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Characterization of the *Histoplasma capsulatum*-Induced Granuloma

Erika Heninger,* Laura H. Hogan,* Jozsef Karman, † Sinarack Macvilay,* Bjork Hill,* Jon P. Woods, ‡‡ and Matyas Sandor‡*‡

Rising rates of *Histoplasma capsulatum* infection are an emerging problem among the rapidly growing population of immunocompromised individuals. Although there is a growing understanding of systemic immunity against *Histoplasma*, little is known about the local granulomatous response, which is an important component in the control of infection. The focus of this article is the characterization of *Histoplasma*-induced granulomas. Five days after i.p. infection, infected macrophage appear in the liver and lung; however, no granulomas are apparent. Two days later, well-formed sarcoid granulomas are abundant in the lung and liver of infected mice, which contain all visible *Histoplasma*. Granulomas are dominated by macrophage and lymphocytes. Most of the *Histoplasma* and most of the apoptotic cells are found in the center of the lesions. We isolated liver granulomas at multiple time points after infection and analyzed the cellular composition, TCR gene usage, and cytokine production of granuloma-infiltrating cells. The lesions contain both CD4⁺ and CD8⁺ T cell subsets, and T cells are the primary source of IFN-γ and IL-17. The main source of local TNF-α is macrophage. Chemokines are produced by both infiltrating macrophage and lymphocytes. Dendritic cells are present in granulomas; however, T cell expansion seems to occur systemically because TCR usage is very heterogeneous even at the level of individual lesions. This study is the first direct examination of host cellular responses in the *Histoplasma*-induced granuloma representing the specific interface between host and pathogen. Our studies will allow further analysis of key elements of host *Histoplasma* interactions at the site of chronic infection. The *Journal of Immunology*, 2006, 177: 3303–3313.

The granuloma is a form of delayed-type hypersensitivity. Localized inflammatory lesions composed of infected macrophages and fused giant cells can subsequently form granulomas with the help of CD4⁺ T lymphocytes. CD4⁺ T cells are very important for initiating and regulating granuloma function, but macrophage is the dominant cell type (1). A mature granuloma consists of an inflammatory interface surrounded by extracellular matrix that contains both inflammatory cells and the pathogen or inducing agent. The benefit of granuloma formation for the host is that it isolates the inflammation, protects the surrounding healthy tissue, controls the growth of pathogens, and prevents systemic dissemination. At the same time, the microorganism may also benefit from localization to the granuloma. As an isolated microenvironment, granuloma presents a special ecosystem for the pathogen in the host. The chronic granulomatous lesion may be the reservoir from which surviving pathogens emerge to reactivate the infection after a long-term latency is broken by failures of the immune system (2, 3).

*Department of Pathology and Laboratory Medicine, ‡Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706

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‡‡P.W. and M.S. are co-senior authors of this study.

*Address correspondence and reprint requests to Dr. Matyas Sandor, Department of Pathology and Laboratory Medicine, University of Wisconsin, 5460 MSC, 1500 University Avenue, Madison, WI 53706. E-mail address: msandor@wisc.edu

Histoplasma capsulatum is a thermally dimorphic fungal pathogen and is an opportunistic pathogen residing in the macrophage phagolysosome (4). *Histoplasma* infection commonly results in mild or unapparent clinical symptoms in immune-competent individuals and is endemic throughout large areas of the American Midwest (5). However, in immune-compromised individuals deficient in CD4⁺ T cell function, failure of adequate granuloma function allows the fungus to disseminate systemically and can lead to a serious, life-threatening disease course (6, 7). In endemic areas, histoplasmosis affects a growing population of patients with secondary immune defects, arising from HIV infection, immune suppression after transplantation, anti-TNF-α immunotherapy of rheumatoid arthritis, and inflammatory bowel diseases (6, 8–11).

*Histoplasma* infection of macrophage induces granuloma formation in different tissues. As in other granuloma-inducing infections, granuloma formation is required to contain fungal growth, prevent systemic dissemination, and protect the organs from widespread inflammatory tissue damage. Both experimental and clinical data indicate that T cells, IFN-γ, and TNF-α are crucial for protection against *H. capsulatum* infection (12–14).

To better understand the biology of diseases induced by intracellular pathogens, it is important to study the local inflammation site, to dissect and characterize the granuloma, and to expand histopathology data by investigating the granuloma at a cellular level. Despite the growing problems of histoplasmosis in immune-compromised populations, very little is known about the nature of the *H. capsulatum*-induced granuloma.

One reason is that granulomas are notoriously difficult to isolate and study. Previously, we and others have shown that granulomas induced by *Schistosoma mansoni* (15), *Leishmania chagasi* (16), and different species of *Mycobacterium* (17–19) can be readily isolated from soft tissues such as liver. In the present study, we report that *H. capsulatum*-induced liver granulomas can also be...
isolated and describe the cellular composition, cytokine milieu, and TCR gene usage in the lesion during the course of the infection.

These data will allow us to better understand Histoplasma-induced granulomatous inflammation and will serve as a baseline for further studies of how granulomas are regulated by different agents and conditions and how genetic and regulatory processes of the pathogenic yeast and the host contribute to the outcome of the interaction.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory and housed in the University of Wisconsin School of Medicine Animal Care Facility. All experiments were approved by the Institutional Animal Care and Use Committee of the School of Medicine and Public Health of the University of Wisconsin.

Fungal strain and infection of mice

H. capsulatum strain G217B (ATCC 26032) was maintained in the yeast form at 37°C in Histoplasma-macrophage medium (HMM) broth in 5% CO₂. Mice were infected i.p. with 5 × 10⁷ mid-epithelial phase yeast cells in 0.5 ml of HMM. For intranasal infections, mice were anesthetized, then 4 × 10⁷ H. capsulatum yeast was introduced into the nose in 20 µl of HMM.

Organ load CFU

Liver, lung, and spleen were harvested aseptically from infected mice. Tissue samples were homogenized in RPMI 1640 medium, and serial dilutions were plated on Brain Heart Infusion agar containing gentamicin and cultured at 28°C for 2–3 wk. Data are presented both as total CFU per organ and as total CFU per gram tissue. The limit of sensitivity is >100 CFU.

Histology

Small pieces of liver and lung tissue and liver granulomatous clumps were fixed in 10% buffered formalin and embedded in paraffin. Sectioning and staining were performed by the Histopathology Service of Department of Pathology, University of Wisconsin. Thin tissue sections (8–10 µm) were stained with Gomori’s methenamine silver (GMS) to detect H. capsulatum in granulomatous area of the liver by microscopy. For quantitation, GMS-stained yeasts were counted in 30 lesions per group at ×400 total magnification, and the result is presented as the average ± SEM. H&E staining was done for morphometric studies. Liver granuloma burden is the number of lesions per grid at ×100 magnification using an Olympus reticular eyepiece and is presented as the average of nine grids per group ± SEM. Granuloma size was measured at ×400 magnification using the grid and is presented as the average of 30 granulomatous lesions ± SEM.

Isolation of splenocytes and liver granuloma cells

Total splenocytes were isolated from aseptically removed naive and infected spleens by standard methods. Isolation of granuloma-infiltrating cells from infected livers was a modification of previously published methods used to isolate Mycobacterium-induced liver granulomas (17, 20, 21). Briefly, livers were homogenized with a tissue blender, and liver granulomas were allowed to settle by virtue of their higher density. After decanting the supernatant, settled granulomas were washed in RPMI 1640 medium. The granuloma pellet was digested with 5 mg/ml type I collagenase (cat. no. 0130; Sigma-Aldrich) at 37°C for 40 min with shaking. Granulomas were disrupted using a syringe, followed by filtering through a 70-µm nylon cell strainer (BD Falcon) to remove any tissue debris. The live leukocyte count was determined by trypan blue staining. Bulk granuloma cell preps were derived by pooling and processing a minimum of two to three livers from infected animals, allowing us to isolate ~10–15 million granuloma-infiltrating cells for flow cytometry.

Flow cytometry and Abs

For flow cytometric analysis of cell surface marker expression, splenocytes or granuloma cell suspensions were incubated for 30 min at 4°C with different labeled Abs at saturation, then washed and analyzed. Unlabeled 50 µg/ml anti-FeR Ab (2.4G2) was used to block binding via FeR. Cell surface staining on 20,000–50,000 events was measured using a FACS-Calibur instrument (BD Biosciences) and analyzed using the FlowJo computer program (Macintosh version 6.2.1; Tree Star).

Abs labeled with various fluorochromes and specific for murine LFA-1, CD8, MHC class II (IA α), CD25, γδ TCR (clone GL3), TNF-α, IFN-γ, IL-17, TGF-β1, CD4, B220, and CD8 were purchased from BD Pharmingen. Abs specific for CD3 (clone Lyt-1), Vγ1-1 (clone U2C2), Mac-1, CD11c (clone N418), and DEC205 were purified from hybridoma cell lines and labeled with fluorochromes in our laboratory.

Intracellular cytokine staining

A total of 10⁶ splenocytes or granuloma cells was cultured for 5 h in a 96-well tissue culture plate, in complete RPMI (cRPMI) 1640 medium, containing 10% FCS and 1% Golgi-Stop (BD Pharmingen) ± 5 µg/ml anti-CD3 Ab. Cells were resuspended in 50 µl of FACS staining buffer (1× PBS containing 10% BSA and 0.1% sodium azide) and stained for surface markers CD4, CD8, and Mac-1 for 30 min on ice, with 2 µg of blocking 2.4G2 Ab. Cells were washed three times and fixed by Cytofix/ Cytosperm solution (BD Pharmingen) at room temperature for 20 min. Cells were permeabilized and washed three times in FACS buffer containing 0.1% saponin and incubated for 30 min on ice with anti-TNF-α, anti-IFN-γ, anti-TGF-β1, anti-IL-17, or isotype control rat IgG Ab.

Cytokine and chemokine measurements from cell culture supernatant

A total of 10⁶ granuloma cells was cultured for 72 h in a 96-well tissue culture plate, in cRPMI 1640 medium, containing 10% FCS ± 5 µg/ml anti-CD3 Ab. Cell culture supernatants were harvested and stored at −80°C until analysis. Supernatants were sent to LINCO Research for custom analysis using a 22-plex Mouse Cytokine and Chemokine cytometric bead array. Intra- and interassay variances are <10 and 20%, respectively. For TGF-β measurements, 10⁶ granuloma cells were cultured for 48 h in a 96-well tissue culture plate, in cRPMI 1640 medium without FBS, containing ±5 µg/ml anti-CD3 Ab. Supernatants were stored at −80°C and assayed by TGF-β1, Easo ELISA kit according to the manufacturer’s protocol (Promega).

PCR and primers

Single granulomas were isolated from the preparative suspension before dispersal with collagenase under ×10 magnification using a Fasteur pipette flame-drawn to a finer tip. Individual lesions were stored at −80°C before processing. mRNA from individual granuloma was isolated using a MicroFast Track mRNA isolation kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. cDNA synthesis and RT-PCR was performed as previously described (22, 23) using published primers for CD3 length analysis (24). Products from these reactions were analyzed on 2% agarose gels and visualized by ethidium bromide staining.

Immunohistochemistry and TUNEL staining

Five-micrometer thick cryosections were cut from OCT-embedded liver tissue samples and fixed for 30 min in 4% paraformaldehyde in PBS, then washed three times with PBS and outlined with a Pap pen. Sections were blocked with 40 µg/ml 2.4G2 Ab in 1% BSA for 30 min and then stained for 30 min using CyChrome-labeled anti-CD4 (RM5-4) and biotin-labeled anti-CD11b (Mac-1) in combination with Alexafluor labeled streptavidin. TUNEL staining was performed following the manufacturer’s protocol (Roche Diagnostics). Confocal images were acquired on a Bio-Rad MRC-1024 maintained by the W. M. Keck Laboratory for Biological Imaging (University of Wisconsin).

Results

To investigate granulomatous inflammation induced by H. capsulatum, we established an i.p. infection model of liver granuloma formation. Intraperitoneal infection results in quantitative inoculum delivery, immediate systemic access, and rapid development of disseminated infection. We developed i.p. inoculation as a model to look at systemic, disseminated histoplasmosis at stages beyond the initial respiratory acquisition—specifically dissemination and granulomatous disease in the mononuclear phagocytic system. Liver granulomas are induced naturally after respiratory Histoplasma infection and after intranasal inoculation, but i.p. infection optimizes both yield and synchronization.
Kinetics of Histoplasma capsulatum growth after i.p. inoculation

A. Photomicrographs at ×400 total magnification of GMS stained thin liver sections at days 5, 7, 10, 14, and 50 after infection of C57BL/6 mice. H. capsulatum cells are indicated by arrows and bar represents 10 mm. B. Quantitation of H. capsulatum in randomly selected lesions was used to calculate the number of yeasts per granulomatous lesion. Error bars represent ±SEM (n = 30 lesions). C. Histoplasma capsulatum organ load cultures from spleen, lung, and liver are presented as LOG CFU (represent used to calculate the number of yeasts per granulomatous lesion. Error bars, but is detectable at low levels in the lung as well (see Fig. 3 A). Data represent the average ± SEM (

Histopathology and morphometric analysis

Our next goal was to analyze the kinetics of Histoplasma-induced granuloma formation in the liver. At day 5 after Histoplasma infection, H&E-stained thin liver sections show mild vasculitis and some cellular infiltration (Fig. 2A, top row). Rarely, minor aggregates of inflammatory cells are observed. Higher magnification images (Fig. 2A, bottom row) show macrophage extravasation and focal aggregation of leukocytes. Digital enlargement of the lower left panel of Fig. 2B demonstrates that after extravasation, inflammatory cells migrate in a line (a direct line into the tissue) to H. capsulatum yeasts. A sophisticated well-regulated process of granuloma formation is suggested by the rapid appearance of many well-structured granulomas 2 days later by day 7, which persist through 10 and 14 days (Fig. 2A, top and bottom rows). The appearance of the granulomas is sarcoid, having epithelioid cells in the center. Analysis of the number (Fig. 2C) and size of lesions (Fig. 2D) shows that while the number of granuloma remains high through 14 days, the size of the lesions is down-modulated by day 14 (Fig. 2C). At 50 days postinfection, a few well-formed and small-sized granuloma persist in the liver.

In the lung (Fig. 3, upper two rows), after the initial appearance of smaller inflammatory cell aggregates, well-formed granulomas dominate from day 10. Interestingly, lung granuloma are much more variable in appearance and size than in the liver but tend to be larger overall. In addition, the very few granulomas present after day 50 (less than or equal to three granulomas per lobe) are still large and are not down-modulated in size as in the liver (Fig. 3, A and B).

Phenotypic analysis and cellular composition of liver granuloma-infiltrating cells during the course of Histoplasma infection

Isolation of granulomatous lesions allows us to study these inflammatory reactions, including cellular composition, cytokine milieu,
a large fraction (8%) of the cells are DEC205 infection (data not shown). In the early granuloma (day 7; Fig. 4A), a large fraction (8%) of the cells are DEC205

and gene expression at the local level. This approach has been applied very successfully to granulomas formed in soft tissues like the liver in response to various infectious agents (16, 25–28). Isolation of infiltrating cells after settling and digestion of granulomas from dispersed liver tissue has the advantage over other methods like laser dissection in that it gives a much larger sample size for study. In the present study, we report that H. capsulatum-induced granulomas can be isolated from the liver.

The individual granuloma settles out with a small rim of liver cells. The digested granuloma preparation is enriched for inflammatory cells that can be studied by flow cytometry (Figs. 4A and 5A, lower left insets). For our flow cytometry studies, bulk granuloma cell preps were derived by pooling and processing a minimum of two to three livers from infected animals. The following section will describe the proportion and phenotype of inflammatory cells isolated in this manner.

The proportion of macrophages is somewhat increased in the spleen after the infection; however, macrophages clearly dominate the Histoplasma-induced granulomatous lesion (Fig. 4A, top and middle rows). MHC class II expression on Mac-1+ cells from both spleen and granuloma is elevated (data not shown and Fig. 4A, bottom row). MHC class II expression is the activation marker of macrophages responding to local IPN-γ-mediated signaling. Both total numbers of Mac-1+ cells and MHC class II expression are highest at 10 and 14 days postinfection.

DEC205+CD11c+ dendritic cells represent 1–2% of total spleen cells and that proportion is unaltered during H. capsulatum infection (data not shown). In the early granuloma (day 7; Fig. 4B), a large fraction (8%) of the cells are DEC205+CD11c+ expressing high levels of MHC class II (Fig. 4B, middle row) and intermediate levels of Mac-1 (Fig. 4B, bottom row). The proportion of these cells decreases at later time points but remains consistently higher than in the corresponding spleen samples.

Next, we examined the phenotype of granuloma-infiltrating T cells. Fig. 5A shows that the ratio of CD4+ to CD8+ T cells in splenocytes is essentially unchanged during the course of infection compared with naive splenocytes. In contrast, in granuloma-infiltrating cells, CD4+ T cells are more abundant during the early acute-phase time points, while CD8+ T cells catch up during the chronic stage granuloma at day 50. At the same time, CD4+ T cells exhibit a uniformly high expression of LFA-1high phenotype in the spleen sooner than do CD8+ LFA-1high T cells, but the proportion of LFA-1highCD8+ T cells is somewhat higher by day 50. Both CD4+ and CD8+ T cells exhibit a uniformly high expression of LFA-1 in the granuloma (Fig. 5B), indicating an activated phenotype. Both CD4+ effector T cells with intermediate expression of CD25 and T cells with CD25high expression roughly double in the spleen by day 5 of infection and remain elevated through day 50 (Fig. 5C). In the granuloma, CD25int cells are present at a higher frequency than in

FIGURE 3. Kinetics of Histoplasma capsulatum induced histopathology in the lung. A, Photomicrographs show are of thin lung sections at days 5, 7, 10, 14, and 50 after infection of C57BL/6 mice. H&E-stained sections are shown at ×40 (top row) and ×400 (middle row) total magnification, and GMS stain is shown at ×400 total magnification (bottom row). Yeasts are indicated by arrows. B, Lung lesion size was measured as in Fig. 2. Data represent the average ± SEM from three mice per group.

Granuloma burden was not quantified because less than one granuloma per field was observed.

Macrophage and dendritic cells in liver granulomas. Flow cytometric analysis of splenocyte and granuloma-infiltrating cells during the course of Histoplasma infection. A, Dot plots represent expression of Mac-1 and CD4 surface staining on open gated cells from spleen (top row) and granuloma cells (middle row). Lower left dot plot demonstrates forward and orthogonal scatter of the open gate for a granuloma suspension. Values on dot plots represent the percentage of the gated cells in the indicated regions. Histograms represent MHC class II expression on Mac-1+ granuloma cells, and values are mean fluorescent intensity (MFI) (bottom row). B, Dot plots represent expression of DEC205 and CD11c+ surface staining on open gated cells from granuloma (top row). Indicated values are the quadrant percent of the gated cells. Histograms represent MHC class II expression (middle row) and Mac-1 expression (bottom row) on DEC205+CD11c+ cells. Values are MFI for the gated population. All plots and histograms are representative of three independent experiments.
the spleen, which is consistent with the LFA-1(high) phenotype of the majority of granuloma-infiltrating T cells. In contrast, CD25(high) CD4(+) T cells thought to be regulatory T cells are present at much lower levels in the granuloma relative to both infected spleen and naive spleen, suggesting an exclusion of those cells from the local inflammatory site that persists through day 50. This is especially interesting since not only does the proportion of these cells double during infection, but the infection increases the total number of splenocytes by 2- to 3-fold.

TCR usage in granuloma-infiltrating T cells

TCR V beta-chain repertoire studies for Histoplasma infection models have been a subject of extensive investigation (29–32). A consensus exists that the repertoire is heterogeneous overall, but there is some controversy regarding V beta-chain preferences observed in some models for spleen and lymph node. Our goals were to extend these broad observations to the local inflammatory site.

We looked at the proportional usage of TCR genes in spleen and granuloma during the course of infection using V beta-chain-specific Abs. Although the V beta-chain usage is somewhat different between spleen and granuloma, all tested V beta-chains are present and similarly distributed in spleen and granuloma (Fig. 6A and data not shown). We further studied whether the heterogeneity we observed at the level of pooled cell suspensions (spleens and bulk granuloma) is present in single lesions. Fig. 6B shows a representative single granuloma from a settled preparation before enzymatic digestion. First, the presence of V beta 8.2 message was measured in six single granulomas representing three different time points (Fig. 6C). Although the absolute level of message varied, V beta 8.2 usage was observed in all single granulomas. Then, the usage of various J beta genes in V beta 8.2-specific gene transcripts was tested (Fig. 6D), and the data indicated that there are both similarities (J beta 2.4) and many differences in the usage of J beta genes in different lesions at each examined time point. These data clearly demonstrate TCR heterogeneity even at the single granuloma level and argues that, instead of a few T cells expanding at the inflammatory site, most T cells expand systemically and then home to the granuloma producing a diverse T cell repertoire.

γδ T cells are known to have a role both in controlling infections (33, 34) and in contributing to regulation of granulomas (35). To
granulomas were isolated.

used as a template for RT-PCR using TCR V/H9252

individual isolated liver granuloma from day 10 postinfection at

preparations and further processed for preparation of single-granuloma to-

nification. Individual granulomas are isolated from homogenized liver

pressing the indicated chain from four to six mice per time point. Error bars

values represent the average percentage of total CD4/H11011

gesting an absence of selection for specific

specific Abs were used in combination with CD4- and CD8-specific Abs to
demonstrate the equivalency of the cDNA template for each reaction. PCR

FIGURE 6. TCR V/J-chain usage in Histoplasma-infected spleen and
granuloma cells. A, TCR V/J3-, V/J6-, V/J8.1/8.2-, and V/J11-chain-spe-
cific Abs were used in combination with CD4- and CD8-specific Abs to
detect cell surface expression in total splenocytes or granuloma-infiltrating
cells at the indicated time points after infection of C57BL/6 mice. Plotted
values represent the average percentage of total CD4+ or CD8+ cells ex-
pressing the indicated chain from four to six mice per time point. Error bars
represent ± SEM. B, The photomicrograph illustrates a H&E-stained in-
dividual isolated liver granuloma from day 10 postinfection at ×100 mag-
nification. Individual granulomas are isolated from homogenized liver

investigate whether γδ T cells accumulate in Histoplasma-induced

liver granulomas, we measured the expression of γδ TCR and the
dominant Vγ1.1 chain in both splenocytes and granuloma-infil-
trating cells at time points after infection. Fig. 7 shows that γδ T
cells as a percentage of the total increase very slightly in sple-
cocytes after Histoplasma infection (0.7% at day 7 vs 0.4% in naive

while T cells are in

histoplasma-induced granulomas, and their proportion does not change during the
course of infection. In addition, TCR Vγ1.1 chain is used by

~80% of systemic γδ T cells in uninfected mice. This ratio does not
change either in the infected spleen or in the granuloma, sug-

Cytokines and chemokines in Histoplasma-induced granulomas

Next, we investigated the local cytokine and chemokine levels in

Histoplasma-induced granulomas. Multiplex analysis of cytokines

secreted by Histoplasma-induced granuloma-infiltrating cells dur-
ging 72 h in vitro culture shows that IL-2 production is minimal
from day 7 onward and only above the level of detection in re-
sponse to anti-CD3 stimulation (Table I). Of the Th2 cytokines,

FIGURE 8. B cells in Histoplasma-induced liver granulomas. Flow
cytometric analysis of splenocytes and granuloma-infiltrating cells at vari-
ous times after Histoplasma infection of C57BL/6 mice. A, Dot plots repre-
sent γδ TCR and Vγ1.1 chain-specific cell surface staining on
CD4+ CD8+B220 Mac-1 lymphocyte-gated cells. Values are the per-
centage of the total sample stained with γδ TCR-specific Ab. γδ TCR "Vγ1.1" populations are shown by separate regions. The plots are repre-
sentative of three independent experiments.

and increasing to ~20% of lymphocyte-gated cells at day 50 after
infection (Fig. 8A). Alternative B cells expressing the nonconven-
tional marker CD5 represent 5% of splenic B cells. Although
CD5+ B cells are the dominant B cell type both in peritoneum and
in liver-resident B cells (data not shown), they do not contribute to the B cell pool in the day 10 granuloma (Fig. 8B).
IL-5 is the most abundant, and IL-4, IL-9, and IL-13 are at low to undetectable levels. Both TNF-α and IFN-γ have been shown to be important to control of Histoplasma infection (13, 37). Our data show that both cytokines can be produced at very high levels by cells from the granulomatous inflammatory site, demonstrating the strong Th1 bias of infiltrating cells. Comparison of the levels in the supernatants of cultures with and without T cell activating anti-CD3 suggests that IFN-γ is T cell derived and TNF-α is not. Intracellular staining of TNF-α and IFN-γ (Fig. 9) shows that most TNF-α is produced by Mac-1+ cells (macrophages), whereas IFN-γ is produced both by CD4+ and CD8+ T cells. It is clear that the population of cells in the granuloma that produce TNF-α (Fig. 9A) or IFN-γ (data not shown) is much higher than in the spleen. Like IFN-γ, IL-17 is a strong inducer of macrophage effector function and is associated with chronic inflammation. IL-17 is produced by CD4+ granuloma-infiltrating cells (Table I and see Fig. 11). Macrophages are also influenced by high levels of inflammatory cytokines IL-1 and IL-6, in addition to chemokines and macrophage growth factors. These factors favor the recruitment, proliferation, and activation of macrophage that are the dominant cell type in H. capsulatum-induced granulomas and were detected at high levels in the supernatants from granuloma-infiltrating cells. While the level of these molecules are high at all three time points tested, at day 14, the level of proinflammatory mediators decreases. Interestingly, although IL-10 and TGF-β are known to have immunosuppressive functions, their levels in early granuloma culture supernatants are high. The main cellular source of TGF-β is the macrophage (see Fig. 11). These data illustrate that a large number of mediators operate in the local inflammatory lesions and calls for a functional analysis of this local regulatory network.

### Apoptosis of granuloma-infiltrating leukocytes in Histoplasma infection

Others have suggested that Histoplasma-induced T cell apoptosis is a requirement for developing effective antifungal protection (38). Microscopic examination of Histoplasma-infected liver granulomas by H&E staining did not show frequent central necrosis in C57BL/6 mice. To characterize the presence of apoptotic cells in Histoplasma granulomas, we used immunofluorescent Ab staining and TUNEL staining. Mac-1+ and CD4+ cells (Fig. 10, A and B) were enriched in Histoplasma liver granulomas in proportions consistent with our FACS data (Fig. 4A). In addition, immunofluorescent end-labeling of DNA fragments was enriched within granulomas, suggesting a relatively high occurrence of apoptotic cells (Fig. 10C). Cystained sections demonstrate that both macrophage and T cells contribute to the apoptotic fraction of granuloma-infiltrating cells (Fig. 10D). The granuloma centers are where the large majority of the apoptotic cells are found.

### Comparison of Histoplasma liver granulomas induced via i.p. and intranasal routes

In this article, we used an i.p. route of infection as a model for disseminated histoplasmosis. It is possible that the natural infection route through the airways might induce a different type of disseminated liver granuloma due to the modifying effect of a complex lung infection and altered local APC. We compared the i.p. model granulomas to liver granulomas induced after intranasal infection. Fig. 11A shows that the morphology of the liver granulomas induced after intranasal infection is T cell derived and TNF-α and IFN-γ have been shown to be important to control of Histoplasma infection (13, 37). Our data shows that both cytokines can be produced at very high levels by cells from the granulomatous inflammatory site, demonstrating the strong Th1 bias of infiltrating cells. Comparison of the levels in the supernatants of cultures with and without T cell activating anti-CD3 suggests that IFN-γ is T cell derived and TNF-α is not. Intracellular staining of TNF-α and IFN-γ (Fig. 9) shows that most TNF-α is produced by Mac-1+ cells (macrophages), whereas IFN-γ is produced both by CD4+ and CD8+ T cells. It is clear that the population of cells in the granuloma that produce TNF-α (Fig. 9A) or IFN-γ (data not shown) is much higher than in the spleen. Like IFN-γ, IL-17 is a strong inducer of macrophage effector function and is associated with chronic inflammation. IL-17 is produced by CD4+ granuloma-infiltrating cells (Table I and see Fig. 11). Macrophages are also influenced by high levels of inflammatory cytokines IL-1 and IL-6, in addition to chemokines and macrophage growth factors. These factors favor the recruitment, proliferation, and activation of macrophage that are the dominant cell type in H. capsulatum-induced granulomas and were detected at high levels in the supernatants from granuloma-infiltrating cells. While the level of these molecules are high at all three time points tested, at day 14, the level of proinflammatory mediators decreases. Interestingly, although IL-10 and TGF-β are known to have immunosuppressive functions, their levels in early granuloma culture supernatants are high. The main cellular source of TGF-β is the macrophage (see Fig. 11). These data illustrate that a large number of mediators operate in the local inflammatory lesions and calls for a functional analysis of this local regulatory network.

### Table I. Cytokine and chemokine production of granuloma-infiltrating cells

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<th>Day 7</th>
<th>Day 10</th>
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<td>Media</td>
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<td>IFN-γ</td>
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* All units are pg/ml.
* nd, not done.
* Done by ELISA.
very similar in intranasal and i.p. infection models (data not shown).

Importantly, measurement of intracellular cytokine production by granuloma cells suggests that lesions induced after either infection route contain CD4+ T cells, which produce similar levels of IFN-γ and IL-17 (Fig. 11B). Both cytokines are important for activating macrophage. Lastly, Mac-1+ cells from both lesion types are able to produce TNF-α (Fig. 11C) and TGF-β (Fig. 11D).

Discussion

The success of host immunity in controlling *H. capsulatum* infection is underscored by the wide prevalence of asymptomatic or clinically mild infections despite skin test reactivity rates over 90% in endemic areas along the Ohio and the Mississippi River valleys (5, 39). Granulomas formed in response to macrophages infected by *Histoplasma* are most likely a dominant component of the highly effective antifungal immune response. Conversely, the progressive, disseminated histoplasmosis observed in immune-compromised persons arises in large part from failures of established granulomas and failure to form new inflammatory lesions in response to recently infected macrophages. While there is growing knowledge about the nature of systemic immunity during the course of histoplasmosis (32, 40–42), there is little known about local immune responses within granulomas despite these lesions representing the main interface between the fungi and the host. Previously, one of the limiting factors was lack of an isolation procedure for *Histoplasma*-induced granulomas. Effective techniques for isolating granulomas from the liver have been published for *S. mansoni* (21)-, *Mycobacterium* (17)-, and *Leishmania* (16)-infected mice among others. Subsequent studies of granuloma-infiltrating cells significantly enriched our understanding of similarities and differences between local and systemic immunity in response to these infectious agents (43). The goal of the present studies was to isolate *H. capsulatum*-induced granulomas and describe the cellular composition and cytokine milieu of granuloma-infiltrating cells during the course of infection. From this foundation of basic knowledge, mechanistic studies can be initiated to understand local immune regulatory networks in *H. capsulatum* infection and their relevance in the control of this infectious agent.
As soon as 5 days after i.p. infection, yeast-infected macrophages are detectable in the liver by histopathology. At that early time, no granulomas are visible, although there is an evident vasculitis and evidence for extravasation of leukocytes toward infected macrophages (Fig. 2). Just 2 days later, the liver is marked by an abundance of organized sarcoid-type granulomas. The ability to form granulomas in just 2 days provides a well-timed model to study granuloma formation. The complexity and similarity of these synchronized lesions suggest a well-orchestrated and regulated process.

Morphometric analysis of liver granuloma size showed a dynamic change throughout the time course of the infection, which is mirrored in the kinetics of Histoplasma clearance. The average granuloma size reaches a maximum at day 10 of the infection and subsequently declines. This observation recalls the down-regulation of acute lesion size during chronic disease reported for Schistosoma and Leishmania infections (44, 45). In the latter models, the down-modulation of granuloma size is driven by IL-10 and TGF-β (46–48). Multiplex data show that IL-10 levels are elevated in ex vivo cultures of *H. capsulatum* granuloma-infiltrating cells with and without anti-CD3 stimulation, suggesting that IL-10 is elevated in the *Histoplasma* granuloma. Interestingly, both IL-10 and TGF-β are highest in the early granuloma. The main source of TGF-β is macrophage, according to our intracellular FACs data. In addition to IL-10, fungal clearance leading to decreased antigenic stimuli, inflammatory agents and chemoattractants, and attenuated cellular recruitment may contribute to the time-dependent size decrease of liver granulomas. Interestingly, the size of lung granulomas remains at a maximum for a longer time, perhaps indicating that the host organ affects the phenotype of granulomatous lesions. Another interesting observation is that new granuloma formation is clearly present after organ load cultures indicate clearance of the yeast. It is possible that fragments of the yeast persist and serve as granuloma-inducing irritants. However, it is also possible that granulomatous protection is not completely sterilizing, and very low numbers of yeast survive and serve as a source for reactivation disease during immune deficiency.

While most *Histoplasma* granulomas appear free of necrosis, TUNEL staining (Fig. 10) shows that at day 10 when the granulomas are the largest, the lesion centers are enriched in apoptotic cells. Clearing of apoptotic cells by local macrophages may contribute to the decrease in granuloma size, perhaps as part of a continuous turnover of the cellular composition of the lesion. It may also be important in the control of the yeast by clearing infected cells. An intriguing report (38) shows that apoptosis is a prerequisite for a successful immune response. *Mycobacteria* are also known to evade immune clearance by inhibiting apoptosis in the infected macrophage (49, 50).

Both liver histology and flow cytometry of the granuloma-infiltrating cells indicate that macrophage are the dominant cell type in the lesion reaching up to 70% of the granuloma. Most of them are activated because the levels of IFN-γ and other macrophage-activating factors are high in the lesions. Many of the Mac-1+ macrophage have elevated MHC class II expression and produce TNF-α (Figs. 4A and 9A and Table I). There are very few other myeloid cells in granulomas. H&E-stained sections suggest that most lesions have few, if any, neutrophils, while flow cytometry indicates that only a low percentage of granuloma-infiltrating cells express a dendritic cell phenotype (CD11c+DEC205+) (Fig. 4B). The presence of dendritic cells may provide a local reactivation for the recruited effector T cells and raises the possibility that they sample granuloma Ags and may carry them to draining lymph nodes. The idea that granuloma-contained Ags might prime systemic T cells needs further investigation.

Both CD4+ and CD8+ T cells are recruited to the lesions. At early stages, there are more CD4+ T cells present, but later the ratio is close to 1:1. This temporal change in the CD4/CD8 T cell ratio likely reflects the somewhat earlier systemic activation of CD4+ T cells relative to activation of CD8+ T cells. Both T cells contribute to the cytokine milieu of the granulomas. A subpopulation of cells has IL-2R expression levels above the level on activated effector T cells and consistent with regulatory T cells. Interestingly, the proportion of CD4+IL-2Rhigh cells is higher systematically than in the granuloma, suggesting that this population of cells fails to accumulate in the lesions. The role of regulatory T cells in *Histoplasma*-induced granuloma formation warrants more investigation since these cells are reported to regulate Leishmania- and Schistosoma-induced lesions (51–54). Furthermore, given the limitation of regulatory T cell classification based on CD25 expression alone, additional phenotypic and functional characterization will be needed in further studies of regulatory T cells in *Histoplasma* infection.

The level of γδ T cell is also lower in granulomas compared with systemic sites. γδ T cells have been reported in granulomas (55) affecting granuloma size during the chronic stage of infection (56–58). A low level of B cells is also present having a conventional B cell phenotype (CD5−Mac-1−) characteristic of peripheral blood. The potential for local Ab production in the granuloma is potentially important since there are reports that Abs can protect against intracellular yeasts (58).

The TCR repertoire of systemic T cells during *Histoplasma* infection has been studied before. While some studies reported relative enrichment of T cells using certain TCR Vβ-chains (29–31, 59, 60) and others did not (29, 32, 60), there is a consensus that *H. capsulatum* activates a diverse repertoire of T cells. In the present study, we extend this finding to granuloma-infiltrating T cells. Our data clearly indicate a heterogeneous repertoire in the granuloma with no major superantigen-type shifts in TCR usage. Moreover, using J allele-specific primer pairs, we demonstrated that there is a large level of T cell heterogeneity even at the single granuloma level (Fig. 6D). Additionally, we looked at TCR usage at days 7, 10, and 14 and saw no shifts consistent with clonal dominance or with founder effects through the time course.
This finding argues that instead of a model in which a few T cell founders home to the early granuloma and expand there, the repertoire of the *Histoplasma*-induced granuloma derives from the entire systemically expanded T cell population. This is similar to our previously reported observations for both *Mycobacterium bovis* bacillus Calmette-Guérin- and *S. mansoni*-induced granuloma formation (22, 23), indicating that similar principles drive T cell accumulation in various granulomas. Likewise, TCR repertoire analysis of the low numbers of γδ T cells infiltrating the granuloma indicates that they express the Vγ1.1 receptor allele in a similar proportion those found in the blood. Collectively, B cells, αβ T cells, and γδ T cells in the *Histoplasma*-induced liver granuloma all appear to reflect the peripheral blood populations.

IFN-γ plays a central role in the immunity against *H. capsulatum*. In fact, IFN-γ-deficient animals are killed by *H. capsulatum* infection (12, 37). We extended this finding by showing that IFN-γ is produced at high levels by granuloma-infiltrating cells (Table I and Fig. 9, B and C). The main cellular source of IFN-γ in the granulomas is CD4+ and CD8+ T cells (Fig. 9, B and C). Local IFN-γ activates macrophage to produce reactive radicals crucial to control of the yeast. The granuloma-infiltrating cells also produce high levels of TNF-α. Intracellular staining (Fig. 9A) and the absence of anti-CD3-induced TNF-α production (Table I) show that the primary cellular source of TNF-α is the macrophage, and T cells contribute little if any to local TNF-α levels. Therapeutic anti-TNF-α treatments induced reactivation of latent *H. capsulatum* infection in some patients, emphasizing the role of this mediator in control of the yeast and preventing dissemination (8, 10, 61). The granuloma has strong Th1-type and very little Th2-type cytokine activity (Table I). Interestingly, the newly described Th-17 cells (61) may also be present, as IL-17 levels increase in response to anti-CD3. Intracellular staining also shows the presence of CD4+ IL-17-producing cells in the lesions (Fig. 11B). The Th-17 lineage is proposed to be a major effector population responsible for tissue damage in chronic autoimmune diseases. One of the reported functions of Th-17 cells is in the induction of chemokines. We observed that granuloma-infiltrating cells produce high levels of multiple chemokines capable of mediating the recruitment of macrophages and lymphocytes to the lesion (Table I). The in situ production of myeloid growth factors may help macrophage recruitment and survival in the granulomas.

All of the previous investigations of immune responses to *Histoplasma* infection characterized either systemic responses or acute peripheral responses. To our knowledge, ours is the first study looking at the local inflammatory site at a stage corresponding to *Histoplasma*-induced delayed-type hypersensitivity responses. We compared our granuloma responses to systemic responses represented by the spleen and found a very good correspondence in the systemic parameters we measured with previous characterizations by other investigators. Specifically, other investigators have seen that systemic responses against *Histoplasma* infection are dominated by CD4+ T cell responses and a requirement for IFN-γ and TNF-α production as basic factors of fungal control. Likewise, *Histoplasma* granulomas are dominated by activated macrophage producing TNF-α and infiltrated by IFN-γ-producing CD4+ T cells. Overall, the *Histoplasma*-induced delayed-type hypersensitivity response in the liver is characterized by diffuse sarcoid-type granuloma formation, which contains fungal growth and is associated with a relatively rapid fungal clearance.

There are three nonexclusive paradigms about granuloma formation. Traditionally, granuloma formation is considered a pathology in which host responses to infectious pathogens induce organ damage. Simultaneously, it is widely accepted that granulomas are a protective form of delayed-type hypersensitivity controlling the expansion of infectious agents. The formation of the granulomatous lesion walls off pathogens from further access to the host, thus preventing dissemination and organ damage arising from the host inflammatory response. Finally, in the field of *Mycobacterium* infection, Ramakrishnan and coworkers (62) describe granulomas as ecosystems of mutual benefit to host and infectious organism. They posit granuloma formation as an evolutionary compromise (62) in which the host restricts both dissemination and damaging acute inflammation, while allowing a small number of pathogens to survive for extended intervals. The granuloma/pathogen balance results in relatively little harm to the healthy host, yet dormant bacteria are able to reactivate and disseminate when host immunity wanes and granuloma formation fails. Mutant *Mycobacteria* eliciting an altered host pathology after infection, *pat* mutants, provide support for the concept that the infectious agent participates in forming the granuloma (reviewed in Ref. 63). Studies indicate that during secondary infection, *Mycobacterium* preferentially “home” to pre-existing granulomas where immunity is strong but not sterilizing (62). The occurrence of reactivation disease in immune-deficient hosts for both tuberculosis and histoplasmosis makes it possible that a similar host-pathogen accommodation may be operating during *Histoplasma* infection. All of these paradigms stress the importance of further knowledge regarding *H. capsulatum*-induced granulomas to understand the biology of infection and provide a sound basis for more efficacious therapeutic interventions.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


