<sup>+</sup> T cells during a period in which the parasites within the granuloma were being largely destroyed.

The course of events in athymic (*nu/nu*) mice was strikingly different. The replication of amastigotes within KC proceeded at a logarithmic rate (Fig. 2 A). Even at the earliest stages of infection (2 wk), granuloma size and number lagged behind the euthymic animal (Fig. 4). Between 0 and 4 wk, the migratory cells were largely granulocytes and monocytes, although the influx of these cells was delayed and their total numbers decreased in the maturing granulomas (Fig. 2 B). This was also

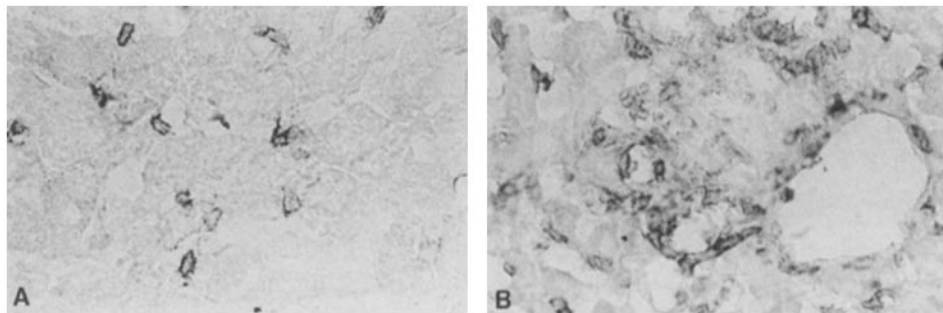


FIGURE 7. Photomicrographs of Ly-2<sup>+</sup> T cells in euthymic livers at 20 wk of infection (A) and after 2 wk of rechallenge (B). Hematoxylin counterstain was omitted ( $\times 108$ ). (A) Ly-2<sup>+</sup> T cells are scattered singly along the sinusoids, persisting after resolution of infection. (B) After 2 wk of rechallenge, numerous Ly-2<sup>+</sup> T cells accumulate within and alongside hepatic granulomas.

manifested by the smaller number and reduced size of the granulomas (Fig. 4). All cells within the granuloma were strongly positive for membrane staining with MHC class II determinants, even in the absence of identifiable T cells (Fig. 6, C, E, and F). Small numbers of B cells were seen in association with heavily parasitized KC (Fig. 6 D). As the infection progressed, the size of the granulomas approached that of their euthymic counterparts at 8 wk of infection (Fig. 4 A). The composition of these structures was, however, entirely different. Instead of the significant T cell contribution, *nu/nu* mouse granulomas consisted primarily of heavily parasitized macrophages, a scattering of granulocytes and monocytes, and an occasional B cell (Figs. 2 and 5 D). Of interest was the strong membrane reactivity of the KC and monocyte-derived macrophages for MHC class II determinants. Most animals survived after 8 wk of infection despite heavy parasite loads.

**Rechallenge.** 18–20 wk after the primary *L. donovani* infection in euthymic animals, the original granulomas were gradually shrinking in size, amastigotes were not visualized, and the major cell type other than macrophages was the Ly-2<sup>+</sup> T cell (Figs. 2 D and 7 A). This process remained unchanged for subsequent weeks in the unchallenged euthymic mouse.

After intravenous rechallenge with  $10^7$  amastigotes, euthymic animals experienced a brief hepatic infection that varied both quantitatively and qualitatively from the primary infection. The cellular parameters of this process can be followed in Figs. 2 and 4. Rechallenge stimulated a rapid increase in both the size and number of granulomas. It was unclear, however, whether the granulomas represented existing or newly developed structures, although it is likely that both types existed. In contrast to the primary infection, parasitized macrophages or KC were never seen after 2 wk of rechallenge (Fig. 2 A). A striking difference was noted in the nature of the T cells present in these granulomas (Fig. 7). As seen in Fig. 2 D, the number of Ly 2<sup>+</sup> T cells increased dramatically, exceeding that observed in the primary response and representing the predominant cell type of the rechallenge granuloma. Smaller numbers of M1/70 positive monocytes (Fig. 2 B) and L3T4<sup>+</sup> T cells (Fig. 2 C) also accumulated. It appeared that the parasites were quickly destroyed and this event was associated with the presence of large numbers of Ly-2<sup>+</sup> T cells and reduced numbers of the L3T4<sup>+</sup> “helper” population.

### Discussion

After the introduction of *L. donovani* into the circulation, large numbers of organisms are sequestered in the sinusoids of liver and spleen and ingested by sessile macrophages of these organs. In the liver, this occurs within KC. This host cell, although competent to carry out many of the endocytic and secretory properties of macrophages, fails to produce toxic oxygen intermediates, one of its main antimicrobial mechanisms (14, 15). As a result, amastigotes attach to the walls of parasitophorous endocytic vacuoles and undergo multiplication. This is accompanied by the aggregation of KC on the sinusoidal surface to form the nuclei of the granulomas that are to be formed.

The second stage in hepatic granuloma formation occurs in proximity to or within the confines of the liver parenchyma. This is dependent upon the generation of chemotactic factors that induce the migration of both granulocytes, lymphocytes, and monocytes from the microvasculature. Initially, granulocytes, both neutrophils and eosinophils, are clustered about infected KC and may serve as an initial antimicrobial screen. Later, monocytes and T cells become more apparent. This probably represents a continuum of migration with a short half-life for the granulocytes and persistence of the mononuclear cells. Chemoattractants may be derived from granulocytes, monocytes, and T cells, as well as endothelial cells, fibroblasts, and macrophages, in the form of leukotriene B, IFN- $\gamma$ , and its induced product  $\gamma$  IP-10 (16).

The rapid influx and early preponderance of helper T cells of the L3T4<sup>+</sup> phenotype may initiate a number of important events. First, the production of IFN- $\gamma$  can serve to maintain the production of toxic oxygen intermediates by competent blood-borne monocytes and monocyte-derived macrophages (17). Such cells in the activated state are able to kill and degrade ingested amastigotes (18). Helper T cells can also make other lymphokines, such as IL-2, IL-3, and IL-4, each of which may have specific tasks in cell-mediated immunity. The helper cells of the L3T4<sup>+</sup> phenotype have a relatively short existence in the granuloma and decline rapidly after the fourth week of infection. The fate of these cells is unclear but may include movement into lymphatics and local nodes or actual destruction within the granuloma. These possibilities will be discussed more fully in the following sections.

Another participant in the cellular response is the Ly-2<sup>+</sup> T cell, which accumulates progressively in the granuloma, reaching peak levels at 4 wk. Thereafter, its levels are maintained at almost maximum levels for many weeks. Its function in the granuloma is uncertain, and its production of lymphokines and/or cytotoxins is unclear. Upon rechallenge, it is the major lymphoid cell of the granuloma. Its presence is associated with the much-enhanced clearance of parasites and the loss of infected cells. We suggest that the Ly-2<sup>+</sup> T cell or a cell controlled by its secretory products, is a potent effector cell. Macrophages, CTL, NK, and LAK cells are all possible candidates for a killer population, either alone or in concert with the Ly-2<sup>+</sup> T cell. Since the heightened response of the Ly-2<sup>+</sup> T cell occurs somewhat after that of the L3T4<sup>+</sup> population, we suspect it may be activated or its function enhanced by lymphokines such as IL-2. Its role in the cell-mediated immune response may be the destruction of parasitized macrophages. Experimentally, it may be possible to dissociate the Ly-2<sup>+</sup> T cells from the hepatic granulomas and examine their role in killing *Leishmania*-infected macrophages. We plan to pursue this investigative approach in the near future.



The central zone of the developing granuloma consists of infected KC, and as the lesion progresses, infected monocyte-derived macrophages are visualized. Since the KC is a permissive host of *L. donovani* amastigotes and fails to respond to IFN- $\gamma$ , a humoral or cell-mediated response may have little influence on the survival of parasites within the KC endocytic vacuole. For this purpose, a mechanism is required to destroy the host cell and liberate its content of organisms into the extracellular milieu. Antibody, complement, and other molecules secreted locally could modify the parasite extracellularly and more importantly prepare it for a second round of endocytosis by a competent phagocyte. As mentioned before, this would include newly immigrated monocytes and activated monocyte-derived macrophages. Such a mechanism exists in the lesions of tuberculoid leprosy and can be induced by the generation of a cell-mediated response in the skin of lepromatous leprosy patients after the local injection of a second party delayed-type antigen (PPD) (19, 20). It may be possible to dissociate the Ly-2<sup>+</sup> T cells from the hepatic granulomas and examine their role in killing leishmania-infected macrophages.

A similar role for activated effector cells may occur in the destruction of the L3T4<sup>+</sup> T cells of the early granuloma. Loss of the helper population may result from cell death or less likely migration to the local lymphatics. This may curtail further local lymphokine production and lead to the down regulation of the cellular response.

After parasite destruction, the stimulus for additional granuloma accretion ceases, and the Ly-2<sup>+</sup> T cell remains in the local environment in a presumably "resting" state. With the introduction of a second bolus of parasites, many hepatic sites are already primed with pre-existing cells, and the ingestion of organisms by KC leads to a cascade of mediators. These in turn result in rapid local cell activation, enhanced cellular migration, and the prompt destruction of amastigotes. Again, the Ly-2<sup>+</sup> T cell is predominant within the granuloma and plays a similar role in the much more rapid and truncated rechallenge response.

### Summary

We have examined the temporal sequence of events leading to the formation of hepatic granulomas after the intravenous injection of *L. donovani* amastigotes into BALB/c mice. Parasite ingestion by permissive Kupffer cells (KC) occurred promptly, and local KC aggregations were the foci about which granulomas were subsequently formed. Infected KC were recognized by the uptake of colloidal carbon and the expression of the macrophage-specific antigen recognized by F4/80 mAb. Peroxidase-positive granulocytes migrated rapidly and were followed by monocytes and L3T4<sup>+</sup> T cells that enclosed the infected KC. Thereafter, Ly-2<sup>+</sup> T cells were prominent members of the granulomatous lymphoid population. Parasites multiplied until 4 wk, and then a prompt reduction in infected cells occurred. This was associated with a sharp decline in the L3T4<sup>+</sup> T cells of the granulomas and the maintenance of the Ly-2<sup>+</sup> subset. In comparison, athymic *nu/nu* mice developed smaller, more slowly appearing granulomas that contained granulocytes and monocytes and exhibited progressive parasite replication. Upon rechallenge, the entire process was completed in 2 wk, and infected KC in the euthymic mice were never observed.

We hypothesize that the effectiveness of the granulomatous response requires the destruction of parasitized host cells (KC), in a lymphokine rich environment. We

further suggest that the Ly-2<sup>+</sup> T cell serves as an important effector cell in this process, either by direct cytotoxicity or by supporting the cytotoxic potential of other cell types in the granuloma.

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