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## MURINE KUPFFER CELLS

### Mononuclear Phagocytes Deficient in the Generation of Reactive Oxygen Intermediates

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Kupffer cells (KC),<sup>1</sup> by virtue of their numbers and sinusoidal exposure, occupy a prominent position in the clearance of blood-borne pathogens. In vivo studies using a variety of test substances indicate KC clearance approaches single pass efficiency (1). Yet, for *Leishmania donovani* and several protozoan (2), bacterial (3), and viral agents (4, 5), the liver represents a site not only for clearance from blood but also for survival and replication.

When macrophages interact with certain soluble or particulate stimuli, the cells normally respond by the secretion of hydrogen peroxide (6), superoxide anion (7), and other reactive oxygen intermediates (ROI) (8). Such toxic oxygen metabolites mediate a substantial portion of macrophage resistance to a number of intracellular pathogens. Mouse peritoneal macrophages elicited by different regimens exhibit a direct correlation between H<sub>2</sub>O<sub>2</sub> release and trypanocidal (9) and toxoplasmatatic activity (10). *L. donovani* promastigotes and amastigotes, while sensitive to oxygen-independent mechanisms (11), are susceptible to H<sub>2</sub>O<sub>2</sub> and are killed by peritoneal macrophages through activities that appear primarily oxygen dependent (12). Exposure of mouse peritoneal macrophages to lymphokine (13) or, more specifically, recombinant murine  $\gamma$  interferon (rIFN- $\gamma$ ) (14) enhances both H<sub>2</sub>O<sub>2</sub> releasing capacity and microbicidal activity.

In this paper, we report that murine KC are impaired in their oxidative response to soluble and particulate stimuli. Lymphokine and rIFN- $\gamma$  failed to induce the capacity to generate ROI, although KC responded by alternate criteria. This deficiency in oxidative metabolism correlates with diminished microbicidal activity against the intracellular protozoal pathogens *Toxoplasma gondii* and *L. donovani*.

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<sup>1</sup> Abbreviations used in this paper: CGD, chronic granulomatous disease; IFN- $\gamma$ ,  $\gamma$  interferon; KC, Kupffer cells; LDP, *Leishmania donovani* promastigotes; LPS, bacterial lipopolysaccharide; NBT, nitroblue tetrazolium; PMA, phorbol myristate acetate; R<sub>10</sub>HIFCS, RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and gentamicin sulfate; rIFN- $\gamma$ , recombinant murine IFN- $\gamma$ ; ROI, reactive oxygen intermediates; RPC, resident peritoneal macrophages; SOD, superoxide dismutase.

### Materials and Methods

*Mice.* 6–8-wk-old female mice were obtained from the Trudeau Institute, Saranac Lake, NY (B6D2F<sub>1</sub>, B6.H2-k, D2CF<sub>1</sub>), the Charles River Laboratories, Wilmington, MA (BALB/c), and The Rockefeller University (NCS).

*Carbon Labeling.* 200  $\mu$ l of a colloidal carbon suspension (5% india ink in normal saline) was administered intravenously 1–2 h before liver isolation. Carbon labeling was used in morphologic and surface marker studies but not in experiments assessing KC function.

*Immunocytochemistry.* Five rat anti-mouse monoclonal antibodies were used as hybridoma culture supernatants. F4/80 (15, 16) is a macrophage-restricted reagent, kindly provided by Dr. J. Austyn and Dr. S. Gordon (Sir William Dunn School of Pathology, Oxford University, Oxford, England). B21-2 (17, 18) is an anti-I-A<sup>b,d</sup> antibody. 1.21J (18) reacts with mouse macrophages and granulocytes but not with most lymphocytes, and appears to be identical to the Mac-1 monoclonal described by Springer et al. (19). 2.4G2 (20) recognizes the IgG1/IgG2b trypsin-resistant Fc receptor; B-5.3 (21) is an anti-Thy-1.2 or pan T cell reagent.

Cryostat sections from mouse liver were fixed in acetone and exposed, successively, to hybridoma culture supernatant, biotinylated mouse anti-rat Ig, and peroxidase-avidin (22). Sections were stained with diaminobenzidine-H<sub>2</sub>O<sub>2</sub> and hematoxylin and examined by light microscopy.

*KC.* KC were obtained by a modification of a protocol used to isolate murine hepatocytes (23). Mice were anesthetized with 2 mg i.p. pentobarbital, and a ventral midline incision exposed the thoracic and peritoneal cavities. A sterile 20-gauge Teflon catheter (Angiocath; Deseret Medical, Inc., Sandy, UT) was inserted through the right atrium into the inferior vena cava. Perfusion was begun with a Ca<sup>++</sup>-Mg<sup>++</sup>-free wash solution, adjusted to pH 7.2–7.4, at 37°C (per liter: 400 mg KCl, 60 mg KH<sub>2</sub>PO<sub>4</sub>, 47.5 mg Na<sub>2</sub>HPO<sub>4</sub>, 1,000 mg dextrose, 1 mM EDTA, 20 mg gentamicin sulfate, and NaCl to 300–320 mosM [ $\sim$ 8.0–8.2 g]). The flow rate was adjusted to 7 ml/min and outflow was established by severing the portal vein. After 2 min, the wash solution was replaced with 50 ml of a prewarmed and filtered collagenase solution (100 U/ml Worthington Class II [Worthington Biochemical Corp., Freehold, NJ] in RPMI 1640 [Gibco Laboratories, Grand Island, NY]). Temperature, pH, osmolarity, and flow rate were rigorously maintained.

After perfusion, the liver was excised, rinsed in warm RPMI 1640, and disaggregated in the same medium by gentle teasing with forceps. Hepatocyte viability was assessed by trypan blue exclusion. Preparations with <70% viable hepatocytes were discarded. Parenchymal cells were then removed by three to five sequential, low speed centrifugations (30 g, 4 min, 4°C). This procedure typically resulted in KC recoveries of 80–90%. Such recoveries were substantially higher than those observed for most isolation procedures using density gradients. Nonparenchymal cells were pelleted and washed twice in RPMI 1640 (400 g, 10 min, 4°C), resuspended in R<sub>10</sub>HIFCS culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [Sterile Systems, Inc., Logan, UT] and gentamicin sulfate), and plated onto plastic 16-mm wells (Costar, Data Packaging, Cambridge, MA) or 12-mm glass coverslips. KC were enriched by adherence; after culture for 45 min at 37°C and 5% CO<sub>2</sub>, nonadherent or weakly adherent cells (including most endothelial cells) were removed by gently pipetting over the culture surface.

*Peritoneal Macrophages.* Resident and casein-elicited peritoneal macrophages were obtained by lavage with RPMI 1640. For casein elicitation, mice were injected intraperitoneally 4 d before sacrifice with 1 ml of 6% sodium caseinate (practical grade; Eastman Kodak Co., Rochester, NY) in 0.9% NaCl. Cells were cultured in R<sub>10</sub>HIFCS on plastic wells or glass coverslips. 4 h after explantation, nonadherent cells were removed by washing and the adherent macrophages cultured in R<sub>10</sub>HIFCS.

*Macrophage Polypeptide Secretory Profiles.* Biosynthetic radiolabeling of macrophage-secreted proteins was performed by the method of Werb et al. (24) with modifications. Briefly,  $5 \times 10^5$  macrophages were washed three times with methionine-free RPMI 1640 (Gibco Laboratories) and radiolabeled for 4 h in 0.5 ml of methionine-free RPMI containing 50  $\mu$ Ci of [<sup>35</sup>S]methionine (500 Ci/mmol; Amersham Corp., Arlington Heights,

IL). Conditioned medium was collected and precipitated with ice-cold trichloroacetic acid (7% final concentration with 100  $\mu$ g/ml carrier bovine serum albumin). The pellet was resuspended in 50  $\mu$ l of electrophoresis sample buffer (25) containing 50 mM dithiothreitol. Polyacrylamide gel electrophoresis and fluorographic visualization were performed as described.

**Lymphokine and rIFN- $\gamma$ .** Spleen cells from B6D2F<sub>1</sub> mice infected 6 d earlier with  $5 \times 10^5$  *Listeria monocytogenes* were incubated at  $5 \times 10^6$  cells/ml for 48 h with 5  $\mu$ g/ml of concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) (26). Lymphokine was obtained as conditioned medium, and IFN- $\gamma$  activity (3,000 U/ml) was titered using a vesicular stomatitis virus cytopathic inhibition assay (27). rIFN- $\gamma$  was provided by Genentech, Inc., South San Francisco, CA, as protein purified to homogeneity from transfected *E. coli* (28) (sp act,  $6 \times 10^6$  U/mg protein).

**Quantitation of Respiratory Burst Activity.** Superoxide anion release was measured spectrophotometrically by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* (29). H<sub>2</sub>O<sub>2</sub> secretion was detected fluorometrically by the horseradish peroxidase-catalyzed oxidation of scopoletin (30). Phorbol myristate acetate (PMA) (100 ng/ml; Sigma Chemical Co.) was used to trigger secretion of ROI. Cell protein was determined by the method of Lowry et al. (31).

Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Macrophages were stirred magnetically at 37°C in 3.0 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 5.5 mM glucose and 1 mM sodium azide. Samples were equilibrated with air and the basal rate of oxygen consumption recorded for each. PMA (100 ng/ml) was then introduced into each sample chamber and oxygen consumption was recorded for an additional 10–15 min.

To measure nitroblue tetrazolium (NBT) reduction, adherent macrophages were exposed for 60 min at 37°C to either  $5 \times 10^6$  toxoplasmas, promastigotes, or opsonized zymosan particles suspended in 1 ml of R<sub>10</sub>HIFCS containing 0.5 mg/ml of NBT (grade III; Sigma Chemical Co.) (13). Coverslips were washed and fixed with 1.25% glutaraldehyde in Dulbecco's modified phosphate-buffered saline, pH 7.4 (Gibco Laboratories). Macrophages were scored as positive if the ingested parasites or particles exhibited deep blue-black formazan staining.

**Parasites.** Virulent trophozoites of the RH strain of *T. gondii* (32), amastigotes of the 1S Sudan strain of *L. donovani* (33), and *L. donovani* promastigotes of the 1S Sudan strain (12) were prepared as described.

**Infection of Macrophages.** Macrophages were incubated for 48 h in the absence or presence of 100 U/ml rIFN- $\gamma$  and then challenged for 30–60 min with RH strain *T. gondii* trophozoites (10), 1S strain *L. donovani* promastigotes (12), or 1S strain *L. donovani* amastigotes (34). Uningested parasites were removed by washing (time zero), and cultures were reincubated in R<sub>10</sub>HIFCS. At time zero and at various times after challenge, the number of parasites per 100 macrophages was scored in Giemsa-stained preparations. For *T. gondii*, the number of toxoplasmas per vacuole was additionally scored at 18 h (10).

**Binding of Monoclonal Anti-Ia Antibody.** B21-2, a monoclonal anti-IA<sup>b,d</sup> reagent, was iodinated with carrier-free Na (<sup>125</sup>I) (New England Nuclear, Boston, MA) by the iodogen method (35) (Pierce Chemical Co., Rockville, IL). Quantitative binding studies were performed on ice using adherent macrophages freshly explanted or cultured for 48 h in the absence or presence of rIFN- $\gamma$ . Triplicate culture wells were exposed to 0.25 ml of antibody at a saturating level of 1.0  $\mu$ g/ml. Equilibrium was achieved by 60 min, after which wells were rinsed through four successive changes of medium. Counts per minute bound per unit cell protein was measured in cells solubilized in 0.4 ml of 0.25 N NaOH (Autogamma 5220; Packard Instrument Co., Downers Grove, IL). Controls for binding specificity (18, 21) included (a) dose-dependent inhibition of monoclonal antibody binding by unlabeled specific antibody, (b) failure to inhibit anti-Ia binding with saturating levels of monoclonal anti-Fc receptor reagent (2.4G2), and (c) minimal binding to macrophages from the inappropriate H-2k haplotype.

## Results

**KC In Situ: Colloidal Carbon Labeling and Phenotype.** Mice were injected intravenously with colloidal carbon, and cryostat sections of liver were stained with the F4/80 macrophage-specific monoclonal antibody (Fig. 1A). As reported (36), carbon granules exhibited a sinusoidal distribution, with the greatest concentration midzonal to peripheral in the liver lobule. Every carbon-laden cell stained positively with F4/80.

This methodology was extended to additional monoclonal antibodies. Carbon-bearing KC in situ were Mac-1-negative, and Ia- and Fc receptor-positive (data not shown). Thy-1-positive T cells were rare and showed no specific association with KC.

**Isolated KC.** Adherent KC were isolated from normal and carbon-labeled livers. Typical of macrophages, the cells were well-spread, with ruffled membranes and numerous lysosomes. In mice receiving colloidal carbon, KC were uniformly carbon laden (Fig. 1B). KC from both normal and carbon-labeled mice bound and ingested antibody-coated sheep erythrocytes and were myeloperoxidase negative (data not shown). On the basis of morphology and the presence of internalized carbon granules, KC yields averaged  $8 \times 10^6$  per gram of liver with >90% purity. Of the remaining adherent cells (those lacking carbon granules), the majority had the morphologic appearance of endothelial cells (37). Hepatocytes represented 0–2% of adherent cells. Inflammatory monocytes (defined by morphologic and functional criteria; 38), dendritic cells (21), and lymphocytes were trace contaminants. In the following metabolic studies, we omitted carbon labeling.

**Polypeptide Secretory Products.** KC were labeled with [ $^{35}\text{S}$ ]methionine, and secreted proteins analyzed by sodium dodecyl sulfate gel electrophoresis and autoradiography (Fig. 2, lane 2). On the basis of molecular weights and comigration with corresponding bands from resident peritoneal macrophages (Fig. 2, lane 1), three products could be tentatively identified (24): Bands at 220,000 mol wt (220 K) (designated A) and 90 K (B) represented fibronectin and factor B, respectively. The band at 33 K corresponded to apolipoprotein E, evidently a major product of resident KC. Additional bands at 34, 35, 40, 55, 58, 68, 73, and 135 K were also shared between the two macrophage populations. The absence of a predominating band at 68 K in the KC population implied little secretion of albumin and thus minimal contamination with biosynthetically active hepatocytes.

**Generation of ROI.** The ability of resident KC to release ROI after triggering with PMA was assessed in five strains of mice (Table I). In three experiments, resident and casein-elicited (activated) peritoneal macrophages were included for comparison. KC were oxidatively deficient relative even to resident peritoneal macrophages. In 22 of 26 experiments, ROI release was below the limits of detection (10 nmol  $\text{H}_2\text{O}_2$  and 20 nmol  $\text{O}_2^-$  per milligram protein). While KC and casein-elicited peritoneal cells exhibited comparable basal levels of  $\text{O}_2$  uptake (Table II), PMA produced only a 3% rise in KC  $\text{O}_2$  consumption, compared with an average 90% increase for casein-elicited peritoneal cells.

Particulate stimuli were also used as triggers of the respiratory burst. In the nitroblue tetrazolium assay, the presence of blue-black formazan around ingested

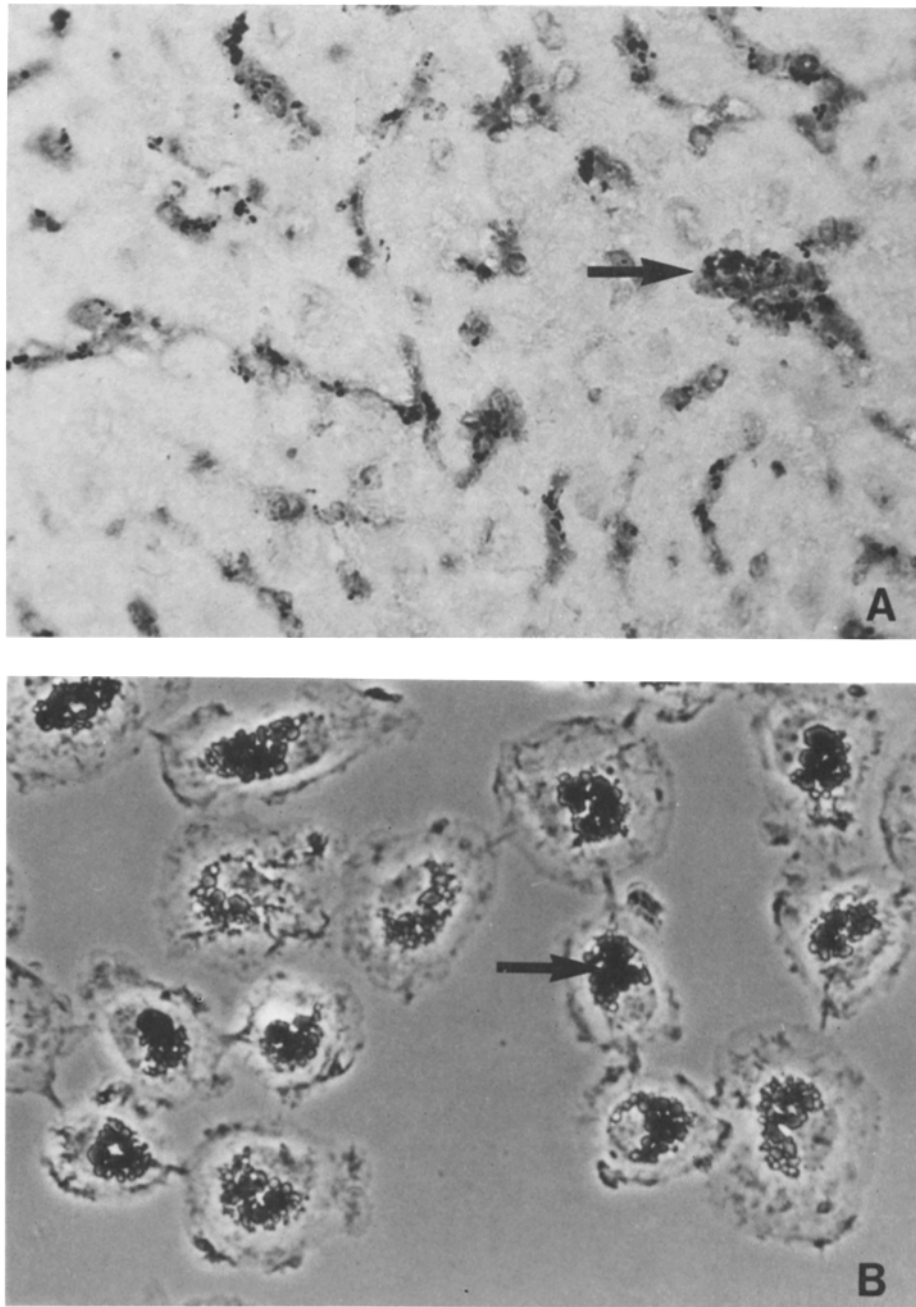
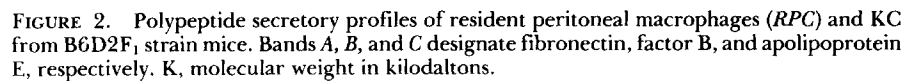


FIGURE 1. Carbon labeling of KC. (A) Cryostat section of carbon-labeled mouse liver stained with F4/80. Cells with black carbon granules (arrow indicates largest of these) also show reaction product (gray) due to bound antimacrophage monoclonal antibody.  $\times 500$ . (B) KC isolated from carbon-labeled liver. Internalized carbon granules (arrow) are present in each of the cells.  $\times 500$ .



Macrophages	Strain	Nanomoles secreted per milligram protein per 60 min	
		H <sub>2</sub> O <sub>2</sub>	O <sub>2</sub> <sup>-</sup>
Kupffer cells	B6D2F <sub>1</sub>	<10, <10, <10, <10, <10, <10, <10, <10, <10, <10, <10, <10, <10, <10, <10, 18.5 ± 4.9, 23.6 ± 4.3, 35.5 ± 7.8, 38.0 ± 2.8	<20
	B6.H2-k	<10	
	RU-NCS	<10, <10, <10	
	BALB/c	<10, <10	
	D2CF <sub>1</sub>	<10	
Resident peritoneal	B6D2F <sub>1</sub>	25.5 ± 7.7, 36.0 ± 10.1, 54.1 ± 18.0	49.2 ± 5.3
Casein-elicited peritoneal	B6D2F <sub>1</sub>	237.4 ± 11.0, 374.5 ± 5.2	216.1 ± 31.1

particles implies a respiratory burst and the generation of superoxide anion (13, 39). KC internalized opsonized zymosan but failed to exhibit the intense formazan staining observed with peritoneal macrophages (Fig. 3). Semi-quantitation of the deficit in NBT staining is provided in Table III, using both opsonized zymosan and *L. donovani* promastigotes (LDP) as particulate triggers.

**Oxidative Response to Lymphokine and *rIFN- $\gamma$* .** IFN-exposed KC also remained oxidatively impaired relative to resident peritoneal macrophages and showed no significant increase in NBT positivity relative to cells cultured in medium alone (Table III). As reported (14), culture of resident mouse peritoneal cells for 48–

TABLE II  
*Kupffer Cell Oxygen Consumption Is Not Increased by PMA*

Cells	O <sub>2</sub> consumption (nmol/[10 <sup>6</sup> cells·min])	
	Basal	Increase after PMA
Kupffer cells*	1.08 ± 0.24 (2) <sup>‡</sup>	0.03 ± 0.02 (2)
Casein-elicited peritoneal cells <sup>§</sup>	1.25 ± 0.52 (3)	1.12 ± 0.30 (3)

\* Tested in suspension without adherence and removal of nonadherent cells, these populations contained 55 ± 4% Kupffer cells; 42 ± 4% lymphocytes, endothelial cells, and hepatocytes; and <1% neutrophils.

<sup>‡</sup> Mean ± standard deviation (number of experiments in parentheses) for duplicate determinations.

<sup>§</sup> Tested in suspension, these populations contained 47 ± 9% macrophages, 51 ± 8% lymphocytes, and 2 ± 1% neutrophils. Results were similar with or without collagenase treatment (see Table IV) and were combined.

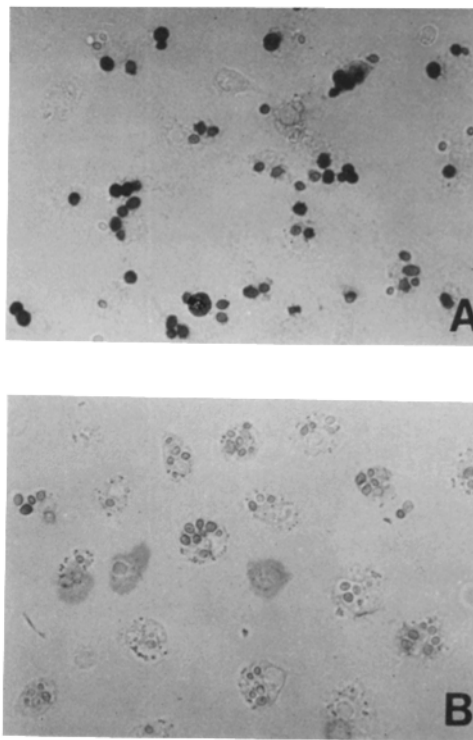


FIGURE 3. NBT staining of resident peritoneal macrophages (A) and KC (B) after internalization of opsonized zymosan.

72 h in rIFN- $\gamma$  produced a dose-dependent enhancement of respiratory burst activity (Fig. 4A). KC, on the other hand, showed no enhancement of H<sub>2</sub>O<sub>2</sub> release in the presence of rIFN- $\gamma$ , even with IFN concentrations ranging to 10<sup>5</sup> U/ml and exposures up to 96 h (not shown). Similar patterns of response were

TABLE III  
Kupffer Cells Are Oxidatively Impaired as Assessed by Nitroblue  
Tetrazolium (NBT) Staining

Exp.	Macrophages	rIFN- $\gamma$ U/ml	Formazan-positive cells*	
			Zymosan %	LDP
1 <sup>‡</sup>	Kupffer cells	0	16	15
	Resident peritoneal	0	62	70
2 <sup>§</sup>	Kupffer cells	0	32	NT <sup>  </sup>
		100	36	NT
	Resident peritoneal	0	85	NT
		100	79	NT
3 <sup>§</sup>	Kupffer cells	0	6	2
		100	9	0
	Resident peritoneal	0	86	83

\* Macrophages were exposed for 1 h to either  $5 \times 10^6$  opsonized zymosan particles or *L. donovani* promastigotes (LDP) in the presence of 0.5 mg/ml NBT. Cells were scored as positive if ingested particles were stained blue-black by precipitated formazan.

<sup>‡</sup> Cells were challenged 3 h after isolation.

<sup>§</sup> Cells were cultured for 48 h in the presence or absence of 100 U/ml rIFN- $\gamma$  before challenge.

<sup>||</sup> Not tested.

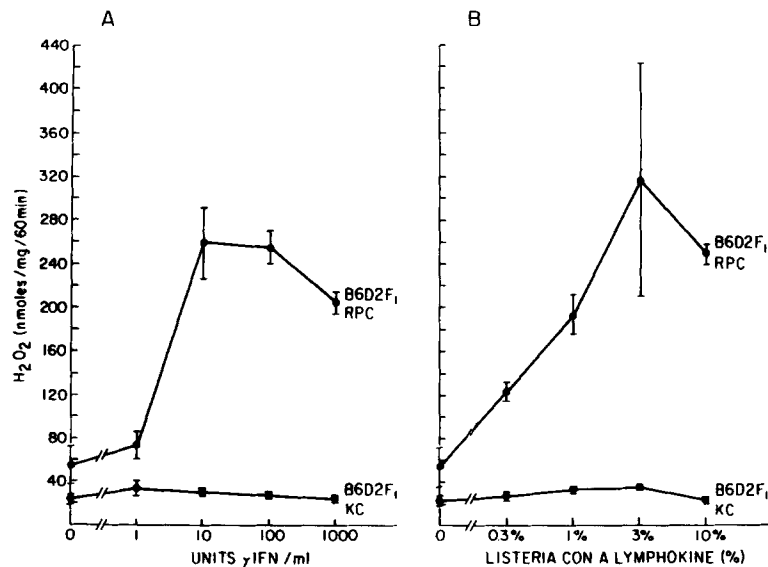


FIGURE 4. PMA-triggered H<sub>2</sub>O<sub>2</sub> secretion by B6D2F<sub>1</sub> strain KC and resident peritoneal macrophages (RPC) after 72 h culture in various concentrations of rIFN- $\gamma$  (A) or concanavalin A-induced lymphokine from splenocytes of listeria-infected mice (B).

observed for cells cultured in Con A-induced lymphokine as a source of IFN- $\gamma$  (Fig. 4B).

To rule out deleterious effects of the isolation procedure in producing an oxidatively impaired KC, resident and casein-elicited peritoneal macrophages were exposed to the solutions used in perfusion. Casein-elicited peritoneal macrophages released comparable levels of  $H_2O_2$  after exposure to collagenase (100 U/ml for 30 min at 37°C) (Table IV). Even with an additional 24 h in culture, the control and collagenase-treated cells showed no decrease in oxidative activity. In addition, rIFN- $\gamma$  continued to activate resident peritoneal macrophages after incubation in a collagenase solution or sequential exposure to the EDTA-containing wash solution followed by collagenase.

**Toxoplasma** Activity. The deficiencies in KC ROI generation were reflected by impaired killing of parasites. Resident KC and peritoneal macrophages supported the replication of *T. gondii* (Table V), leading to a three- to eight-fold increase in both total numbers of parasites and toxoplasmas per vacuole over 18 h. As reported (14), rIFN- $\gamma$  induced toxoplasma activity in peritoneal macrophages (50% killing over 18 h in Table V, Exp. 1) as well as a failure of surviving toxoplasmas to replicate within vacuoles (1.0 toxoplasma per vacuole). KC, however, showed no appreciable cidal or static activity after exposure to rIFN- $\gamma$ . Parasite numbers rose four- to fivefold over 18 h, paralleling an increase in the number of toxoplasmas per vacuole (Table V and Fig. 5A).

**Leishmanicidal Activity.** After infection with *L. donovani* promastigotes, resident peritoneal macrophages killed 40–60% of ingested parasites in 4 h and >90% in 18 h (12). KC only reduced parasite numbers 11–15% at 4 h and

TABLE IV  
Perfusion Media Do Not Affect PMA-triggered  $H_2O_2$  Release by  
Peritoneal Macrophages

Type of macrophage	Treatment	$H_2O_2$ release (nmol/[mg protein · 60 min])	
		2 h culture	24 h culture
Casein-elicited*	None	237 $\pm$ 11	374 $\pm$ 5
	Collagenase	250 $\pm$ 27	378 $\pm$ 1
Resident†		No rIFN- $\gamma$	Plus 100 U/ml rIFN- $\gamma$
	None	23 $\pm$ 11	239 $\pm$ 51
	Collagenase	<10	259 $\pm$ 15
	Wash solution	26 $\pm$ 8	178 $\pm$ 37

\* Casein-elicited peritoneal macrophages were incubated for 30 min at 37°C in RPMI or 100 U/ml collagenase before adherence. At 2 and 24 h after adherence, PMA-triggered  $H_2O_2$  release was determined. Values are means of triplicate determinations  $\pm$  standard deviations.

† Resident peritoneal macrophages were incubated for 30 min at 37°C in RPMI, 100 U/ml collagenase, or EDTA wash solution followed by 100 U/ml collagenase (15 min in each) before adherence. The cells were then cultured for 72 h in the absence or presence of 100 U/ml rIFN- $\gamma$ , and PMA-triggered  $H_2O_2$  release was determined. Values represent means of triplicate determinations  $\pm$  standard deviations.

TABLE V  
*rIFN- $\gamma$  Fails to Induce Toxoplasmacidal Activity in Resident Kupffer Cells*

Population	Exp.	Fold increase in number of toxoplasmas at 18 h			Toxoplasmas per vacuole at 18 h		
		1	2	3	1	2	3
Kupffer cells							
No rIFN- $\gamma$		8.2	3.3	3.7	5.8	4.0	4.9
Plus 100 U/ml rIFN- $\gamma$		5.3	5.6	4.5	5.0	3.5	4.4
Resident peritoneal macrophages							
No rIFN- $\gamma$		7.4	4.5	8.8	6.2	4.7	6.2
Plus 100 U/ml rIFN- $\gamma$		0.5	0.8	1.1	1.0	1.0	1.5

Cells were cultured for 48 h in the presence or absence of 100 U/ml rIFN- $\gamma$  before infection with  $5 \times 10^5$  *T. gondii*. Immediately after the 30-min challenge (time 0) and at 18 h postinfection, the number of toxoplasmas per 100 macrophages were scored. Fold increase in number of toxoplasmas per 100 cells at 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  $\geq 100$  cells.

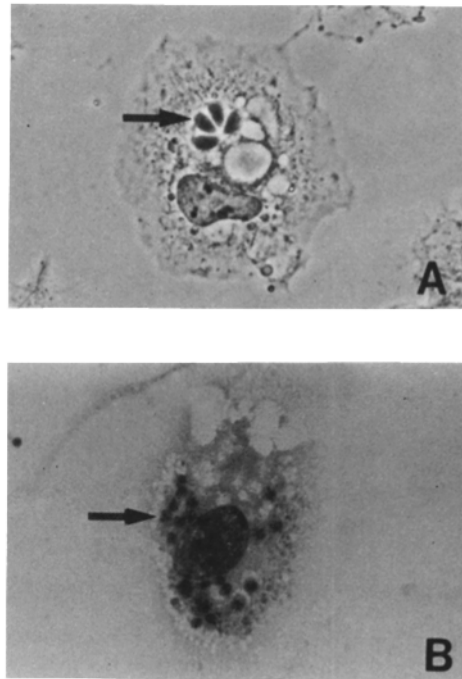


FIGURE 5. rIFN- $\gamma$ -treated KC support replication of *T. gondii* trophozoites and *L. donovani* amastigotes (LDA). (A) rIFN- $\gamma$ -treated KC 18 h after challenge with toxoplasmas (phase contrast,  $\times 790$ ). The parasite has undergone two rounds of replication within the single parasitic vacuole (arrow). (B) Giemsa-stained rIFN- $\gamma$ -treated KC 72 h after challenge with LDA.  $\times 790$ . Internalized amastigotes (arrow) survive and replicate within the KC.

TABLE VI  
Kupffer Cells Exhibit a Limited Capacity to Kill *Leishmania donovani*  
Promastigotes

Exp.	Macrophages	rIFN- $\gamma$ U/ml	LDP per 100 cells		
			0 h	4 h	18 h
1	Kupffer cells	0	73	62 (15)	28 (62)
		100	61	51 (16)	37 (39)
	Resident peritoneal	0	106	48 (55)	9 (92)
		100	81	48 (41)	8 (90)
2	Kupffer cells	0	45	40 (11)	16 (64)
		100	34	27 (21)	10 (71)
	Resident peritoneal	0	84	50 (40)	4 (95)
		100	81	32 (60)	4 (95)

Cells were cultured for 48 h in the presence or absence of 100 U/ml rIFN- $\gamma$  before challenge with  $5 \times 10^6$  *L. donovani* promastigotes (LDP). Immediately after the 2 h infection (time 0) and at the indicated times, the number of LDP per 100 macrophages was assessed. Numbers in parentheses denote the percentage killing relative to time 0 for each population.

TABLE VII  
Kupffer Cells Support the Replication of *L. donovani* Amastigotes

Exp.	Macrophages	rIFN- $\gamma$ U/ml	LDA per 100 cells			LDA killed at 72 h %	Fold increase in LDA per 100 cells at 72 h
			0 h	48 h	72 h		
1	Kupffer cells	0	267	—	478	0	1.8
		100	234	—	403	0	1.7
	Resident peritoneal	0	101	—	90	11	—
		100	114	—	13	89	—
2	Kupffer cells	0	73	181	242	0	3.3
		100	80	178	243	0	3.0
	Resident peritoneal	0	87	86	68	22	—
		100	83	10	7	92	—
3	Kupffer cells	0	502	751	914	0	1.8
		100	494	626	906	0	1.8
	Resident peritoneal	0	58	—	129	0	2.2
		100	261	—	130	50	—

Cells were cultured for 48 h in the presence or absence of 100 U/ml rIFN- $\gamma$  before infection with  $5 \times 10^6$ /ml *L. donovani* amastigotes (LDA). After the 2 h infection (time 0) and at the indicated times, the numbers of LDA per 100 macrophages was scored. Percent LDA killed and fold increase in LDA per 100 cells represents the ratio of LDA per 100 cells at 72 h vs. time 0.

62–64% at 18 h (Table VI). Exposure to rIFN- $\gamma$  produced no elevation in the already appreciable killing by peritoneal macrophages, nor did it activate KC for enhanced microbicidal activity.

When the handling of *L. donovani* amastigotes was examined (Table VII), more striking qualitative and quantitative differences emerged in the rIFN- $\gamma$

responses of peritoneal macrophages and KC. Exposure to rIFN- $\gamma$  enhanced peritoneal macrophage amastigote clearance from 0–22% to 50–92%. KC, however, supported the replication of amastigotes, with a 1.8–3.3-fold increase in parasite numbers at 72 h even in the presence of rIFN- $\gamma$  (Table VII and Fig. 5B).

**Induction of Ia Antigen.** To assess a generalized defect in the response of KC to lymphokine, surface Ia antigen expression was quantitated in a binding assay using an iodinated monoclonal anti-Ia<sup>b,d</sup> antibody. In the B21-2-reactive strain B6D2F<sub>1</sub> (Ia<sup>b,d</sup>), KC showed a dose-dependent increase in Ia expression, with a plateau at 10–100 U/ml rIFN- $\gamma$  (Fig. 6). This increase represented a twofold induction of surface Ia antigen between 0 and 48 h of culture (not shown). Controls for nonspecific binding (see Materials and Methods) included a lack of antibody binding to nonreactive B6.H2-k (Ia<sup>k</sup>) KC (Fig. 6).

### Discussion

A major goal of this study was the development of an isolation procedure maximizing KC yield and purity while minimizing phenotypic alterations. A simplified collagenase perfusion technique was used, based on a method for the isolation of murine hepatocytes (23). Efforts to maintain hepatocyte viability were associated with increased KC yields and a reduction in ingestible, potentially toxic cellular debris. Pronase treatment, a method often used for purifying KC through destruction of hepatocytes (37, 40), was avoided. Also omitted was the reportedly harmful use of ammonium chloride (41) and certain separatory materials, such as metrizamide (42). Instead, hepatocytes were removed through low speed differential centrifugation and KC purified by short-term adherence to plastic or glass with meticulous removal of nonadherent cells.

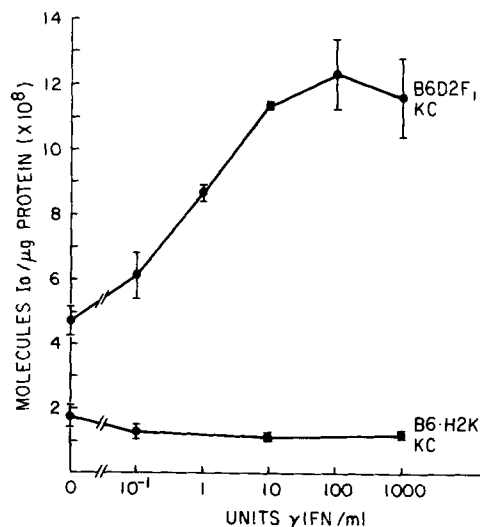


FIGURE 6. Induction of surface Ia antigen on KC after 48-h culture in various concentrations of rIFN- $\gamma$ . Binding of iodinated monoclonal anti-Ia<sup>b,d</sup> antibody was assessed on reagent-specific (B6D2F<sub>1</sub>) and reagent-nonreactive (B6.H2-k) KC.

In our approach to defining resident KC, we used in situ labeling with intravenously administered colloidal carbon. The identity of carbon-laden cells was confirmed with the F4/80 monoclonal antibody (15). However, Mac-1 did not stain KC in frozen sections; this may represent blocking of the anti-C3b $\alpha$  receptor in situ (44, 45). In situ carbon labeling was then coupled to our isolation protocol to provide a quantitative appraisal of cell isolates. KC yields averaged  $8 \times 10^6$  per gram of liver with >90% purity. The KC so isolated were typical tissue macrophages by several criteria: cytology, cytochemistry, phagocytic capacity, and surface markers (F4/80, Ia, and IgG1/IgG2b Fc receptor antigens). Secretory polypeptide profiles revealed a marked similarity to resident peritoneal macrophages. At least 11 co-migrating bands were observed, including those previously identified as fibronectin, factor B, and apolipoprotein E (24). The production of apolipoprotein E by KC has recently been reported in rats (46) and mice (47). From the intensity of the 33 K band in our gels, we conclude that apolipoprotein E represents a major secretory product, which suggests that there may be a significant role for KC in hepatic lipoprotein metabolism.

The most intriguing property of KC and their most striking difference from peritoneal macrophages was a deficiency in the generation of ROI. In three assay systems ( $H_2O_2$  secretion monitored by scopoletin oxidation, and  $O_2^-$  generation assayed by ferricytochrome  $c$  reduction and semi-quantitative NBT staining), KC were deficient relative to resident peritoneal macrophages. Similar results were noted using both soluble (PMA) and particulate triggering agents (opsonized zymosan, toxoplasmas, *Leishmania* promastigotes). Moreover, exposure to PMA produced negligible changes in KC oxygen consumption, arguing for a true defect in respiratory burst activity rather than extensive catabolism of its products or interference with their detection.

Hashimoto et al. (48) have reported diminished chemiluminescence by phagocytizing KC compared with resident peritoneal macrophages. They speculated that such differences might arise from their isolation protocol. To explore this possibility, we subjected peritoneal macrophages to the same enzyme treatment and subsequent handling as KC. The peritoneal cells remained fully active in the generation of ROI.

Superoxide release by rat KC has been reported by Bhatnagar et al. (49). In that study, KC were isolated by incubating pronase-perfused liver fragments in a pronase bath, a method which, in our studies, favors release of any inflammatory monocytes. Inflammatory monocytes are highly activated for the production of ROI and may account for the observed superoxide release (38). However, species differences between murine and rat KC can not be excluded.

While resident peritoneal macrophages responded to lymphokine or rIFN- $\gamma$  by an enhanced capacity to secrete ROI, KC did not. However, by two independent criteria (induction of surface Ia antigen [Fig. 6] and down-regulation of apolipoprotein E secretion [not shown]), KC did respond to rIFN- $\gamma$ , suggesting IFN- $\gamma$  receptors were present and functional.

The basis for the diminished KC oxidative metabolism is a question for ongoing research. At issue are differences between KC and other macrophages in terms of genetic programming vs. environmental influences, such as local circulation, soluble factors, and cell-cell interactions. Pretreatment of peritoneal macro-

phages with PMA inhibited antimicrobial activity (50) and blunted the respiratory burst in response to subsequent challenge (50, 51). Exposure of KC to serum proteins, immune complexes, bacterial products, or senescent erythrocytes may likewise induce a similar prolonged refractory state. Bacterial lipopolysaccharide (LPS) has variously been implicated in macrophage activation through priming for superoxide release (52), serving as a second signal in activation (53), or suppressing macrophage activation by rIFN- $\gamma$  (14). Experiments are underway to assess the role of LPS in KC deactivation by examining germ-free and LPS-resistant mice. In addition, since KC have been shown to regulate hepatocyte fibrinogen synthesis (54) and cytochrome P-450-dependent drug transformations (55), it is possible that hepatocytes, in turn, can interact directly or via soluble products to influence KC oxidative metabolism. A cell-derived protein that can suppress macrophage oxidative metabolism is currently under study (56, 57). Indeed, the diminished ROI generation may prevent injury to hepatocytes or erythrocytes during KC's frequent phagocytic encounters (58, 59). Distinctions among these possibilities may be aided by study of the NADPH oxidase of KC and its cytochrome  $b_{559}$  cofactor. These experiments are underway.

Deficient KC oxidative metabolism correlated with impaired parasite handling. Resident KC supported the replication of internalized *T. gondii* trophozoites. Lymphokine and rIFN- $\gamma$  failed to induce toxoplasmaicidal activity.

*L. donovani* might provide a clinically more relevant model. Parasites (either promastigotes or amastigotes) leave the cutaneous site of introduction and localize in liver macrophages in vivo. In vitro, the promastigote form of *L. donovani* is exquisitely sensitive to  $H_2O_2$  and to oxidative killing by peritoneal macrophages (12). Nonoxidative mechanisms have also been implicated, in that oxidatively impaired monocytes from chronic granulomatous disease (CGD) patients can slowly reduce a promastigote challenge (11). Resident KC did exhibit a limited capacity to kill promastigotes. The pattern of reduced leishmanicidal activity coupled with impaired kinetics of killing resembled that of a CGD monocyte. Lymphokine and rIFN- $\gamma$  failed to enhance KC leishmanicidal activity.

A somewhat different picture emerged for the *L. donovani* amastigote, which is more resistant than the promastigote to oxidative injury (60). Whereas resident peritoneal macrophages displayed limited static activity against amastigotes, leishmanicidal activity was markedly enhanced after exposure to rIFN- $\gamma$ . KC, on the other hand, supported the replication of amastigotes, with a similar two- to threefold increase in parasite numbers in the absence or presence of rIFN- $\gamma$ .

*Leishmania* promastigotes or amastigotes entering the circulation are cleared by fixed tissue macrophages, particularly in the liver and spleen. The specific targeting of blood-borne parasites may be in part a consequence of impaired host cell oxidative response as well as of circulatory dynamics and host cell phagocytic capacity. For internalized promastigotes, a competition may ensue between KC killing of parasites by limited nonoxidative means and the promastigote's ability to transform into an amastigote. Amastigotes can replicate within KC despite the elaboration of macrophage-activating factors by T lymphocytes. Host resistance would therefore depend upon an influx of inflammatory monocytes that possess active oxidative and antimicrobial capacities (38).

### Summary

Murine Kupffer cells (KC) were isolated by a high yield collagenase perfusion technique. The morphology, surface markers, and secretory products were typical of macrophages in other tissues. However, KC released negligible levels of  $H_2O_2$  and  $O_2^-$ , in contrast to peritoneal macrophages. KC oxygen consumption was not increased by agents triggering a respiratory burst in peritoneal cells. Moreover, KC capacity to secrete reactive oxygen intermediates (ROI), in contrast to Ia antigen expression, was not enhanced by exposure to lymphokines or recombinant  $\gamma$  interferon. The selective defect in KC oxidative response was paralleled by impaired in vitro killing of *Toxoplasma gondii* trophozoites and *Leishmania donovani* promastigotes and amastigotes. Deficient secretion of ROI by KC might protect hepatocytes and erythrocytes from injury during endocytosis by KC, but might render the liver more susceptible to parasitization by organisms that are primarily killed through oxygen-dependent mechanisms.

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