Neutrophils Sequestered in the Liver Suppress the Proinflammatory Response of Kupffer Cells to Systemic Bacterial Infection[1]

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The liver plays a major role in clearing bacteria from the bloodstream. Rapid clearance is primarily the function of fixed tissue macrophages (Kupffer cells) that line the hepatic sinusoids. Although Kupffer cells play a critical role in blood clearance, the actual elimination of the bulk of bacteria taken up by the liver depends upon the accumulation of bactericidal neutrophils. Subsequent experiments demonstrating neutrophils inside Kupffer cells derived from infected animals prompted our speculation that neutrophils modulate the proinflammatory response of Kupffer cells to bacteria cleared from the bloodstream. Indeed, we report here that neutrophils accumulated in the liver sinusoids suppress cytokine and chemokine mRNA expression and protein production by Kupffer cells. Using listeriosis in mice as an experimental model, we found that IL-1β, IL-6, IL-10, IL-12, TNF-α, MIP-1α, keratinocyte-derived chemokine, and MCP-1 mRNA levels were $\geq$10-fold more in the livers of *Listeria*-infected, relative to noninfected control, mice at 0.5–2 h after i.v. infection. Most message levels were sharply diminished thereafter, correlating inversely with increased neutrophil sequestration. Relative to intact animals, mice rendered neutrophil deficient exhibited marked increases in cytokine/chemokine mRNA expression and protein production in the liver subsequent to infection. Moreover, purified Kupffer cells derived from infected, neutrophil-depleted mice produced significantly more IL-6, IL-10, IL-12, TNF-α, keratinocyte-derived chemokine, and MCP-1 in culture. These findings document the critical role of neutrophils in moderating the proinflammatory response of Kupffer cells to bacteria taken up by the liver. *The Journal of Immunology,* **2009, 183: 3309–3316.**

The liver plays a major role in clearing systemic bacterial infections (1–4). Rapid clearance of bacteria from peripheral blood is often attributed to fixed liver macrophages, i.e., Kupffer cells, that line the hepatic sinusoids (1, 2). Kupffer cells are found in greatest number in the periportal area where they constitute the first macrophage population to come in contact with bacteria, endotoxin, and microbial debris derived from the gastrointestinal tract via the portal vein (5, 6). Consequently, they are constantly exposed to substances known to activate macrophages. The factors that prohibit the deleterious consequences that would otherwise follow the unrestrained proinflammatory response of Kupffer cells to such substances remain to be delineated (5–7).

Previously, we reported that the bulk of bacteria taken up by the liver is bound by Kupffer cells and killed by immigrating neutrophils (3). Neutrophils constitute the first line of defense against most microorganisms. Their effector functions include phagocytosis, production of toxic metabolites, and release of proteolytic enzymes (8). Although facilitating the elimination of invading organisms, these functions can also cause severe tissue injury (8, 9). Neutrophils sequestered in the liver sinusoids are a distinguishing feature of endotoxemia and sepsis. Reactive oxygen intermediates and proteolytic enzymes released by neutrophils are a central cause of severe hepatic injury (10, 11).

Eliminating neutrophils from infectious sites is essential to resolving inflammation (12). There is little evidence to suggest that inflammatory neutrophils return to the circulation or that lymphatic channels provide a route of egress (13). Rather, limited histological evidence to date indicates that they undergo apoptosis and are subsequently removed by macrophages in situ, thus preventing the discharge of cytolytic contents into the extracellular milieu (12, 14, 15). Activated neutrophils transfused into rats or mice localize primarily in the liver, suggesting that the liver is actively involved in the retention and disposal of neutrophils and in maintaining a balanced population (16, 17). The number of transfused neutrophils is increased in the peripheral blood and decreased in the livers of animals rendered Kupffer cell deficient before transfusion, supporting the role of Kupffer cells in neutrophil disposal. Shi et al. (18, 19) found neutrophils inside Kupffer cells in liver sections derived from rats inoculated with bacterial endotoxin or the *Streptococcus*-derived immunomodulator OK-432. Similarly, macrophages recovered from the livers of mice infected with *Listeria monocytogenes* contained intracellular neutrophils (4). These findings suggest that Kupffer cells play a critical role in eliminating neutrophils that accumulate in the liver sinusoids subsequent to clearance of bacteria, bacterial endotoxin, and microbial debris from the blood.

In vitro studies indicate that phagocytosis of apoptotic neutrophils exerts a significant, anti-inflammatory effect on macrophages. The ingestion of neutrophils suppresses production of proinflammatory

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cytokines (IL-1β, IL-12, and TNF-α), chemokines (IL-8), and lipid mediators (thromboxane B_2 and leukotriene C_4) by endotoxin-stimulated, human monocyte-derived macrophages (20, 21). Additionally, soluble factors synthesized and secreted by neutrophils suppress the production of proinflammatory cytokines (i.e., TNF-α and IL-6) by macrophages in culture (22). Elevated TNF-α mRNA expression in the livers and increased IL-1β and TNF-α levels in the sera of neutropenic rats or mice inoculated with endotoxin demonstrate the potential role of neutrophils in regulating proinflammatory cytokine production by macrophages in vivo (6, 23–25). Elevated serum levels of IL-1β, IL-6 and TNF-α in neutropenic patients indicates the clinical relevance of these observations (26).

L. monocytogenes is a Gram-positive, facultative intracellular bacterial pathogen that can cause severe, sometimes fatal, infections in humans (27). Listeriosis in mice is an experimental model often used to study host defenses to systemic infections and the factors that effect resistance to intracellular bacterial pathogens (27). Like other pathogens that enter the bloodstream, the bulk of *Listeria* inoculated i.v. into mice is cleared quickly and recovered in the liver (3). Between 10 min and 6 h after infection, the number of listeriae in the liver declines markedly, dependent upon the rapid accumulation and antimicrobial activity of neutrophils (3). Subsequent experiments demonstrating neutrophils inside Kupffer cells obtained from infected animals prompted us to speculate that neutrophils modulate the proinflammatory response of Kupffer cells to bacteria cleared from the bloodstream (4, 28). In this study, we report the critical role of neutrophils in down-regulating cytokine and chemokine production by Kupffer cells subsequent to *Listeria* infection.

Materials and Methods

Animals

Specific pathogen-free female, C57BL/6J mice were purchased from The Jackson Laboratory and used at 8–12 wk of age in the experiments described. The animals were treated in accordance with National Institutes of Health publications entitled *Principles for Use of Animals* and *Guide for the Care and Use of Laboratory Animals* and a protocol approved by the Rhode Island Hospital Animal Care and Use Committee. The mice were housed in well-ventilated rooms maintained at 22°C and an alternating 12-h light and dark cycle; food and water were provided ad libitum.

L. monocytogenes

L. monocytogenes (EGD strain) was routinely passed in mice to maintain its virulence. Stock preparations suspended in 1% gelatin were stored at −80°C as reported previously (4). Listeriae growing exponentially in trypticase soy broth cultures inoculated with an aliquot of frozen stock were used in the experiments described. Mice were infected i.v. with 2×10^7 listeriae estimated spectrophotometrically; the actual inoculum was subsequently quantified from the number of colonies that grew on trypticase soy agar plates. Similarly, the number of organisms recovered in the livers of infected animals was calculated from the colonies enumerated on trypticase soy agar plates inoculated with tissue homogenates.

Cell preparation and culture

Nonparenchymal liver cells (NPCs).[3] The NPC population was isolated and purified following perfusion of the liver with collagenase via the portal vein using methods we reported previously (3, 4, 29). The perfused liver was dissected and teased apart; the resultant cell suspension was centrifuged at $40 \times g$ for 15 min at 4°C to remove the parenchymal cells recovered in the pellet. The NPCs remaining in suspension were centrifuged, resuspended in cold Histodenz (20% final concentration in Ca- and Mg-free HBSS; Sigma-Aldrich,), overlaid with cold HBSS, and centrifuged at $1500 \times g$ for 20 min at 4°C. The NPCs banding at the Histodenz-HBSS interface were collected and washed in HEPES-buffered RPMI 1640 medium (Mediatech) supplemented with 5% heat-inactivated FBS (Sterile Systems). The resultant purified NPC population was 99% viable and free of hepatocyte contamination.

Kupffer cells. Kupffer cell-enriched populations were also prepared as we described previously (29). Briefly, the purified NPCs were incubated simultaneously with biotin-conjugated anti-CD115 (clone AFS98) and biotin-conjugated anti-F4/80 (clone BM8) mAb purchased from eBioscience. The labeled cells were then washed and incubated with streptavidin-conjugated magnetic microbeads (Miltenyi Biotec). Subsequently, the $CD115^+$ $F4/80^+$ Kupffer cells with beads attached were separated from bead-negative cells by passing the suspension over a MS^+ selection column (Miltenyi Biotec) placed in a magnetic field; the Kupffer cell-enriched population was eluted and washed. Purified Kupffer cell populations were cultured in HEPES-buffered RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM L-glutamine, 1% essential and nonessential amino acids, 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium).

Neutrophils. The peritoneal exudate cells were obtained by lavage from mice inoculated i.p. 10–12 h previously with 1 ml of 10% protease peptone. The cells were suspended in 2 ml of Ca- and Mg-free HBSS supplemented with 0.5% FBS, layered onto a two-step (54.8%/70.2%) discontinuous Percoll gradient, and centrifuged at $1000 \times g$ for 30 min and 22°C. The cells (96% neutrophils, $\sim 1 \times 10^7$ neutrophils/mouse) recovered at the lower interface were treated with 0.83% ammonium chloride to eliminate contaminating RBC. To assess the capacity of *Listeria* to induce apoptosis, 3.0×10^6 purified neutrophils/1 ml of antibiotic-free culture medium/well in 24-well tissue culture plates coated with 0.1% polyhydroxyethyl methacrylate to reduce cell attachment were incubated in the presence or absence of 3.0×10^7 listeriae (i.e., 10:1::bacteria:cell). After a 6-h incubation period, the cells were collected and the apoptotic neutrophils were enumerated by staining with PE-annexin V and 7-aminoactinomycin (7-AAD) (described below).

Cell depletion

Kupffer cells were eliminated in accordance with methods we described previously (4, 29, 30). Multilamellar liposomes containing dichloromethylene diphosphonate (Cl_2MDP-L; a gift from Roche Diagnostics) were prepared according to methods we described previously (30). Mice were inoculated i.v. with 200 μl of 1 mg/ml Cl_2MDP-L suspended in saline on day 3 before infection. Mice administered 200 μl of liposome-encapsulated saline served as the control (29). To deplete neutrophils, mice were inoculated i.p. with 500 μg of rat IgG2b anti-mouse Gr-1 (RB6–6C5 hybridoma obtained from R. L. Coffman, DNAX Research Institute, Palo Alto, CA) 2–3 days before experimental use (described previously in Ref. 3). Control mice received an equivalent amount of normal rat IgG (Sigma-Aldrich).

Flow cytometry

Flow cytometry was conducted in accordance with methods previously described by us (31). Dye-conjugated mAb specific for the following determinants were purchased and used: anti-F4/80 (clone BM8; eBioscience), anti-CD11b (clone M1/70; BD Biosciences), and anti-Ly6G (clone 1A8; BD Biosciences). To detect neutrophils inside Kupffer cells, NPCs were stained with PE-labeled anti-F4/80 mAb, fixed, and permeabilized with a BD Biosciences Cytofix/Cytoperm kit at 4°C for 16 h and then stained with FITC-labeled 1A8 mAb. Apoptotic and necrotic $Ly\text{-}6G^+$ neutrophils were enumerated by staining with PE-annexin V and 7-AAD according to the protocol provided by the supplier (BD Biosciences).

RNA extraction, purification, and quantitative real-time RT-PCR

Total cellular RNA in representative liver samples was extracted and purified using TRIzol (Invitrogen). Real-time RT-PCR was conducted using a slight modification of the methods we described previously (29). Briefly, purified RNA was reverse transcribed with Quantitec Reverse Transcriptase (Qiagen). Quantitative analysis of gene expression was performed using the Eppendorf Mastercycler ep realplex system and SYBR Green technology. iTaq SYBR Green Super Mix (Bio-Rad) was mixed with an equal volume of RNase-free water containing 0.6 μM of both the forward and reverse primers and cDNA corresponding to 500 ng of total RNA input. The plates were heated for 3 min at 95°C to activate the HotStartTaq DNA polymerase. The ratios of mRNA to 18S ribosomal RNA (the housekeeping standard) copies were estimated from the PCR cycle number required for the fluorescent signal for each to reach a fixed intensity, the threshold cycle. The mean number of targeted gene mRNA copies per 10^3 18S rRNA

[3] Abbreviations used in this paper: NPC, nonparenchymal liver cell; Cl_2MDP-L, liposome-encapsulated dichloromethylene diphosphonate; KC, keratinocyte-derived chemokine; MPO, myeloperoxidase; 7-AAD, 7-aminoactinomycin D.

Table I. *Primers*

Gene	GenBank Accession No.	Orientation	Primer Sequence	Amplicon Size
IL-1β	NM_008361	Forward	TCACAGCAGCACATCAACAA	112
		Reverse	TGTCCTCATCCTGGAAGGT	
IL-6	NM_031168	Forward	AGTTGCCTTCTTGGGACTGA	103
		Reverse	CAGGTCTGTTGGGAGTGG	
IL-10	NM_010548	Forward	CCAAGCCTTATCGGAAATGA	162
		Reverse	TTCACAGGGGAGAAATCG	
IL-12p40	NM_008352	Forward	GGAAGCACGGCAGCAGAATA	180
		Reverse	AACTTGAGGGAGAAGTAGGAATGG	
TNF-α	NM_013693	Forward	TCCCCAAAGGGATGAGAAGT	139
		Reverse	CTCCTCCACTTGGTGGTTT	
MIP-1α (CCL3)	NM_011337	Forward	ACCATGACACTCTGCAACCA	202
		Reverse	CCCAGGTCTCTTTGGAGTCA	
KC (CXCl1)	NM_008176	Forward	GCTGGGATTCACCTCAAGAA	169
		Reverse	TGGGGACACCTTTTAGCATC	
MCP-1 (CCL2)	NM_011333	Forward	CCCACTCACCTGCTGCTACT	164
		Reverse	TCTGGACCCATTCCTTCTTG	
18S rRNA	XR_000144	Forward	AATGGTGCTACCGGTCATTCC	192
		Reverse	ACCTCTCTTACCCGCTCTC	

copies ± SEM for samples derived from five or six mice treated comparably is reported. The PCR primers listed in Table I were designed from published sequences using Primer3 software (Whitehead Institute, Cambridge, MA) and purchased from Operon Biotechnologies).

Cytokine and chemokine analyses

The cytokines and chemokines present in culture supernatants or representative tissue samples were quantified using multiplex cytokine bead arrays. In the latter case, liver wedges were homogenized (50–100 mg/ml) in calcium- and magnesium-free HBSS containing 0.5% Triton X-100 and 2× complete protease inhibitor mixture (Roche Diagnostics). Homogenates were centrifuged at 13,500 × *g* to remove cell debris, and supernatants were stored at −20°C until analyses. Bio-Plex cytokine assay kits were purchased and used with the Bio-Plex 200 system in accordance with the manufacturer's instructions (Bio-Rad). The following panel of cytokines and chemokines were quantified: IL-1β, IL-6, IL-10, IL-12, TNF-α, keratinocyte-derived chemokine (KC), MIP-1α, and MCP-1.

Statistical analysis

The results were analyzed using the SigmaStat statistics program (Jandel Scientific). Individual means were compared using a nonpaired Student's *t* test or a Mann-Whitney rank sum test. Data derived from three or more groups were compared by one-way ANOVA-Tukey ($p < 0.05$).

Results

Elevated cytokine/chemokine mRNA expression and protein production in livers of Listeria*-infected mice*

Previously, Vogel and coworkers (32, 33) reported Kupffer cell-dependent increases in cytokine and chemokine message expression in the livers of mice inoculated with bacterial endotoxin. Message levels peaked and were substantially higher between 1 and 3 h after inoculation than at 6 h after inoculation and were sharply diminished in Kupffer cell-depleted mice. In this study, mice inoculated i.v. with *Listeria* exhibited similar rapid and transient increases in mRNA expression. Mean cytokine (i.e., IL-1β, IL-6, IL-10, IL-12, and TNF-α) and chemokine (KC, MIP-1α, and MCP-1) mRNA levels were ≥10-fold higher in the livers of infected, relative to noninfected control, mice within a relatively short period of time, peaking at 0.5–2 h after infection (Fig. 1). MIP-1α and MCP-1 mRNA levels stayed elevated while the remaining mRNAs were sharply diminished ≥2-fold by 6 h after infection. The protein products of these messages were also higher in the livers of infected animals, albeit to varying degrees. Notably, the tissue concentrations of IL-1β, IL-12, KC, MIP-1α, and MCP-1 were at maximum levels and significantly greater at 2 h after infection relative to the noninfected (0 time) controls (Fig. 2).

Kupffer cells ingest neutrophils sequestered in the liver sinusoids

Previously, we reported that the bulk of listeriae inoculated i.v. into mice and taken up by the liver were initially killed by immigrating neutrophils (3). As shown in Fig. 3, ~70% of the inoculum (1.8×10^7 listeriae/mouse) was recovered in the liver at 30 min after i.v. infection. Subsequently, the listerial burden of the liver decreased significantly; at 6 h after infection, only 15–20% of the inoculum remained. Mice rendered neutrophil deficient before infection exhibited a similar ability to clear listeriae from the bloodstream; the numbers of listeriae in the livers of control and neutrophil-deficient mice were equivalent at 0.5 and 2 h after infection. In contrast to nondepleted mice, neutrophil-depleted mice exhibited an approximate 3-fold increase in liver listeriae between 2 and 6 h after infection. Consequently, at 6 h after infection, 20-fold more bacteria were recovered from the livers of neutrophil-depleted animals.

$Ly6G^+$ neutrophils constitute <1% of total hepatic NPCs in normal, noninfected mice (Fig. 4). Flow cytometric analysis demonstrated the rapid accumulation of neutrophils in the livers of mice infected i.v. with *Listeria*. The percentage of neutrophils contained within the hepatic NPC population peaked at 2 h after infection and then declined thereafter. Concurrently, a marked increase in the percentage of $F4/80^+$ Kupffer cells that contained $Ly6G^+$ neutrophils occurred with the accumulation and subsequent disappearance of hepatic neutrophils. Thus, at 2 and 6 h after infection, ~7% of total Kupffer cells were $F4/80^+Ly6G^+$, substantiating the role of Kupffer cells in eliminating neutrophils that accumulate in the liver subsequent to systemic bacterial infection.

Notably, the percentage of $F4/80^+$ Kupffer cells that comprised the NPC population quantified here by flow cytometry was significantly less than the approximate 30% of NPCs estimated by other methods (34, 35). This finding concurs with a previous report by ten Hagen et al. (35) who concluded that Kupffer cell expression of F4/80 was inherently low. Alternatively, we speculate that F4/80 is inordinately sensitive to degradation during collagenase/protease treatment and cell purification. Regardless, while these data substantiate our previous findings demonstrating the role of Kupffer cells in removing apoptotic neutrophils accumulated in the livers of *Listeria*-infected mice (4), they provide little insight into the contribution of individual cells. Rather, the critical role of Kupffer

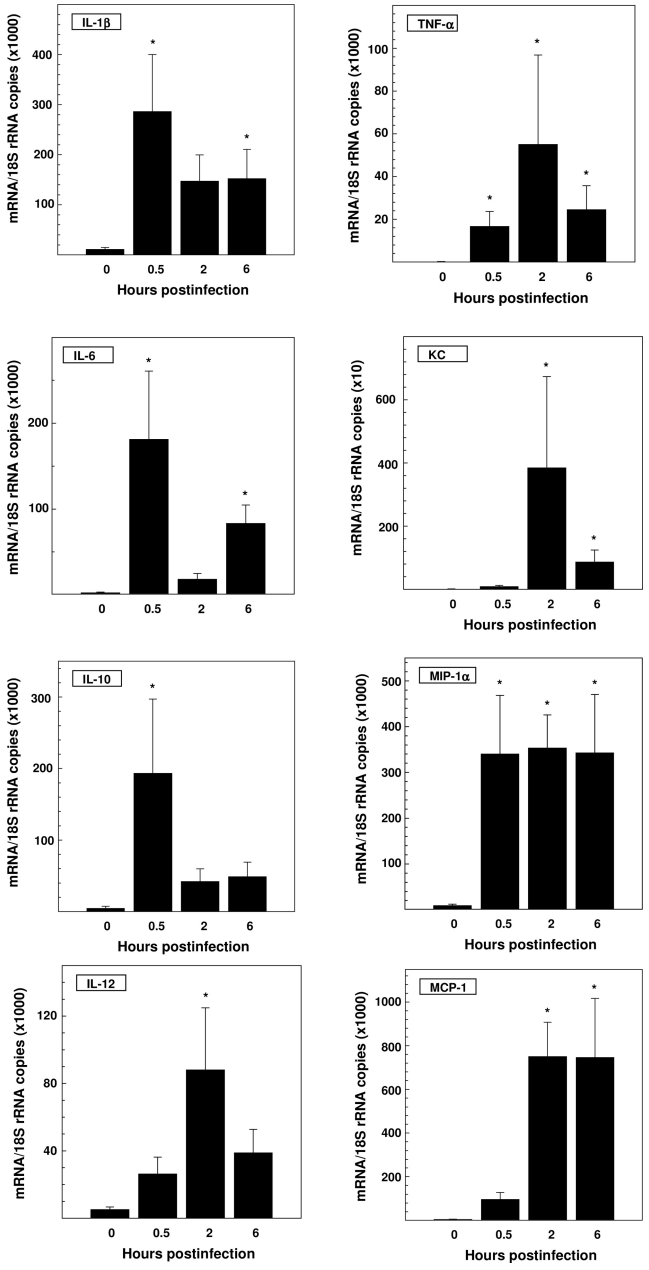

FIGURE 1. Cytokine and chemokine mRNA expression is rapidly upregulated in the livers of *Listeria*-infected mice. Groups of mice were inoculated i.v. with 1.8×10^7 listeriae. Representative liver samples were obtained at 0, 0.5, 2, and 6 h after infection and the cytokine and chemokine mRNAs listed were quantified by real-time RT-PCR. Data are the means ± SEM derived from two experiments and six mice treated comparably. ∗, Significantly greater than the noninfected control group ($p < 0.05$; ANOVA).

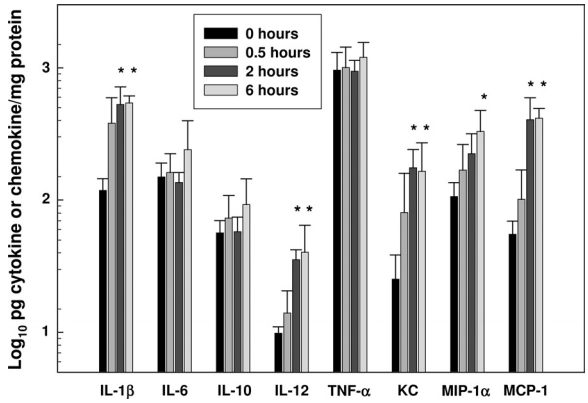

FIGURE 2. Inflammatory cytokine and chemokine protein levels are elevated in the livers of *Listeria*-infected mice. Groups of mice were inoculated i.v. with 3.3×10^7 listeriae. Representative liver samples were obtained at the times indicated postinfection and the cytokines/chemokines listed were quantified by multiplex bead array analysis. Data are the means ± SD derived from two experiments and six mice treated comparably. ∗, Significantly greater than the noninfected, 0 time group ($p < 0.05$; ANOVA).

cells in eliminating neutrophils is supported by a significant reduction in number of neutrophils recovered from the livers of normal, but not Kupffer cell-depleted, animals between 2 and 6 h after infection (Fig. 5). Indeed, despite finding far fewer neutrophils in the livers of Kupffer cell-depleted mice at 2 h (shown previously and correlating directly with an approximate 5-fold reduction in bacterial burden at this time (4)), there was no significant difference in the numbers of neutrophils recovered from the livers of depleted and nondepleted mice at 6 h after infection. This latter finding appears to differ from our previous report demonstrating more myeloperoxidase (MPO) activity, an indictor of neutrophil recruitment, in the livers of control mice at 6 h (4). It is relevant to note in this regard that MPO is released by activated neutrophils and remains in the tissues bound by vascular endothelial cells (36). Consequently, the level of MPO activity is not a strict correlate of neutrophil number.

Early apoptotic neutrophils accumulate in the livers of Listeria*-infected mice*

Large percentages of neutrophils accumulated in the livers of *Listeria*-infected mice at 2 and 6 h after infection were undergoing apoptosis; the majority of these cells were in the early (annexin V^+7-AAD^-) phase regardless of the presence or absence of Kupffer cells (Table II). Far fewer neutrophils found in the livers were annexin V^+7-AAD^+ (late apoptotic); approximately 2% were annexin V^-7-AAD^+ (necrotic). These findings differ sharply from the results of in vitro experiments demonstrating directly the capacity of *Listeria* to induce apoptosis by neutrophils (Fig. 6).

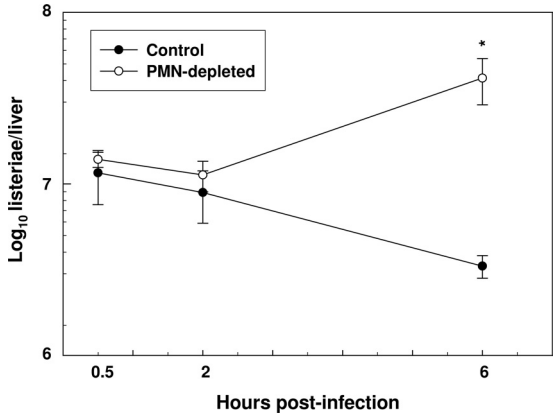

FIGURE 3. Neutrophil depletion exacerbates bacteria replication in the liver. Groups of control and neutrophil-depleted mice were inoculated i.v. with 1.8×10^7 listeriae. The mice were euthanized at the times indicated postinfection, the livers were dissected and homogenized, and the bacterial burden was quantified by growth on agar plates. Values are the means ± SD obtained from three mice treated identically in a single representative experiment. ∗, Control and neutrophil-depleted are significantly different ($p = 0.006$; Student's t test).

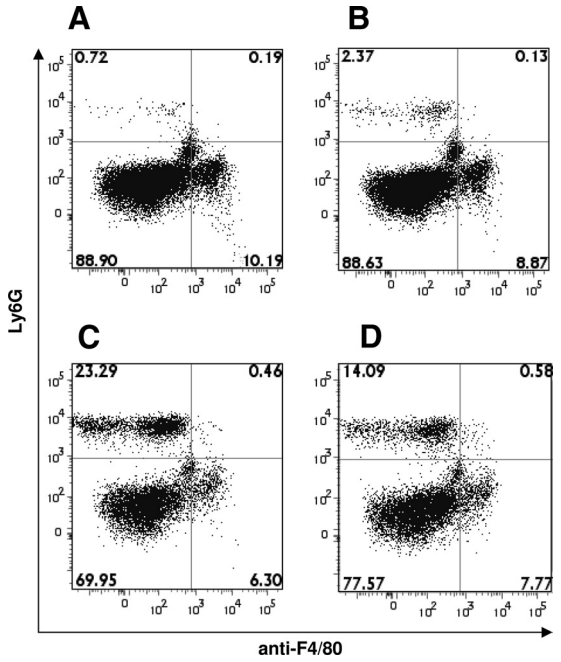

FIGURE 4. Kupffer cells ingest inflammatory neutrophils sequestered in the liver. Mice were inoculated i.v. with 2.6×10^7 listeriae. $Ly6G^+$ neutrophils, $F4/80^+$ Kupffer cells, and $Ly6G^+F4/80^+$ neutrophil-containing Kupffer cells in the liver were quantified by flow cytometry at 0 (*A*), 0.5 (*B*), 2.0 (*C*), and 6 (*D*) h after infection. Data at each time point were generated from three mice treated identically. A second experiment yielded comparable results.

Relative to neutrophils cultured alone, a far greater percentage of cells retrieved after a 6-h incubation with *Listeria* were annexin $V^+7\text{-}AAD^+$; moreover, a large portion of these cells was necrotic. In contrast to neutrophils purified from the livers of *Listeria*-infected mice, comparatively few cells recovered after culture either in the presence or absence of listeriae were in the early phase of apoptosis. Thus, although listeriae induced apoptosis by neutrophils in vitro, as well as in vivo, the physiologic outcomes evident at 6 h differed sharply.

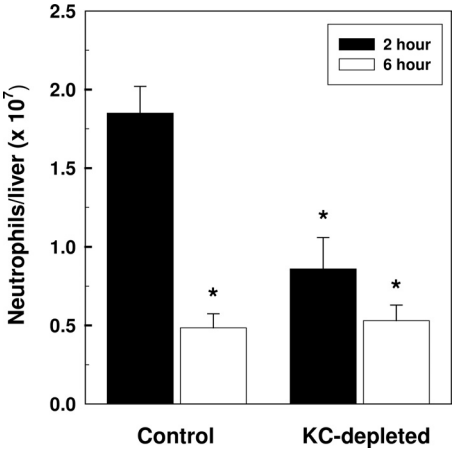

FIGURE 5. Kupffer cell-dependent elimination of neutrophils sequestered in the liver. NPCs were obtained from groups of four control and four Kupffer cell-depleted mice at 2 and 6 h after i.v. infection with 1×10^7 listeriae. The data are the means ± SD number of neutrophils per liver calculated from the percentage of neutrophils determined in cytospin smears times the total number of NPCs per liver. ∗, Significantly less than the 2 h control group, but not significantly different from each other. A second experiment yielded comparable results.

Table II. *Apoptotic neutrophils accumulate in the livers of* Listeria*-infected mice*[a]

Pretreatment	Viability	% of Total Neutrophils: 2 h Postinfection	% of Total Neutrophils: 6 h Postinfection
Control	Early apoptotic	35.2 ± 7.7	48.7 ± 3.3[b]
	Late apoptotic	8.9 ± 3.1	10.1 ± 2.1
	Necrotic	0.1 ± 0.0	0.9 ± 0.5
Kupffer cell depleted	Early apoptotic	26.8 ± 4.4	50.4 ± 5.2[b]
	Late apoptotic	8.8 ± 2.6	8.5 ± 1.1
	Necrotic	0.2 ± 0.1	0.7 ± 0.4

[a] Groups of control and Kupffer cell-depleted mice were infected i.v. with an approximate 2×10^7 listeriae. The hepatic NPCs were obtained and purified from individual mice at the times indicated postinfection. The data are the means ± SD percentages of annexin $V^+7\text{-}AAD^-$ (early apoptotic), annexin $V^+7\text{-}AAD^+$ (late apoptotic), and annexin $V^-7\text{-}AAD^+$ (necrotic) $Ly6G^+$ neutrophils derived from four mice treated identically. A second experiment yielded comparable results.

[b] Significantly greater than the percentage found at 2 h after infection.

Neutrophils suppress cytokine/chemokine mRNA expression and protein production in the liver

Neutrophils exert a significant effect on cytokine and chemokine production by endotoxin-stimulated macrophages in vitro (20, 21). To determine the effect of neutrophils on cytokine and chemokine production by Kupffer cells in vivo, mice were not treated or neutrophil-depleted before *Listeria* infection. Without exception, cytokine/chemokine message expression was substantially (≥2-fold) higher in livers obtained from neutrophil-depleted mice at 2 h after infection, i.e., when the numbers of listeriae in the livers of depleted and nondepleted mice were equivalent and not a contributing factor (Fig. 7). In the absence of infection, message expression was comparable in the livers of neutrophil-depleted and nondepleted mice and was equivalent to that expressed in the livers of the noninfected animals shown in Fig. 1. The protein products of

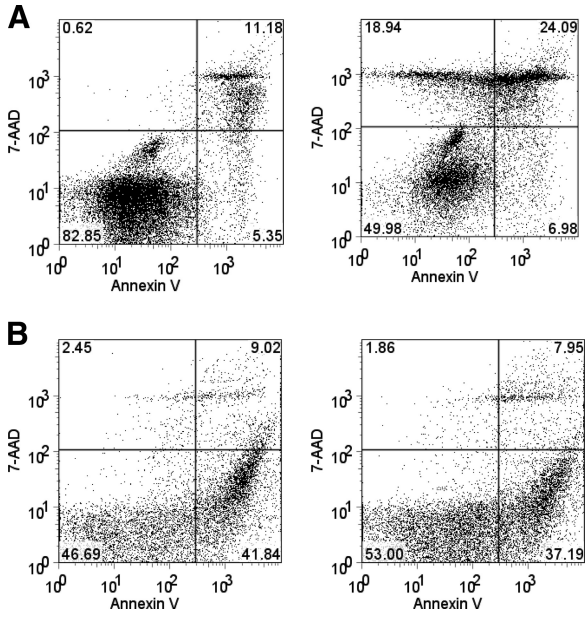

FIGURE 6. *Listeria* induces late phase apoptosis and necrosis by neutrophils in culture. *A*, Neutrophils cultured 6 h without (*top left panel*) or with listeriae (*top right panel*) were stained with PE-annexin V and 7-AAD and subjected to flow cytometric analysis. *B*, Dot plots of stained neutrophils derived from untreated (*bottom left panel*) and Kupffer cell-depleted (*bottom right panel*) mice at 6 h after infection, representative of the data depicted in Table II, are provided for comparison purposes.

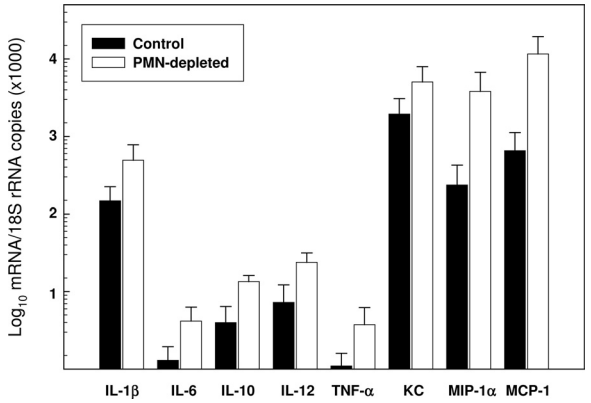

FIGURE 7. Cytokine and chemokine mRNA expression is elevated in the livers of neutrophil-depleted mice infected with *Listeria*. Liver samples were obtained from control and neutrophil-depleted mice at 2 h after i.v. infection with 3.1×10^7 listeriae; mRNA concentrations of the cytokines and chemokines listed were quantified by real-time RT-PCR. Data are the means ± SD derived from groups of five mice treated identically. Two additional experiments yielded comparable results. In all cases, cytokine/chemokine message expression was significantly greater in the livers of neutrophil-depleted mice (data shown, $p \leq 0.05$) and greater in infected than noninfected animals (data not shown).

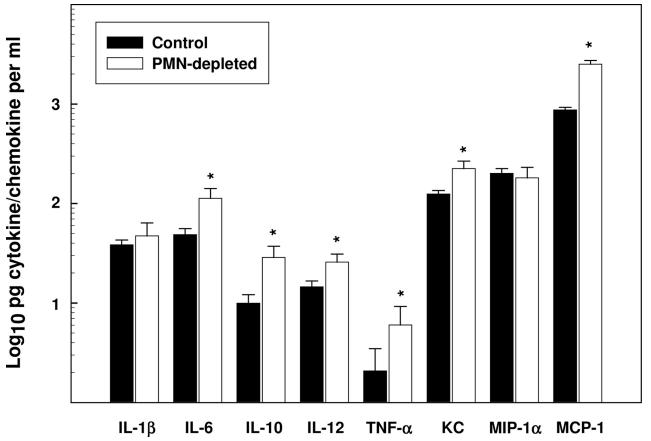

FIGURE 8. Neutrophils accumulated in the liver suppress cytokine/chemokine production by Kupffer cells. Purified Kupffer cells derived from the livers of nontreated and neutrophil-depleted mice at 2 h after infection with 2.5×10^7 listeriae were cultured for ~40 h. Cytokines/chemokines in the culture supernatants were quantified by multiplex bead array analysis. Data are the means ± SD obtained in a single experiment representative of two experiments. *, Significantly greater than that generated in culture by Kupffer cells derived from nontreated (non-neutrophil-depleted) mice.

most of these cytokine/chemokine messages were similarly elevated (2- to 6-fold) and significantly greater in the livers of neutrophil-depleted animals (Table III). Indeed, although the concentrations of IL-10 and TNF-α were equivalent in nondepleted mice at 0 time and 2 h after infection (illustrated in Fig. 2), they were elevated substantially in the livers of neutrophil-depleted mice at 2 h after infection. Likewise, the concentrations of IL-6 and KC in the livers of neutrophil-depleted mice appeared elevated albeit variability within the groups precluded statistical significance.

To demonstrate directly the effect of neutrophils on cytokine and chemokine production by Kupffer cells, the NPC populations were obtained from groups of nondepleted and neutrophil-depleted mice at 2 h after infection. The Kupffer cells purified from both populations were subsequently cultured for ~40 h. Significantly greater concentrations of IL-6, IL-10, IL-12, TNF-α, KC, and MCP-1 were determined in supernatants collected from the culture of Kupffer cells derived from neutrophil-depleted animals (Fig. 8).

Table III. *Neutrophils sequestered in the liver suppress cytokine/chemokine protein production*[a]

Factor	Mice		Significance[b]
	Control	Polymorphonuclear Leukocyte-depleted	
IL-1β	78.5 ± 6.7	213.9 ± 59.1	$p = 0.016$
IL-6	40.0 ± 11.1	69.6 ± 39.3	$p > 0.05$
IL-10	4.5 ± 0.6	8.2 ± 3.0	$p = 0.032$
IL-12	3.0 ± 0.5	14.9 ± 7.7	$p = 0.016$
TNF-α	71.8 ± 17.9	144.0 ± 59.8	$p = 0.035$
KC	41.6 ± 14.4	166.7 ± 133.3	$p > 0.05$
MIP-1α	18.4 ± 3.9	111.2 ± 62.0	$p = 0.016$
MCP-1	224.9 ± 40.4	1089.4 ± 841.1	$p = 0.016$

[a] Representative liver samples were obtained from control and neutrophil-depleted mice at 2 h after i.v. infection with 3.1×10^7 listeriae. The cytokines and chemokines listed were quantified by multi-plex bead array analysis. Data are the means ± SD pg of cytokine or chemokine per mg protein derived from groups of five mice treated identically in a single experiment. An additional experiment yielded comparable results.

[b] Cytokine/chemokine concentration is significantly greater in livers dissected from *Listeria*-infected, neutrophil-depleted group.

Discussion

Bacterial sepsis and the complications that ensue affect 751,000 patients in the United States each year; more than one-third of these patients die (37). Mortality is even higher in cases where sepsis occurs secondarily to liver disease. Most bacteria that enter the bloodstream are taken up and eliminated in the liver (1, 2, 38). Consequently, the liver is the most important organ in the body for blood clearance and prevention of septicemia and sepsis. When sepsis occurs, the liver represents a major target for development of multiple organ dysfunction syndrome (39). Indeed, proinflammatory cytokines and chemokines (e.g., IL-1β, TNF-α, and MCP-1) produced by Kupffer cells activated by materials derived from the gut via the portal vein have been implicated in hepatocellular dysfunction (32, 40). The factors that moderate the response of Kupffer cells to such materials like bacterial endotoxin, a common component of the portal blood, are largely unknown. Like resident tissue macrophages that reside in other organs, however, Kupffer cells differ phenotypically from activated macrophages recently recruited to the liver having seemingly adapted to the local environment to perform certain tasks, e.g., trophic interactions with other cell types such as NK and NKT cells (41). In this regard, Kupffer cells exhibit a diminished capacity to: generate reactive oxygen intermediates; produce proinflammatory cytokines/chemokines; and ingest and kill microorganisms (3, 42, 43). The factors that modulate (down-regulate) these activities wait to be determined.

Vogel et al (32, 33) reported rapid and transient increases in the expression of cytokine (i.e., IL-1β, IL-6, IL-10, IL-12, and TNF-α) and chemokine (e.g., MIP-1α and MIP-1β) messages in the livers of mice inoculated i.p. with bacterial endotoxin. Message levels were 3- to 10-fold higher at earlier peak time points (1–3 h) than at 6 h after inoculation, and dependent upon Kupffer cells, mice pretreated with Cl_2MDP-L exhibited a 75–95% reduction in peak message level expression. Although suggestive, depletion studies such as these fail to provide direct, definitive evidence identifying Kupffer cells as the cell source of cytokines and chemokines produced. Moreover, they provide little insight into the factors that modulate cytokine/chemokine mRNA expression or protein production by Kupffer cells.

Like animals inoculated with bacterial endotoxin, mice infected i.v. with *L. monocytogenes* exhibit rapid and significant increases in expression of IL-1β, IL-6, IL-10, and TNF-α messages in the liver (44, 45). As reported here, message expression peaks within 0.5 and 2 h after infection and declines sharply thereafter (Fig. 1). The decline in expression of most, albeit not all, messages assessed correlated inversely with increased neutrophil sequestration in the liver and ingestion by Kupffer cells (Fig. 4, *C* and *D, upper left* and *right quadrants*, respectively). Clearly, the disparate kinetics with which different cytokine/chemokine messages are expressed could reflect inherent differences in the factors that regulate the synthesis of multiple messages within a single cell type (i.e., Kupffer cells). Alternatively, expression of the same message by multiple cell types, e.g., Kupffer cells and hepatocytes, could account for the disparate kinetics observed.

Elevated message levels in the livers of neutrophil-depleted, relative to nondepleted, mice at 2 h after infection evidence the inhibitory effect of neutrophils on cytokine/chemokine mRNA expression (Fig. 7). These data are substantiated by experiments demonstrating increased protein concentrations of the same cytokines and chemokines in livers dissected from neutrophil-depleted animals (Table II). Bacterial burden was not a factor contributing to the differences in cytokine/chemokine production observed; equivalent numbers of listeriae were recovered from the livers of neutrophil-depleted and nondepleted mice at 2 h after infection (Fig. 3). Finally, the data presented herein demonstrate directly the inhibitory effect of neutrophils on the production of cytokines and chemokines by Kupffer cells. Relative to cells derived from *Listeria*-infected nondepleted animals, Kupffer cells obtained from infected neutrophil-depleted mice produce increased amounts of IL-6, IL-10, IL-12, TNF-α, KC, and MCP-1 in culture (Fig. 8).

The factors that govern the inhibitory effect of neutrophils on cytokine and chemokine production by Kupffer cells remain to be clarified. Neutrophils circulating in the peripheral blood of humans exhibit a relatively short (6–7 h) half-life; turnover rate is $\sim 10^{11}$ cells/day (46). Spontaneous apoptosis and subsequent removal by macrophages contribute to this high rate of turnover and are essential mechanisms by which neutrophil homeostasis and inflammation are regulated (12, 47). A myriad of bacterial species including *L. monocytogenes* induce apoptosis by neutrophils; reactive oxygen species generated during phagocytosis are critically involved (48, 49). As described in the Introduction, the ingestion of neutrophils induced to undergo apoptosis by UV irradiation, for example, suppresses proinflammatory cytokine production by endotoxin-stimulated human macrophages in culture (20, 21). On the other hand, recent studies report that the ingestion of neutrophils induced by bacterial pathogens to undergo apoptosis promote the production of these same cytokines, e.g., TNF-α (50, 51). In this regard, it was suggested that noninfected apoptotic neutrophils contribute to the resolution of the inflammatory response once the bacteria are cleared from the site of infection (51). Although the literature regarding the ability of apoptotic neutrophils to modulate cytokine production by macrophages in vitro is extensive, there is little evidence to suggest that neutrophils exert the same effect on macrophages in vivo. Only recently, Ren et al. (52) reported a marked increase in the survival rate of mice challenged with endotoxin and subsequently administered apoptotic human neutrophils. Increased survival correlated inversely with serum IL-12, TNF-α, and IFN-γ levels and liver injury.

Although initial studies suggested that the ingestion of apoptotic neutrophils suppressed proinflammatory cytokine production by macrophages (20, 21), more recent experiments indicate that cell-cell contact, not phagocytosis, is essential. Lucas et al. (53) reported that the production of TNF-α, IL-10, and IL-12 by LPS-stimulated macrophages in culture was sharply diminished by apoptotic neutrophils present under conditions where phagocytosis was inhibited. In addition to mechanisms that involve direct contact between cells, soluble factors secreted by neutrophils, including prostaglandin E_2 and adenosine, have the capacity to down-regulate cytokine production by macrophages (22, 54, 55). Thus, despite results demonstrating neutrophils inside Kupffer cells during the course of *Listeria* infection (shown here and reported previously (4)), the mechanisms that underlie the effects of neutrophils on Kupffer cell-dependent cytokine/chemokine production await clarification. Consequently, a series of in vitro experiments was initiated in an effort to obtain insight into those factors involved. Aside from the viable cells that survived, however, the population collected following culture of purified neutrophils with *Listeria* was comprised largely of late apoptotic and necrotic cells and bore little resemblance to the neutrophil population recovered from the livers of *Listeria*-infected mice (Fig. 6). The heterogeneous and seemingly unrelated character of the neutrophil population generated in vitro, in addition to the disparate effects of viable, apoptotic and necrotic neutrophils on inflammatory cytokine production by macrophages (discussed above), precluded conducting any meaningful coculture experiments to delineate the effect of neutrophils on cytokine/chemokine production by Kupffer cells following *Listeria* infection in animals. Nonetheless, the alleged role of Kupffer cells in maintaining neutrophil homeostasis and the presence of neutrophils inside Kupffer cells residing in both healthy and diseased livers support the role of neutrophils in suppressing the response of Kupffer cells to inflammatory substances such as bacteria, endotoxin, and microbial debris cleared from the bloodstream by the liver (16–19).

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Disclosures

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