PROPERTIES OF ISOLATED RED PULP MACROPHAGES FROM MOUSE SPLEEN

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Studies of the murine Kupffer cell have indicated that this sinusoidal lining cell has many of the endocytic and secretory properties typical of the peritoneal macrophage. Yet, isolated Kupffer cells fail to produce toxic or reactive oxygen intermediates (ROI), e.g., O$_2^-$, H$_2$O$_2$, and fail to respond to IFN-γ by increasing ROI production and microbicidal function (1, 2). In turn, Kupffer cells are permissive hosts for intracellular parasites. Recent data show that although Kupffer cells fail to respond to IFN-γ in situ, they recover oxidative capacity after 7 d of culture (3).

We have examined another organ, mouse spleen, in which most of the macrophages resemble Kupffer cells in their close approximation to sinusoids in the marginal zone and red pulp. To analyze functional phenotypes, a technique was required to prepare sufficient numbers of spleen macrophages for biochemical analyses. We have isolated resident spleen macrophages and compared their properties with Kupffer cells, with recruited monocytes induced by Listeria monocytogenes, and with resident peritoneal macrophages.

Materials and Methods

Mice. Specific pathogen-free female BALB/c mice (18–25 g) were bred at our facility.

Tissue Culture. The medium was RPMI 1640 (KC Biologicals, Lenexa, KS) with 10% FCS (HyClone Laboratories, Logan, UT) heated for 30 min at 56°C, and 20 μg/ml gentamicin. The cells were maintained adherent to 12–32-mm glass coverslips, which were presoaked in 70% ethanol for 48 h before use.

Macrophages. Peritoneal cells were harvested by lavage with Ca/Mg-free Dulbecco's phosphate-buffered saline (PD). Resident macrophages were isolated from spleen after perfusion with collagenase (120–170 U/ml, type CLS II; CooperBiomedical, Malvern, PA) and DNase (20 μg/ml, type I; Sigma Chemical Co., St. Louis, MO). Enzymes were dissolved in a modified Dulbecco's PBS (DPBSG) containing per liter: 131.7 mM NaCl, 8.06 mM Na$_2$HPO$_4$ · 7H$_2$O, 1.5 mM KH$_2$PO$_4$, 5.5 mM D-glucose, 2.7 mM KCl, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$ · 6H$_2$O adjusted to pH 7.3. Spleens were held at the hilum with blunt forceps and perfused with 40 ml of enzyme solution at 37°C using a syringe and a 21-gauge needle. The tissue was passed through a 60-mesh stainless steel sieve into a 50-ml Teflon beaker and the contents were gently pipetted at 37°C for 7–8 min. All subsequent steps were on ice. The suspension was passed through a 40-μm nylon mesh, sedimented at 250 g for 10 min, and resuspended in 63.5% isotonic Percoll in PD with 9% FCS and 36 μg/ml DNase. The gradient was overlaid with 1.5 ml PD and centrifuged at 1,800 g for 15 min. The floating

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interface was collected, washed twice in DPBSG + 1% FCS, and plated in droplets on glass
coverslips (0.1 ml, 40 x 10^6/ml, 12-mm slips; 0.5 ml, 30 x 10^6/ml, 32-mm slips). After 1-2 h
at 37°C, nonadherent cells were washed by pipetting over the coverslips. The cells were
cultured overnight before assay. Inflammatory spleen macrophages were obtained 1 wk after
infection intravenously with 2-3 x 10^3 live Listeria monocytogenes (strain EGD, a gift of Dr.
R. J. North, Trudeau Institute, Saranac Lake, NY).

Morphology. Cells were examined by phase contrast, live and fixed in 2% glutaralde-
yhyde/PBS. Diff-Quick (Harleco, Gibserville, NJ), a modified Giemsa stain, was used to detect
neutrophil contamination. Cytochemistry for peroxidase (4) used benzidine dihydrochloride
as substrate, and for nonspecific esterase (5), a-naphthyl butyrate. For indirect immunofluores-
cence for surface antigens we used a panel of rat anti-mouse leukocyte hybridoma superna-
tants (6), followed by biotin rabbit anti-rat Ig and fluorescein avidin (both from Vector Labora-
tories, Burlingame, CA).

Hydrogen Peroxide Release. H_2O_2 release was measured using the reduction of scopoletin
fluorescence catalyzed by horseradish peroxidase and H_2O_2 (7). Values were expressed as
H_2O_2 release per milligram adherent protein (Bradford assay kit; Bio-Rad Laboratories,
Richmond, CA) (8).

Ligand-coated Erythrocytes. Sheep erythrocytes (E) were coated with C3b, C3bi, or IgG (9)
to an average of 80,000 C3 or Ig/E (9). The effect of fibronectin on binding and uptake of
E was monitored as described (10).

Quantitation of [3H]20:4 Metabolites. Cells were labeled with [3H]20:4 (70 Ci/mM, 0.5
µCi/ml; New England Nuclear, Boston, MA) to equilibrium overnight, washed in PD, and
overlaid with serum-free RPMI 1640. The calcium ionophore A23187 (10 ng/ml) was then
added. 20:4 metabolites in the medium were extracted and identified by reverse-phase HPLC
(11, 12).

Levels of Surface Ia Antigens. 125I anti-Ia mAb B21-2 was used to quantitate surface class
II MHC molecules (1, 13).

FIGURES 1 and 2. (Fig. 1) Phase contrast microscopy of splenic red pulp macrophages. After overnight
culture, all cells are well spread and contain many lysosomal granules. x500. (Fig. 2) Binding of ligand-
coated erythrocytes (E). Spleen macrophages avidly bind ElgG (A). On glass, only a rare cell binds
EC3b (B). Peritoneal macrophages bind IgG (not shown) and EC3b (C). x375.
Results and Discussion

Enriched Populations of Splenic Red Pulp Macrophages. The red pulp of mouse spleen, but not the white pulp, contains large phagocytes that stain for the F4/80 antigen (14). These could be released by collagenase digestion, flotation on Percoll, and adherence to glass (Fig. 1). The Percoll gradient was helpful for sedimenting red cells and enriching adherent phagocytes about threefold, so that dense monolayers could be obtained. All adherent cells were reactive for nonspecific esterase but not peroxidase. If colloidal carbon was given intravenously (1, 2) 1–24 h before isolation, all cells were laden with carbon and had been in contact with the vasculature.

Receptors for Opsonized Erythrocytes. All splenic macrophages bound and ingested many ElgG (Fig. 2 A). Only a small subpopulation bound EC3b (Fig. 2 B) and EC3bi (not shown). Peritoneal macrophages in contrast bound both ElgG and EC3b (Fig. 2 C). The attachment and endocytosis of EC3b and EC3bi were enhanced on Fn-coated plastic surfaces, consistent with previous data on blood monocytes (Table I) (10).

Cell Surface Antigens. Immunofluorescence (not shown) indicated that splenic macrophages expressed the macrophage antigen F4/80, the leukocyte common antigen, and both class I and II MHC products. The adherent cells did not stain with mAbs to the lymphocyte antigens B220 and Lyt-2 (CD8). Binding studies with 125I-B21-2 anti-Ia mAb revealed that spleen macrophages had higher levels of Ia than peritoneal cells (Table II). Culture in rIFN-γ, 100 U/ml (Genentech, Inc., San Francisco, CA) for 48–72 h did not increase spleen macrophage Ia substantially, but did enhance peritoneal macrophage Ia (Table II).

Arachidonic Acid Metabolism. Resident peritoneal and splenic macrophages were labeled to equilibrium with [3H]20:4 and challenged with zymosan. While peritoneal cells released 16–25% (three experiments) of total cellular 20:4 as 20:4 metabolites, spleen cells did not release (data not shown). Since uptake of zymosan by spleen cells was low, and since prior data had shown that calcium ionophore A23187 could bypass the signal transduction pathway leading to 20:4 metabolism (3, 15, 16), we challenged the cells with A23187. Both splenic and peritoneal macrophages released ~25% of cellular 20:4 in 30 min (not shown), but the macrophages differed in the

### Table I

**Attachment and Phagocytosis of Ligand-coated Erythrocytes by Splenic and Peritoneal Macrophages**

| Coated surface | Attachment index | Phagocytic index |
|----------------|-----------------|-----------------|
|                | EC3b | EC3bi | ElgG | EC3b | EC3bi | ElgG |
| Spleen         |       |       |      |       |       |      |
| HSA            | 388  | 940   | TNTC | 6    | 106   | 303  |
| Fn             | 2312 | 2640  | TNTC | 323  | 326   | 412  |
| Peritoneal     |       |       |      |       |       |      |
| HSA            | 868  | 783   | TNTC | 75   | 103   | 295  |
| Fn             | 3298 | 1978  | TNTC | 807  | 640   | 417  |

Cells were plated in Terasaki wells coated with HSA or Fn for 1 h. Binding and phagocytosis of ligand-coated erythrocytes was then assayed (8). Data are presented as attachment or phagocytic index, i.e., the number of erythrocytes bound or phagocytosed per 100 macrophages. TNTC, too numerous to count; Fn, fibronectin; HSA, human serum albumin.
Table II

Influence of IFN-γ on Ia Expression of Splenic and Peritoneal Macrophages

| Source of macrophages | Level of Ia (cpm/μg adherent protein) |
|-----------------------|--------------------------------------|
|                       | Exp. A | Exp. B | Exp. C |
| Peritoneal             | 63     | 18     | 65     |
| Peritoneal + 10 U/ml rIFN-γ | 626 (19.9×) | 270 (115×) | 1968 (15.7×) |
| Spleen                | 386    | 93     | 3696   |
| Spleen + 10 U/ml rIFN-γ | 485 (11.3×) | 139 (11.5×) | 6908 (11.9×) |

Macrophages from BALB/c mice (Iaα) were exposed to 125I-anti-Ia mAb B21-2 at 4°C. Background binding was determined in the presence of a 20-fold excess of unlabeled B21-2 and was subtracted from the values shown. The values in parentheses are the fold increase due to rIFN-γ. Peritoneal and spleen cells were obtained from the same animals and studied fresh or after 2 d of culture in rIFN-γ. Experimental variations in cpm are due to separate batches of 125I-mAb.

A23187 usually leads to the preferential production of lipoxygenase products (15–18). Exposure of peritoneal cells to a sham spleen isolation protocol did not modify their secretory profile (not shown).

Reactive Oxygen Intermediate Production. An important microbicidal mechanism of

Table III

Arachidonic Acid Metabolites Released by Macrophages in Response to Calcium Ionophore A23187

| Product          | Percent of total recovered radiolabeled products |
|------------------|--------------------------------------------------|
|                  | Peritoneal | Spleen   |
| 6-Keto-PGF₁₀     | 12.1       | 5.0      |
| PGE₂             | 8.1        | 23.0     |
| LTC              | 19.2       | 0.9      |
| 12-HETE + HHT    | 39.1       | 12.4     |
| 20:4             | 21.1       | 58.2     |

Freshly isolated macrophages were labeled with 14H]20:4 for 12-18 h, washed, and challenged with A23187 ionophore at 10 μg/ml for 30 min. 20:4 metabolites in the medium were extracted, and the profiles were analyzed on Ultrasphere C-18, 4.6 mm x 25 cm columns (Altex Scientific Inc., Berkeley, CA). HHT and HETE migrated close together and are shown as a unit. Values are expressed as percent of total radiolabeled products identified (73% of the released 20:4), and calculated on the basis of peaks above background. The data are from two experiments in which the variation was <5%. The peritoneal macrophages were exposed to a sham spleen cell isolation protocol and exhibited the previously described profile of metabolites (11).
macrophages is to form various reduced products of molecular oxygen. Circulating monocytes, pulmonary macrophages, and macrophages obtained from serous cavities all may produce O$_2$ and H$_2$O$_2$ with appropriate stimuli (19), but liver Kupffer cells do not. When splenic macrophages were examined, the resident cells released very little ROI (Fig. 3A). Exogenous rIFN-γ at 10 and 100 U/ml for 1-4 d did not enhance ROI production (not shown). The latter experiment was not readily interpretable, since we found that exposure of control peritoneal cells to the spleen isolation procedure reduced responsiveness to rIFN-γ. The number of peritoneal and spleen macrophages recovered from Listeria-infected animals was severalfold higher than controls, and both sources could make high levels of H$_2$O$_2$ (Fig. 3B). The data are consistent with prior work showing that oxidative competence in Listeria-infected livers is associated with an influx of inflammatory cells (2).

**Summary**

Dense monolayers of large, adherent macrophages were prepared from the red pulp of mouse spleen. These sinus-lining phagocytes resembled liver Kupffer cells in morphology, as well as expression of F4/80 and class II MHC antigens and receptors for IgG. C3-coated red cells attached at low levels to spleen macrophages, but attachment and endocytosis were enhanced on fibronectin-coated surfaces. The ionophore A23187 induced spleen macrophages to synthesize prostaglandin E$_2$, but like Kupffer cells, spleen macrophages did not synthesize leukotrienes and made relatively small amounts of HETE and 12-hydroxyheptadecanoic acid. Resident spleen macrophages did not produce H$_2$O$_2$, but splenic inflammatory cells, induced by infection of animals with *Listeria monocytogenes*, actively released H$_2$O$_2$. We conclude that the functional properties of resident, sinusoidal-lining macrophages in liver and spleen are similar to one another but distinct from other pools of phagocytes.

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