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LIVER MACROPHAGES IN MURINE LISTERIOSIS

Cell-mediated Immunity Is Correlated With an Influx of Macrophages Capable of Generating Reactive Oxygen Intermediates

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Resident liver macrophages (Kupffer cells) are typical of macrophages in other sites in several respects: phagocytosis; expression of Ia antigens, Fc receptors, and the F4/80 macrophage-restricted antigen; and secretion of several polypeptides, including apolipoprotein E (1). However, Kupffer cells are deficient in the capacity to release reactive oxygen intermediates (ROI) (hydrogen peroxide and superoxide anion) and to dispose normally of intracellular protozoan parasites in vitro, even after exposure to murine γ interferon (IFN-γ) (1). This deficit raises two questions: Do Kupffer cells fail to show enhanced oxidative capacities during a cell-mediated immune response? If so, how does the host cope with hepatic infection by facultative or obligate intracellular pathogens?

We have chosen murine listeriosis as a model of an effective cell-mediated immune response to a facultative intracellular pathogen. It is known (2) that hepatic resistance to Listeria monocytogenes depends upon an influx of monocytes. The immigrant monocytes, derived from the marrow, congregate in infectious foci that initially contain proliferating organisms, neutrophils, and necrotic debris. Over a period of ~10–14 d, the lesions fully resolve. In this paper, we describe the separation of immigrant macrophages and resident Kupffer cells from listerotic mouse livers and the ability of isolated populations to secrete H₂O₂. Kupffer cells from inflamed livers, as well as those isolated from normal livers and exposed to inflammatory mediators in vitro (1), secrete low levels of H₂O₂. In contrast, immigrant macrophages secrete abundant H₂O₂ and account, quantitatively, for the emergence of H₂O₂ secretory capacity in the liver as a whole during clearance of listeria.

This work was supported by research grants AI 07012, AI 13013, AI 16963, CA 22090, and CA 30198 from the U. S. Public Health Service. Dr. Steinman is an Established Investigator of the American Heart Association; Dr. Nathan is a recipient of a research career development award from the Rita Allen Foundation; Dr. Murray is the recipient of a Research Career Development Award in Geographic Medicine from the Rockefeller Foundation (RF 78021). 1 Abbreviations used in this paper: IFN-γ, γ interferon; PMA, phorbol myristate acetate; RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and gentamicin sulfate; ROI, reactive oxygen intermediates.
Materials and Methods

**Mice.** 6-8-wk-old female (C57BL/6 × DBA/2)F1 mice were obtained from the Trudeau Institute, Saranac Lake, NY.

**Bacteria.** *Listeria monocytogenes* (strain EGD, serotype 3b) was generously provided by Dr. Robert North (Trudeau Institute). Mice were inoculated intravenously with $5 \times 10^5$ bacteria in 0.2 ml of 0.9% saline. In vivo *Listeria* clearance was followed by plating 10-fold serial dilutions of splenic homogenates on trypticase soy broth-agar (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, MD); bacterial colonies were counted after 24 h. Three mice were used for each time point.

**Liver Macrophages.** Liver macrophages were obtained from *Listeria*-infected mice by a high yield collagenase perfusion technique (1). Macrophages were cultured in 16 or 35 mm plastic wells (Costar, Data Packaging, Cambridge, MA) at 37°C with 5% CO2 in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Sterile Systems, Inc., Logan, UT) and 20 mg/l gentamicin sulfate (R10HIFCS).

Separation of immigrant macrophages from Kupffer cells was performed after 30-50 h in culture. By this time, immigrant macrophages, but not Kupffer cells, proved to be loosely adherent. 5 ml of 37°C RPMI 1640 was added to total liver macrophage populations, cultured at 2-3 × 10^6 cells per 35-mm Costar well. Immigrant macrophages were separated by gently pipetting over the culture surface and removing detached cells. These cells were washed twice in RPMI 1640 (400 g, 10 min, 4°C) and returned to R10HIFCS for readherence to plastic wells and cultivation. Cells remaining firmly adherent after removal of immigrant macrophages provided an enriched Kupffer cell population that was then cultured in R10HIFCS.

**H2O2 Secretion.** Cells were rinsed in saline, triggered with 100 ng/ml of phorbol myristate acetate (PMA) (Sigma Chemical Co., St. Louis, MO), and assayed for H2O2 release by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin (3).

**Histology and Immunocytochemistry.** Cryostat sections from carbon-labeled, *Listeria*-infected livers were fixed in acetone and stained with hematoxylin or examined for immunoperoxidase staining with antimacrophage (F4/80), anti-Mac-1, anti-Ia, or anti-Thy-1 monoclonal antibodies (1, 4).

**Infection With Toxoplasma gondii.** Virulent trophozoites of the RH strain of *T. gondii* were prepared as described (5). Macrophages were challenged for 30-60 min with $5 \times 10^5$ *T. gondii* trophozoites (6). Uningested parasites were removed by washing (time zero), and cultures were reincubated in R10HIFCS. At time zero and at various times after challenge, the number of parasites per 100 macrophages and the number of toxoplasmas per vacuole were scored in Giemsa-stained preparations.

Results

**H2O2 Secretion by Liver Macrophages in Listeriosis.** We confirmed previous work on the growth cycle of *Listeria* in situ by performing bacterial counts on organ homogenates throughout the course of infection. As described by Mackaness and others (7, 8) (Fig. 1A), ~90% of a sublethal intravenous challenge can be recovered from liver and spleen within 10 min of inoculation. Organ *Listeria* counts increased logarithmically for 2-3 d, after which bacterial growth ceased and microbicidal activity ensued.

Adherent liver mononuclear cells (>85% phagocytes) were isolated from these same animals and examined for PMA-triggered H2O2 secretion (Fig. 1B). In contrast to Kupffer cells from uninfected mice (reference 1 and Fig. 1B, time zero), macrophages from listeriotic livers did secrete H2O2. H2O2 production paralleled the course of infection, exceeding 300 nmol/mg protein on commencement of log-linear bacterial clearance. At the resolution of infection, on days 10-12, H2O2 production returned to the levels in normal livers. Neutrophil
contamination (1–4%) was too low to account for the observed increase in H$_2$O$_2$ secretion during infection. Indeed, the capacity to secrete H$_2$O$_2$ was maintained over 24–48 h in culture, by which time adherent neutrophils were virtually undetectable (<1%).

Identification and Separation of Immigrant Macrophages. The presence of immigrant macrophages in listeriotic liver granulomata was confirmed by immunoperoxidase staining. From the 2nd of infection, large numbers of Mac-1-positive immigrant macrophages were found both within and at the periphery of discrete infectious foci (Fig. 2A). These cells stained positively with monoclonal anti-Ia and macrophage-specific F4/80 antibodies (not shown), and were thus distinct from an earlier neutrophilic response. Small numbers of Thy-1-bearing T cells were also seen at the periphery of the foci on days 2–6, but were outnumbered at least 10–100-fold by Ia$^+$ macrophages (not shown).

Not only Mac-1 positivity distinguished immigrant macrophages from Kupffer cells in situ (1, 9); these two populations also exhibited differential labeling with intravenously administered colloidal carbon (Fig. 2B). Carbon granules were excluded from immigrant macrophages, but continued to label Kupffer cells in the adjacent liver parenchyma.

The difference in carbon labeling was used to identify resident and immigrant macrophages in dissociated livers. Two morphologically distinct macrophage populations were evident in the adherent monolayers (Fig. 3). One population was circumferentially spread and heavily labeled with carbon, and fully resembled the Kupffer cells from uninfected livers (Fig. 3, A and B). The other was irregular or spinous in shape, had little or no carbon, and presumably corresponded to the immigrant macrophages in infectious foci (Fig. 3, A–C). >90% of both immigrant and resident hepatic macrophages bound (Fig. 3D) and internalized (not shown) antibody-coated erythrocytes. Only 6–8% of the immigrant macrophages were strongly myeloperoxidase positive by Kaplow staining (10).

Total hepatic mononuclear cell yield increased approximately twofold during the first 4 d of infection (Table I), parallel with the development of infectious
FIGURE 2. (A) Infectious focus at 120 h of infection stained for Mac-1. Immigrant macrophages within the focus and at its periphery show reaction product (dark gray) due to the bound anti-Mac-1 monoclonal antibody. × 125. (B) Infectious focus (f) at 96 h of infection along with adjacent liver parenchyma (LP) from a carbon-labeled animal. The animals were given 200 μl of colloidal carbon intravenously 2 h before sacrifice. Carbon granules (arrow) label Kupffer cells in the liver parenchyma but were not found within the focus. Hematoxylin stain. × 200.
FIGURE 3. Liver macrophages isolated from carbon-labeled, 4-d *Listeria*-infected mice. (A and B) Total liver macrophage population, showing irregularly spread, carbon-negative immigrant macrophages (arrow) and carbon-positive Kupffer cells. × 500. (C) Immigrant macrophage. × 790. (D) Both immigrant macrophages (arrows) and Kupffer cells bind opsonized sheep erythrocytes. × 500. Cells were photographed using phase contrast (A, C, D) or bright field optics (B).

### Table 1

**Hepatic Mononuclear Cell Yield Parallels the Course of *Listeria* Infection**

| Day*  | n  | Total hepatic mononuclear cell yield (×10⁶)‡ |
|-------|----|------------------------------------------|
| 0     | 2  | 7.2 ± 0.2                                |
| 2     | 2  | 8.4 ± 3.9                                |
| 4     | 11 | 14.1 ± 3.6                               |
| 5     | 2  | 15.0 ± 2.8                               |
| 6     | 5  | 10.6 ± 3.3                               |
| 8     | 2  | 6.1 ± 0.1                                |
| 10    | 2  | 7.3 ± 1.6                                |

* Days after intravenous inoculation with 5 × 10⁴ live *Listeria*.

‡ Total hepatic mononuclear cells were enumerated in suspension and include Kupffer cells, immigrant macrophages, and lymphocytes. Among the adherent populations on day 4, there were 55 ± 7% Kupffer cells, 36 ± 6% immigrant macrophages, 2 ± 1% lymphocytes, 3 ± 1% neutrophils, and 6 ± 3% endothelial cells (mean ± SE for four experiments). In comparison, corresponding values for uninfected mice were 91 ± 3% Kupffer cells, 9 ± 4% endothelial cells, and <1% monocytes, neutrophils, and lymphocytes (See reference 1).
Much of this increase was due to immigrant macrophages, which accounted for 30–40% of the adherent macrophage yield at day 4. At the resolution of infection on days 10–12, mononuclear cell yields returned to baseline values, and irregularly spread immigrant macrophages were no longer observed.

Assessing the activation status of Kupffer cells and immigrant macrophages in listeriosis required an enrichment protocol. The two types of hepatic macrophages were separated in substantial yield and purity on the basis of differential adherence. After 30–50 h in culture, carbon-poor immigrant cells were dislodged from the monolayer by gentle pipetting, while carbon-laden Kupffer cells remained firmly adherent. The dislodged cells quickly readhered when plated on plastic, providing two types of monolayers: one rich in immigrant macrophages (Fig. 4, A and B) and the other rich in Kupffer cells (Fig. 4, C and D). In subsequent functional studies, colloidal carbon was not used as a marker, but differences in cell shape and adherence properties of resident and immigrant macrophages persisted.

**Inflammatory Macrophages Are the Oxidatively Activated Population in Listeriotic Livers.** After 40 h in culture, total macrophage populations from 4-d *Listeria-*infected livers (containing 30–40% immigrant macrophages and 60–70% Kupffer cells) released substantial levels of H$_2$O$_2$ (90 nmol/mg cell protein; Fig. 5). Enriched, firmly adherent Kupffer cells exhibited little respiratory burst activity.

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**Figure 4.** Separated immigrant macrophage and Kupffer cell populations from carbon-labeled, 4-d *Listeria*-infected mice. (A and B) Enriched immigrant macrophages separated after 30 h in culture. (C and D) Enriched Kupffer cells. Cells were photographed using phase contrast (A, C) or bright field optics (B, D). $\times$ 500.
However, the enriched immigrant macrophage fraction displayed a two- to threefold enhancement of \( \text{H}_2\text{O}_2 \) secretion, proportional to the degree of enrichment, and appeared to account quantitatively for the \( \text{H}_2\text{O}_2 \) release of unseparated populations.

Toxoplasmacidal Activity. The failure of Kupffer cells from listerotic livers to generate appreciable levels of \( \text{H}_2\text{O}_2 \) was paralleled by a failure to limit replication of internalized \textit{T. gondii} (Table II). Kupffer cells from both uninfected and 4-d listerotic livers supported a similar three- to eightfold increase in total numbers of parasites and toxoplasmas per vacuole over 18 h. Attempts to assess toxoplasmacidal activity of the immigrant macrophages were unsuccessful due to a poor initial uptake of toxoplasmas.

Discussion

While isolated KC are impaired in the generation of ROI, in vivo challenge with inflammatory stimuli has been recently reported to enhance liver macrophage oxidative metabolism (11). In the present study, we found that during the course of \textit{Listeria} infection, isolated liver macrophages exhibited an enhanced capacity to secrete \( \text{H}_2\text{O}_2 \). However, as in reference 11, we noted a significant increase in hepatic mononuclear cell yield after in vivo challenge.

Resistance to systemic murine listeriosis (2) is associated with an influx of bone marrow-derived phagocytes into morphologically discrete infectious foci in liver and spleen. These immigrant macrophages appear at the periphery of foci by the second day of infection (after an earlier neutrophilic response) and are present in greatest numbers at days 4–5.
TABLE II
Kupffer Cells in Listeriosis Support the Replication of Toxoplasma gondii

| Exp. | Liver status* | Fold increase in number of toxoplasmas over 18 h‡ | Toxoplasmas per vacuole at 18 h§ |
|------|---------------|-----------------------------------------------|----------------------------------|
| 1    | Normal        | 3.7                                           | 4.9                              |
|      | Listeria-infected | 3.6                                           | 3.5                              |
| 2    | Listeria-infected | 7.8                                           | 7.3                              |

* Hepatic macrophages from normal and day-4 listeria-infected mice were cultured for 36 h, and firmly adherent (Kupffer cell-enriched) populations were prepared.
‡ Cells were infected in vitro with $5 \times 10^5$ T. gondii. Immediately after the 30 min challenge (time 0) as well as at 18 h postinfection, the number of toxoplasmas per 100 macrophages was scored. Fold increase in number of toxoplasmas per 100 cells over 18 h represents the ratio of values at 18 h vs. time 0.
§ The number of toxoplasmas per parasitic vacuole was scored at 18 h and averaged for ≥100 cells. Values >1 represent replication of the organism.

We assessed criteria for separating immigrant (infectious foci) from resident (sinusoidal lining Kupffer cell) macrophages. In immunocytochemical studies of cryostat sections (12), both types of cell have Ia and F4/80 antigens, but immigrant macrophages appeared much richer in the Mac-1 antigen present on the C3bi receptor. The exclusion of intravenously administered colloidal carbon from Listeria granulomata suggested carbon as a marker for distinguishing populations. Macrophages isolated from carbon-labeled listerotic livers were of two types: carbon-laden, circumferentially spread cells resembling resident Kupffer cells, or carbon-poor macrophages, spread in a dendritic fashion and presumably granuloma-associated immigrant macrophages. Both populations were phagocytic and rich in Fc receptors in vitro.

At the peak of infection (day 4), immigrant macrophages accounted for 30–40% of total hepatic macrophage yield. Van Furth et al. (13) reported a murine monocyte production of $3.43 \times 10^6$ cells during the first 48 h of an acute inflammatory reaction in the peritoneal cavity, and even greater production during hepatic infection (14). Our recovery of up to $4 \times 10^6$ immigrant macrophages from listerotic livers suggests that a considerable proportion of newly produced monocytes enter into hepatic granulomata.

When carbon-poor immigrant macrophages were separated and enriched by differential adherence, they proved to be the oxidatively activated population in listerotic livers. Remarkably, Kupffer cells in infected livers remained impaired in the generation of $H_2O_2$ and supported replication of internalized toxoplasmas, much as with cells from normal livers.

An important issue is the role of T cells in the activation of immigrant macrophages. Previous studies have shown that antilisterial resistance is mediated by antigen-specific T cells (15, 16), and that the protective capacity of T cells is paralleled by an ability to release IFN-γ (17). The infectious foci in our studies exhibited relatively few T cells in immunocytochemical stains of liver sections,
and we are currently testing whether the function of these T cells can be characterized in vitro. Some evidence of T cell involvement in macrophage activation may be inferred from the proximity of T lymphocytes and immigrant macrophages in infectious foci and the finding that IFN-γ sustains viability and oxidative activation of immigrant macrophages over at least 72 h after readherence (not shown).

Other questions arise from this study regarding host resistance mechanisms: Are monocytes that enter infectious foci already activated? Would monocytes that fail to encounter T cells or T cell products assume the properties of Kupffer cells and eventually become resistant to oxidative activation by IFN-γ? Are the ROI in infectious foci essential to the sterilization observed in situ? Are the oxidative responses observed during listeria infection similar to those accompanying infection with other facultative or obligate intracellular pathogens?

In conclusion, our data indicate that resident and inflammatory cells that coexist in the same organ can be qualitatively very different. Heightened cell-mediated resistance may require not only T cells and IFN-γ, but also responsive monocytes.

Summary

Sublethal infection of mice with Listeria monocytogenes was accompanied by an influx of immigrant macrophages into the liver and the generation of substantial H₂O₂ by isolated liver macrophages. H₂O₂ production paralleled the course of infection and, after resolution of granulomata, returned to the low levels seen in normal livers. To assess the activation status of Kupffer cells and immigrant macrophages in listeriosis, a separation protocol was developed based on the differential adherence properties of the two macrophage populations. As in the steady state, Kupffer cells in listeriosis failed to generate significant levels of H₂O₂ and did support the replication of internalized toxoplasmas. Immigrant macrophages produced substantial levels of H₂O₂ and could quantitatively account for H₂O₂ production by total liver macrophages. Our findings suggest distinct functions for Kupffer cells and immigrant macrophages.

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