Brief Definitive Report

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_2O_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 $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, 5.5 mM D-glucose, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$ adjusted to pH 7.3. Spleens were held at the hila 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 63.6% 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 × 10^6/ml, 12-mm slips; 0.5 ml, 30 × 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 × 10^3 live *Listeria monocytogenes* (strain EGD, a gift of Dr. R. J. North, Trudeau Institute, Saranac Lake, NY).

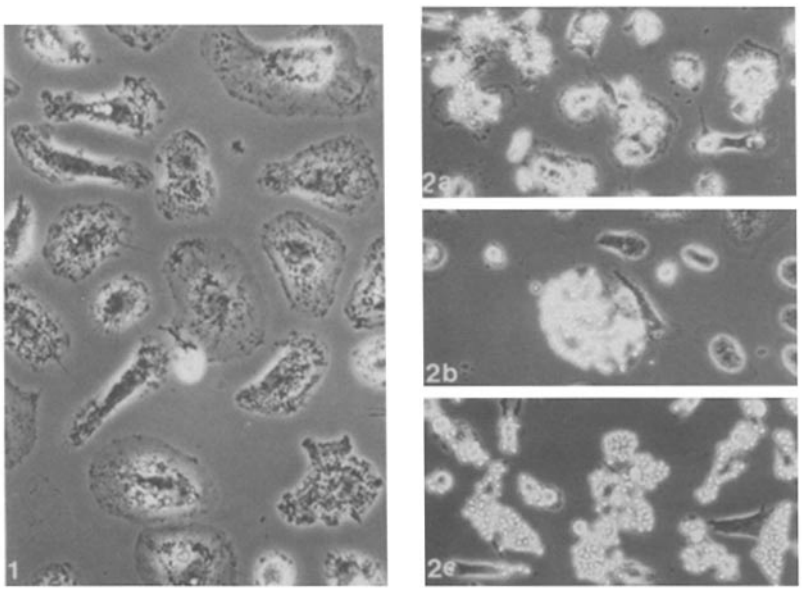
Morphology. Cells were examined by phase contrast, live and fixed in 2% glutaraldehyde/PBS. Diff-Quick (Harleco, Gibbsville, NJ), a modified Giemsa stain, was used to detect neutrophil contamination. Cytochemistry for peroxidase (4) used benzidine dihydrochloride as substrate, and for nonspecific esterase (5), α-naphthyl butyrate. For indirect immunofluorescence for surface antigens we used a panel of rat anti-mouse leukocyte hybridoma supernatants (6), followed by biotin rabbit anti-rat Ig and fluorescein avidin (both from Vector Laboratories, 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 [^{3}H]20:4 Metabolites. Cells were labeled with [^{3}H]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. ^{125}I 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. ×500. (*Fig. 2*) Binding of ligand-coated erythrocytes (E). Spleen macrophages avidly bind EIgG (A). On glass, only a rare cell binds EC3b (B). Peritoneal macrophages bind IgG (not shown) and EC3b (C). ×375.

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 EIgG (Fig. 2 *A*). Only a small subpopulation bound EC3b (Fig. 2 *B*) and EC3bi (not shown). Peritoneal macrophages in contrast bound both EIgG 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 ^{125}I-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 [^{3}H]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

	Attachment index			Phagocytic index		
Coated surface	EC3b	EC3bi	EIgG	EC3b	EC3bi	EIgG
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 (↑9.9 ×)	270 (↑15 ×)	1968 (↑5.7 ×)
Spleen	386	93	3696
Spleen + 10 U/ml rIFN-γ	485 (↑1.3 ×)	139 (↑1.5 ×)	6908 (↑1.9 ×)

Macrophages from BALB/c mice (Ia^d) were exposed to ^{125}I-anti-Ia^d 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 ^{125}I-mAb.

proportion of cyclooxygenase to lipoxygenase products. Spleen cells formed three times more prostaglandin E_2 (PGE_2) but 20-fold less leukotriene C (LTC) and threefold less 12-HETE than peritoneal cells (Table III). The data suggest that splenic macrophages, like Kupffer cells (3), cannot synthesize leukotrienes and other products of the lipoxygenase pathway. This point is reinforced by the findings that 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-$PGF_{1\alpha}$	12.1	5.0
PGE_2	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 [^{3}H]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 × 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).

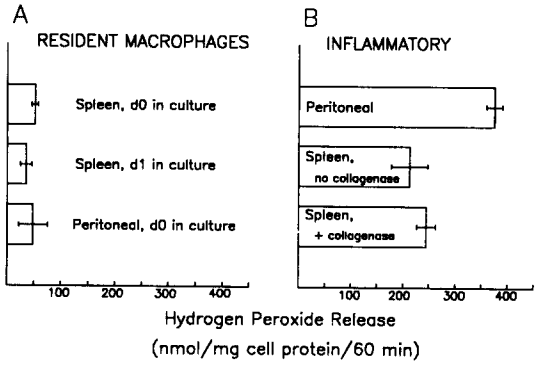


FIGURE 3. Release of reactive oxygen intermediates in response to PMA. Resident populations of spleen and peritoneal macrophages show little release of ROI (A), while macrophages from mice infected with *L. monocytogenes* release high levels (B). The ROI value for control peritoneal cells from uninfected animals in *B* was 125 (not shown).

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_2O_2 with appropriate stimuli (19), but liver Kupffer cells do not. When splenic macrophages were examined, the resident cells released very little ROI (Fig. 3 *A*). 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_2O_2 (Fig. 3 *B*). 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_2O_2, but splenic inflammatory cells, induced by infection of animals with *Listeria monocytogenes*, actively released H_2O_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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