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*J Immunol* 2009; 183:7557-7568; Prepublished online 16 November 2009;
doi: 10.4049/jimmunol.0901786
http://www.jimmunol.org/content/183/11/7557

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Supplementary Material  
http://www.jimmunol.org/content/suppl/2009/11/16/jimmunol.0901786 DC1

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Selective Down-Regulation of Neutrophil Mac-1 in Endotoxemic Hepatic Microcirculation via IL-10

Gustavo Batista Menezes,* Woo-Yong Lee,† Hong Zhou,* Christopher Curtis Matchett Waterhouse,* Denise Carmona Cara,‡ and Paul Kubes2

Hepatic neutrophil adhesion during endotoxemia is an integrin-independent, CD44-dependent process. Because integrins function in other endotoxemic vasculatures, we used spinning disk confocal intravital microscopy to assess whether LPS down-modulated integrin functions in sinusoids. First, we applied fMLP onto the liver surface, and compared it with systemic LPS administration. Local fMLP caused neutrophil adhesion, crawling, and emigration for at least 2 h. Surprisingly, the number of adherent and crawling neutrophils was markedly reduced in Mac-1−/− and ICAM-1−/− mice, but not in mice treated with anti-CD44 mAb. By contrast, systemic LPS injection induced a robust accumulation of neutrophils in sinusoids, which was dependent on CD44, but not on integrins. Strikingly, local fMLP could not induce any integrin-dependent adhesion in endotoxemic mice treated with anti-CD44 mAb, indicating that Mac-1-dependent neutrophil adhesion was inhibited by LPS. This response was localized to the hepatic microvasculature because neutrophils still adhered via integrins in brain microvasculature. ICAM-1/ICAM-2 levels were not decreased, but following LPS treatment, Mac-1 was down-regulated in neutrophils localized to liver, but not in the circulation. Mac-1 down-regulation in neutrophils was not observed in IL-10−/− mice. In vitro neutrophil incubation with IL-10 induced direct decrease of Mac-1 expression and adhesivity in LPS-stimulated neutrophils. Therefore, our data suggest that Mac-1 is necessary for neutrophil adhesion and crawling during local inflammatory stimuli in sinusoids, but during systemic inflammation, neutrophils are exposed to high concentrations of IL-10, leading to a CD44-dependent, integrin-independent adhesion. This may be a mechanism to keep neutrophils in sinusoids for intravascular trapping.

Liver dysfunction during systemic, uncontrolled inflammation is a common clinical finding, which has been well defined as a direct consequence of neutrophil accumulation in liver parenchyma (1). Neutrophil-mediated liver injury has been widely studied using a number of experimental models, including cecal ligation and puncture (2), ischemia-reperfusion (3), alcohol (4), endotoxin (5), and other insults (6). In these situations, neutrophils can be attracted to the liver parenchyma by a variety of inflammatory mediators, including TNF-α and IL-1, which produce increased levels of chemokines (7). The emigrated neutrophils release reactive oxygen species (8), peroxidases (9), and proteases (10), which are molecules that cause liver parenchymal damage.

In contrast to the neutrophil recruitment cascade derived from mesentery, muscle, brain, and skin in vivo and flow chambers in vitro, the liver displays a distinct neutrophil recruitment paradigm (11). It is well accepted that in tissues like brain, selectins play an important role in initial neutrophil tethering and rolling, and integrins (Mac-1 and LFA-1, mainly) are crucial to promote firm adhesion of neutrophils to the vessel wall (12). In contrast, neutrophil accumulation within liver sinusoids during systemic inflammation is independent of selectins (13) and β2 integrins (7). In fact, the participation of these adhesion molecules in neutrophil recruitment into liver has only been described for postsinusoidal venules, but not sinusoids (14). Although originally it was hypothesized that neutrophil accumulation within inflamed sinusoids was a consequence of mechanical trapping of these cells in these narrow vessels (15), blockade of adhesion molecules such as CD44 and its ligand hyaluronan (HA)3 has recently been reported to prevent both neutrophil recruitment and the progression of neutrophil-derived liver injury (5, 16). CD44 avidity for HA did not increase in endotoxemia, but rather serum-derived HA-associated protein (SHAP), a molecule known to increase HA adhesivity, was noted to bind to HA in sinusoids in response to stimuli-like endotoxin (5). Clearly, there is a dominant role for neutrophil adhesion in the liver, albeit not via integrins. This is surprising in light of data suggesting that basal recruitment of lymphocytes into liver occurred via integrins (17). This raises the following question: why do the neutrophils not use integrins to adhere in liver sinusoids?

In addition to releasing many proinflammatory acute-phase proteins, the liver has the capacity to release IL-10 and TGF-β as tolerogenic cytokines (18, 19). These regulatory cytokines can protect the liver against severe injury and failure during acute inflammatory processes, displaying an anti-inflammatory

3 Abbreviations used in this paper: HA, hyaluronan; SHAP, serum-derived HA-associated protein.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901786
response under these conditions (20, 21). The constant expression of regulatory cytokines, such as IL-10 in the liver, may account for the proposed tolerogenic role of the liver. Indeed, endotoxin and other bacterial products constantly arrive from the intestine into the liver, where they are cleared from the circulation without promoting overt inflammatory responses (22). This raises the possibility that even during endotoxemia or sepsis, the liver releases factors that continuously reduce integrin adhesivity on neutrophils.

Using spinning disk confocal microscopy, we investigated differences in adhesion following exposure of neutrophils to stimuli with different activating properties. Interestingly, direct local stimulation of neutrophils with formyl peptide agonist revealed that integrins and, more specifically, Mac-1 were essential for the early neutrophil recruitment into the liver. In sharp contrast, endotoxin induced a CD44-dependent, integrin-independent mechanism of neutrophil recruitment into the liver. Our data also demonstrate that integrin function was abolished specifically in the liver during endotoxemia via an IL-10-dependent mechanism, allowing CD44 to become the dominant adhesive mechanism.

Materials and Methods

Mice

C57BL/6 and IL-10−/− mice were purchased from The Jackson Laboratory. ICAM-1−/− mice were a gift of D. Bullard (University of Alabama, Birmingham, AL). Lysm-enhanced GFP mice, in which the enhanced GFP gene was knocked into the murine lysozyme M locus (greater than 80% are neutrophils), were provided by T. Graf (Albert Einstein College of Medicine, Bronx, NY) and generated, as previously described (23). Mac-1−/− mice (24) were provided by Dr. C. M. Ballantyne (Methodist DeBakey Heart Center and Baylor College of Medicine, Houston, TX). All mice were maintained in a specific pathogen-free, double-barrier unit at the University of Calgary. The protocols used were in accordance with the guidelines drafted by the University of Calgary Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. IL-10−/− mice were age matched (7 wk) to avoid time-related spontaneous inflammatory bowel disease complications, and other strains were used between 6 and 12 wk of age.

Blocking Abs to study involvement of adhesion molecules

mAbs (eBiosciences) against ICAM-1 (100 µg/mouse), Mac-1 (CD11b; 50 µg/mouse), LFA-1 (CD11a; 50 µg/mouse), and CD44 (20 µg/mouse) were injected i.v. 30 min before the experiments (5, 25).
Induction of local and systemic inflammatory responses

We developed a novel model of local liver inflammation using a 1-mm² filter (paper filter grade 410; VWR Scientific) gently placed onto the liver surface impregnated with the synthetic peptide (WKYMV(D-Met)-NH₂; Phoenix Pharmaceutical), which functions as a formyl peptide receptor agonist (called fMLP in this study). Liver samples were collected for histological analysis using Leder esterase stain.

LPS (Escherichia coli LPS; 0111:B4; Calbiochem) was used to induce endotoxemia (systemic inflammation; 0.5 mg/Kg; i.p.). In one set of experiments, E. coli (Xen14; Bioware; 10⁷ CFU) were injected i.p., and the liver was prepared for intravital microscopy to determine which adhesion pathway dominates.

Spinning disk confocal intravital microscopy: visualization of liver microvasculature

Murine liver intravital microscopy was performed, as previously described (5). Briefly, mice were anesthetized by i.p. injection of a mixture of 10 mg/kg xyazine hydrochloride (MTC Pharmaceuticals) and 200 mg/kg ketamine hydrochloride (Rogar/STB). The right jugular vein was cannulated to provide additional anesthetic and for i.v. administration of Abs. Body temperature was maintained at 37°C using an infrared heat lamp. Mice

FIGURE 2. fMLP induces neutrophil adhesion, polarization, and emigration. A and B, Lower magnification (×4 objective) showing filter positioning on liver surface. Neutrophil (in green) accumulation in sinusoids due to local stimulus (A, saline; B, fMLP) was mainly observed adjacent to the filter in fMLP-treated mice. fMLP-stimulated neutrophils exhibited polarized cell shape in comparison with controls (C, D, and G), with elongated axis (D, white arrow). E and F, Histological confirmation of neutrophil accumulation by esterase staining (Leder). Neutrophils (F, black arrows) were significantly increased in liver parenchyma following 2 h of fMLP local stimulus (H). *, Indicates statistically significant difference compared with controls (saline). p < 0.05.
were placed in a right lateral position on an adjustable microscope stage. A lateral abdominal incision along the costal margin to the midaxillary line was made to exteriorize the liver, and all exposed tissues were moistened with saline-soaked gauze to prevent dehydration.

The liver was prepared for in vivo microscopic observation. Briefly, the liver was placed on the pedestal of an upright microscope and continuously superfused with warmed buffer. The liver surface was then covered with a coverslip to hold the organ in position. The liver microvasculature was visualized using a spinning disk confocal head, and images were acquired with an Olympus BX51 upright microscope (Olympus) using a ×4/0.16 UplanSapo objective, ×10/0.30 UplanFL N objective, and ×200/0.45 LUcplanFL N objective, as previously described (5). The microscope was equipped with a confocal light path (WaveFx; Quorum) based on a modified Yokogawa CSU-10 head (Yokogawa Electric). Lysm-enhanced GFP mice were used to visualize neutrophils in the hepatic vasculature and parenchyma. FITC anti-GR-1 (eBiosciences; 10 μg/mouse) was injected i.v. to visualize neutrophils (in knockout mouse strains). PE-coupled anti-PECAM-1 (CD31) was used to stain liver sinusoidal endothelial cells. PE-coupled anti-ICAM-1 (2 μg/mouse) and Alexa Fluor 488-coupled anti-ICAM-2 (15 μg/mouse; Invitrogen) were used to quantify the expression of these adhesion molecules during endotoxia. Two laser excitation wavelengths (Cobalt) were used in rapid succession and visualized with the appropriate long-pass filters (Semrock). A 512 × 512 pixel back-thinned electron-multiplying charge-coupled device camera (C9100-13; Hamamatsu) was used for fluorescence detection. Velocity software (Improvision) was used to drive the confocal microscope and to render three-dimensional reconstructions. Sensitivity settings were 232–240, and auto contrast was used. Images were captured at 16 bits/channel in red, green, and blue. Red, green, and blue channels were overlaid, when necessary, using brightest point settings before export in .tiff or .avi format (for movies).

Visualization of brain microvasculature

Intravitral microscopy of the neutrophil-endothelium interactions in brain microcirculation was performed and analyzed, as previously described (26). Briefly, a craniotomy was performed using a high-speed drill (Fine Science Tools), and the dura mater was removed to expose the underlying pial microvasculature. To observe leukocyte endothelial interactions, leukocytes were fluorescently labeled by i.v. injection of PE anti-GR1 Abs (10 μg/mouse). Neutrophil-endothelium interactions in the brain microcirculation were observed using a BX51W1 spinning disk confocal microscope, and movies were recorded for further analysis following similar parameters to liver microscopy analysis.

Analysis of liver intravitral imaging videos

Cells were tracked and counted using ImageJ software version 1.41 (National Institutes of Health). Time-lapse video exported from Velocity was imported in .avi files, and then converted to 8-bit greyscale. Fluorescent cells were adjusted using threshold control, and noise particles less than 2.0 pixels were removed by despeckle filter or median filter. Movement of cells was measured using manual tracking, and was calculated for each track by manipulation of ImageJ output spreadsheet. The cell velocity was expressed in μm/min. Tracks of 15 min, covering all 2-h videos, were analyzed in each mouse. To quantify neutrophil accumulation, fluorescent cells that adhered in liver sinusoids were counted. In experiments using local stimulation, the whole field was divided in two halves (distal and adjacent to the filter; Fig. 1, E and F), and cells were counted in each half to measure directional neutrophil accumulation toward the chemotactic gradient. Neutrophils that adhered or were immobilized in the vasculature for more than 30 s were considered as adherent cells. The expression of ICAM-1 and ICAM-2 during endotoxia was assessed by measuring the integrated density of the fluorescence in liver three-dimensional reconstructions using ImageJ software.

ELISA

TNF-α, IL-6, and IL-10 were measured in liver homogenates and serum by ELISA. Liver tissue was homogenized in PBS (pH 7.4) containing protease inhibitors (Roche Diagnostics), and protein concentration was quantified using the dextran dinitrophenyl protein assay (Bio-Rad), according to the manufacturer’s protocol. Samples of liver homogenate and serum were measured in duplicate for each of the above cytokines using OptEIA ELISA kits for each (BD Biosciences), according to the manufacturer’s instructions. Liver cytokine expression was expressed in pg/mg total protein in each sample. Serum cytokines were expressed in pg/ml.

In vitro incubation of neutrophils for assessment of adhesion molecule expression and Mac-1/fibrinogen binding

To investigate the effect of IL-10 directly on LPS- and/or FMLP-stimulated neutrophils, wild-type mice were anesthetized and blood was collected by cardiac puncture with heparinized syringes (27). Blood samples were immediately placed in sterile tubes kept on ice, and LPS (100 ng/ml), FMLP (0.1 μM), or IL-10 (100 ng/ml; Cedarlane Laboratories) was added to the samples, as indicated. Samples were incubated for up to 4 h in a humidified incubator (37.5°C; 5% CO₂), and then prepared for flow cytometry analysis, as described below. Alexa Fluor 488-coupled fibrinogen (15 μg/ml; Invitrogen) was added to the samples at the end of the incubation process for measurement of Mac-1 adhesivity (28). In one set of experiments, bone marrow-derived neutrophils were purified (5) and incubated in HBSS (added to 10% of plasma) with LPS and/or IL-10 under the same conditions.

Leukocyte harvest for flow cytometry analysis

Four hours after i.p. LPS or saline injection, mice were anesthetized and the peritoneal and thoracic cavities were opened for liver excision and blood
collection by cardiac puncture. LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), CD44 (Ly-24), and GR-1 (Ly-6G) expression were measured in circulating and in liver-infiltrating neutrophils in response to LPS using flow cytometry, as previously described (29). Extraction of neutrophils from the liver was performed, as described previously (30), adapted to neutrophil purification using Percoll gradient. Samples of 100 μl of whole blood or 1 million liver-derived leukocytes were incubated with 1 μg of FITC-conjugated mAb anti-GR-1, 3 μg of PE-conjugated mAb against LFA-1 or CD44, and 3 μg of PE-Cy5-conjugated against Mac-1 mAb or nonspecific isotype controls (all purchased from BD Biosciences) for 30 min at room temperature. Blood samples were added to 100 μl of Optilyse B (Immunotech) to lyse RBC. Cells were washed and resuspended in a PBS/0.5% BSA/20 mM glucose solution, read by a BD FACScan flow cytometer (BD Biosciences) using CellQuest Pro software (BD Biosciences), and analyzed using FlowJo software (Tree Star). In all blood and liver neutrophil studies, flow cytometry was performed with parameters setting to gate only granulocytes. Sequentially, 40,000 events were analyzed for each sample and gated using side/forward scattergram to select...
only polymorphonuclear cells and FLH-1 histogram to select only GR-1high-expressing cells (predominantly neutrophils). In a set of experiments, intracellular levels of integrins were also assessed by flow cytometry. The cells were incubated with saturating concentrations of nonfluorescent (blocking) Abs for 30 min (10 times the regular dose; 30 μg of anti-LFA-1 and anti-MAC-1) to block the majority (99%) of surface Ags. These cells were then fixed and permeabilized (Cytofix/Cytoperm; BD Biosciences) to stain intracellular integrins with the fluorescent Abs. Mean of fluorescence intensity of these populations was obtained and compared with control and experimental groups.

Statistical analysis
All data are displayed as mean ± SEM or percentage of control. Data were analyzed using standard statistical analysis (ANOVA and Student’s t test).

FIGURE 5. A, Integrin-independent, CD44-dependent E. coli induced neutrophil accumulation in liver sinusoids. E. coli (10⁷ CFU) induces neutrophil (green) accumulation 4 h after i.p. administration. This is blocked with anti-CD44 mAb (B and C), but not with anti-Mac-1 mAb (C). *, Indicates statistically significant difference compared with saline (UT; E. coli injection alone), and †, in comparison with vehicle-injected mice (UT). p < 0.05.

FIGURE 6. LPS decreases Mac-1-dependent neutrophil adhesion in liver. A, Neutrophil adhesion following 2 h of local fMLP or 4 h of i.p. LPS. Treatments were indicated by the + sign. *, Indicates statistically significant difference compared with controls (saline injection), and †, in comparison with LPS injection alone. B and C, Flow cytometry analysis of blood and liver neutrophils showing selective down-regulation of Mac-1 in neutrophils that home to the liver. Intracellular staining (D) for Mac-1 and LFA-1 in liver neutrophils revealed that these molecules are not internalized during systemic (LPS) inflammation. *, Indicates statistically significant difference compared with controls (saline injection). p < 0.05.
with Bonferroni’s correction for multiple comparison, where appropriate). Statistical significance was set at $p < 0.05$.

**Results**

*Local fMLP stimulus induces Mac-1/ICAM-1-dependent, CD44-independent neutrophil adhesion and crawling in liver sinusoids*

To study the features related to local liver inflammation, an fMLP impregnated filter was placed on the liver surface, and neutrophil accumulation was visualized using spinning disk confocal microscopy. To better visualize neutrophil accumulation, we used three-dimensional computer reconstruction of liver Z-stacks (total depth of 80 μm) from liver microvasculature. Local administration of fMLP (2 μg/filter) induced significant neutrophil adhesion and emigration to liver parenchyma in comparison with controls (Fig. 1, A–D; supplemental video 1). Neutrophil adhesion was observed within 30 min of local exposure to fMLP with continued cell recruitment up to 2 h (Fig. 1C). The greatest quantity of cells was present immediately adjacent to the filter (Figs. 1, D–F, and 2, A and B), and close to 100% of adherent neutrophils exhibited crawling movement and polarized cell shape toward fMLP, as assessed by the longest cell axis (Fig. 2, C, D, and G). Many neutrophils were seen to emigrate out of the sinusoids. The presence of neutrophils in liver parenchyma was confirmed by histological staining for neutrophil esterase (Fig. 2, E, F, and H). Using time lapse videos, we observed that neutrophils were able to adhere and to crawl inside sinusoids toward the fMLP gradient (supplemental video 2), a behavior not observed with saline-soaked filters (supplemental video 3). Observation of these cells for longer times (up to 4 h) did not reveal any changes in adhesion and crawling profile, indicating that 2 h of observation was adequate for subsequent studies (data not shown). Some increase in neutrophil adhesion could be noted even at 200 μm away from fMLP (labeled as distal in Fig. 1F). The majority of these cells migrated toward the chemottractant (supplemental video 2). Macroscopically, no visual lesions were observed at the end of the experiment on the liver surface after filter placement, and sinusoidal morphology and perfusion were not visually altered.

The number of adherent neutrophils was significantly decreased in ICAM-1−/− and Mac-1−/− mice, but not in anti-CD44 or anti-LFA-1 mAb-treated mice (Fig. 3A). These data showing significant inhibition were confirmed using blocking Abs against ICAM-1 and Mac-1 in wild-type mice (data not shown). The percentage of adherent cells that crawled was close to 100% and was not affected by either anti-LFA-1 or anti-CD44 mAb, but was abolished by ICAM-1- or Mac-1 deficiency (Fig. 3B). The few cells that still adhered and/or crawled in Mac-1−/− or ICAM-1−/− mice did so at extremely low velocity (Fig. 3C). Although anti-LFA-1 did not alter the percentage of crawling cells, there was a partial reduction in crawling velocity, indicating a contribution of LFA-1 to this parameter (Fig. 3C). Treatment with anti-CD44 mAb did not alter the percentage of crawling neutrophils nor velocity of neutrophil crawling, suggesting that this adhesion molecule has no detectable role in this model of chemotactic-induced neutrophil recruitment (Fig. 3, B and C).

**Systemic LPS injection induced CD44-dependent neutrophil accumulation in liver**

Previous studies have reported no role for β2 integrins in neutrophil adhesion in liver sinusoids following systemic LPS administration (14). LPS induced a marked increase in neutrophil adhesion in the sinusoids (Fig. 4A) compared with saline-injected mice (Fig. 4B). Several blanched areas were macroscopically observed on the liver surface (sign of malperfusion), and histological analysis confirmed significant accumulation of neutrophils in liver vessels (data not shown). The adhesion in this model was entirely independent of Mac-1, LFA-1, or ICAM-1 (Fig. 4C) and CD18 (data not shown). In striking opposition to fMLP, the neutrophil adhesion induced by LPS was blocked 70% with the anti-CD44 mAb. More than 90% of adhered neutrophils were static, and did not crawl or crawled for a very short distance in response to LPS (Fig. 4D) with erratic crawling movements (supplemental video 4). The majority of neutrophils remained within the vasculature and did not emigrate.
To evaluate the role of integrins in neutrophil adhesion during endotoxemia in a nonhepatic tissue, the brain microvasculature was observed using spinning disk confocal microscopy under the same conditions as those described for the liver. We observed that systemic LPS injection induced a significant increase in adherent neutrophils in brain venules in comparison with controls (Fig. 4, E–G). Treatment with anti-LFA-1 mAb reduced the number of adherent neutrophils, but no statistically significant difference was observed in Mac-1−/− mice. However, the treatment of endotoxic Mac-1−/− mice with anti-LFA-1 mAb further decreased the number of adherent neutrophils, suggesting that both integrins mediated neutrophil adhesion in brain during systemic LPS inflammation.

To compare LPS and fMLP responses to a bacterial infection, we inoculated mice i.p. with *E. coli* (10⁷ CFU; 4 h). It could be argued that in this model both LPS and fMLP pathway might be activated. Surprisingly, *E. coli* infection caused marked neutrophil adhesion in liver sinusoids (Fig. 5A) that was inhibited with anti-CD44 mAb, but not with anti-Mac-1 mAb (Fig. 5, B and C). This suggests that *E. coli* infection causes a profile of hepatic neutrophil adhesion more similar to that seen with LPS than with fMLP treatment, or that the former pathway deactivated the latter.

**Up-regulation of Mac-1 expression on circulating neutrophils induced by systemic LPS injection is not maintained by neutrophils that home to the liver**

To test our hypothesis that LPS dampens Mac-1-mediated adhesion in liver, we tracked the expression of Mac-1, LFA-1, and CD44 on both circulating and liver-harvested neutrophils after LPS treatment. Systemic LPS induced a significant increase in Mac-1 expression, but not in LFA-1 nor CD44, on circulating neutrophils (Fig. 6B). In sharp contrast, neutrophils harvested from the liver of LPS-injected mice had reduced Mac-1 compared with neutrophils from untreated mice (Fig. 6, C and D); however, no significant changes were observed in LFA-1 nor in CD44 expression (Fig. 6, B and C).

Next, we investigated whether the decreased expression of Mac-1 in liver neutrophils after LPS administration was due to internalization of this integrin or an inability to mobilize Mac-1 to the membrane surface. Interestingly, local fMLP (2 h) did not induce any further adhesion (Fig. 6A) and could not induce crawling of neutrophils when LPS was present (data not shown). Anti-CD44 mAb inhibited 70% of neutrophil adhesion during endotoxemia in liver sinusoids (Fig. 6A). Adding the fMLP-laden filter under these conditions did not induce any additional neutrophil adhesion (Fig. 6A) nor crawling (data not shown), indicating that a Mac-1-dependent mechanism could not be induced in the liver in the presence of a systemic LPS injection.

**Up-regulation of Mac-1 expression on circulating neutrophils induced by systemic LPS injection is not maintained by neutrophils that home to the liver**

![FIGURE 8. IL-10 as a candidate for Mac-1 down-regulation in liver neutrophils. Dosage of IL-10, TNF-α, and IL-6 levels in liver tissue (A) and blood (B). Disproportionate amounts of IL-10 are expressed in liver tissue, in contrast to the lower levels in blood. C, Flow cytometry analysis of Mac-1 expression on neutrophils following LPS incubation (100 ng/ml) in the presence and absence of IL-10 (10 ng/ml). *, Indicates statistically significant difference compared with incubation with LPS alone. p < 0.05. D, Flow cytometry analysis of Mac-1 adhesivity. Blood samples were incubated with LPS (100 ng/ml), fMLP (0.1 µM), or IL-10 (100 ng/ml) for 4 h. Alexa Fluor 488-coupled fibrinogen was added to the samples at the end of the incubation process for measurement of Mac-1 adhesivity. †, Indicates statistically significant difference compared with incubation with saline, and ‡, compared with fMLP plus LPS incubation. p < 0.05.](http://www.jimmunol.org/)

**Systemic LPS-induced liver inflammation modifies local hepatic inflammatory response**

To determine whether β₂ integrins could be activated in endotoxemic liver, we examined whether β₂ integrin-dependent adhesion with fMLP could still occur in endotoxemic mice. Interestingly, local fMLP (2 h) did not induce any further adhesion (Fig. 6A) and could not induce crawling of neutrophils when LPS was present (data not shown). Anti-CD44 mAb inhibited 70% of neutrophil adhesion during endotoxemia in liver sinusoids (Fig. 6A). Adding the fMLP-laden filter under these conditions did not induce any additional neutrophil adhesion (Fig. 6A) nor crawling (data not shown), indicating that a Mac-1-dependent mechanism could not be induced in the liver in the presence of a systemic LPS injection.
For completeness, we assessed by confocal intravital microscopy whether the endothelial ligands for Mac-1 (ICAM-1 and ICAM-2) were down-regulated during endotoxemia. Interestingly, we found no differences in the expression of both adhesion molecules during endotoxemia (Fig. 7).

IL-10 as a candidate for Mac-1 suppression in liver neutrophils

Our data suggested that the low levels of Mac-1 expression are restricted to liver neutrophils, because circulating neutrophils expressed elevated levels of Mac-1 after LPS stimulation. These findings led us to hypothesize that a local mediator produced in high amounts selectively in the liver may be affecting adherent and emigrated neutrophils within the liver microenvironment, thereby maintaining low Mac-1 expression in these cells. Data using Luminex array revealed that serum levels of many proinflammatory cytokines and chemokines (such as IL-1α, IFN-γ, TNF-α, MIP-1α, RANTES, KC, and IL-6) are up-regulated during endotoxemia (data not shown). In addition, we noted IL-10 as the one anti-inflammatory cytokine significantly up-regulated during this process (Fig. 8). To further assess the production of cytokines in liver during LPS challenge, we collected liver samples. Interestingly, we found that disproportionately high amounts of IL-10 (~650 pg/mg tissue) were constitutively expressed in liver when compared with IL-6 and TNF-α (~25 pg/mg; Fig. 8A), the two highest proinflammatory cytokines. Within the liver, high levels of IL-10 were observed over the 4 h of LPS injection. In striking contrast, serum levels of IL-10 were much lower than IL-6 over the 4 h of LPS injection (Fig. 8B). Clearly, whereas disproportionately high levels of IL-10 were seen in the liver tissue, serum IL-10 levels increased notably, but less than IL-6 and TNF-α in blood (Fig. 8B).

Because IL-10 is described as an anti-inflammatory cytokine, its elevated expression in liver tissue led us to investigate the effect of this cytokine on Mac-1 expression by neutrophils. Blood samples from wild-type mice were collected and incubated in vitro with LPS in the presence or absence of IL-10. Incubation of blood cells with LPS (100 ng/ml) induced a significant increase in Mac-1 expression on neutrophils by 1 h, which was further increased at 4 h after incubation. Strikingly, IL-10 directly inhibited LPS-induced Mac-1 up-regulation by neutrophils (Fig. 8C). Interestingly, IL-10 in the absence of LPS caused a decrease in Mac-1 expression. As a negative control, incubation of blood cells with sterile PBS for 4 h did not alter Mac-1 expression levels (data not shown).

In an additional set of experiments, we examined the adhesivity of Mac-1 by measuring fibrinogen binding to neutrophils, a specific Mac-1 ligand. IL-10 was able to block the increased adhesivity of Mac-1 induced by LPS and fMLP (Fig. 8D).

We also investigated the effect of IL-10 on purified bone marrow-derived neutrophils. We confirmed that IL-10 was also able to significantly reduce LPS-induced Mac-1 up-regulation in bone marrow-derived neutrophils. However, the magnitude of the reduction was much smaller (20%). Moreover, the down-regulation of constitutively expressed Mac-1 by IL-10 was not observed (data not shown).

Low Mac-1 expression in liver neutrophils during LPS challenge is not observed in the absence of IL-10

To investigate the role of IL-10 in the regulation of Mac-1 expression on neutrophils in vivo, we harvested neutrophils from blood and liver of IL-10−/− mice treated with LPS. We observed that after systemic LPS inflammation, blood neutrophils from IL-10−/− mice displayed a similar phenotype to neutrophils from wild-type mice, in that Mac-1 expression was up-regulated (Fig. 9A). However, Mac-1 levels in liver neutrophils from IL-10−/− mice were not low after LPS administration, in contrast to liver neutrophils from wild-type mice (Fig. 9B). CD44 levels were not significantly altered by the presence or absence of IL-10 (Fig. 9, A and B).

Because Mac-1 levels did not decrease in neutrophils harvested from livers in endotoxemic IL-10−/− mice, we examined whether the presence of Mac-1 had a physiologic function and now contributed to neutrophil adhesion in liver sinusoids during endotoxemia. Interestingly, anti-Mac-1 Ab blocked LPS-induced neutrophil adhesion in IL-10−/− mice by more than 60%, indicating that the retained expression of Mac-1 in neutrophils in IL-10−/− livers in response to LPS injection can now contribute to neutrophil adhesion within sinusoids (Fig. 9C). Although some adhesion via CD44 was retained, the 70% inhibition seen in wild-type mice using anti-CD44 mAb (Fig. 6A) was of much smaller magnitude (Fig. 9C) in IL-10−/− mice. Anti-Mac-1 mAb could further reduce neutrophil adhesion in anti-CD44 mAb-treated IL-10−/−.
mice (data not shown). Addition of anti-Mac-1 and anti-CD44 Abs to wild-type mice did not reduce adhesion any more than anti-CD44 Ab treatment alone (data not shown).

**Discussion**

Neutrophil accumulation in liver may be paradoxical in that these cells play a vital role in promoting effective bacterial clearance (31), but neutrophil infiltration in the liver can also result in serious and progressive parenchymal damage, leading to liver failure and severe clinical outcomes. Acute and chronic diseases of the liver are not uncommon, and in worst case scenarios may even require liver transplantation as a life-saving procedure (32). In this study, it was our goal to investigate the mechanisms involved in hepatic neutrophil accumulation during endotoxemia, compared with a local stimulus that directly activated neutrophils. Unexpectedly, the mechanisms were drastically different, in that an acute stimulus like fMLP that directly activates neutrophils made use of standard neutrophil-adhesive mechanisms, including Mac-1, an adhesion molecule that played no role following LPS administration. In fact, when fMLP was superimposed onto LPS-induced neutrophil recruitment, Mac-1-mediated neutrophil adhesion could no longer be observed. The underlying mechanism involved a novel inhibitory role for IL-10 down-regulating Mac-1 expression on the neutrophil surface, while permitting CD44 to retain neutrophils in the liver sinusoids, where they may enhance bacterial trapping.

To date, no role for integrins as contributors to neutrophil recruitment into liver sinusoids has been described. In particular, no integrins are involved in endotoxin or sepsis-related neutrophil accumulation into sinusoids (14). In this study, for the first time, we have demonstrated in vivo that adherent neutrophils can adhere and crawl directionally toward a chemotactic molecule within sinusoids using the integrin Mac-1. Up-regulation of Mac-1 expression by fMLP-stimulated neutrophils has been described in vitro (33), and Mac-1-mediated neutrophil crawling is an important step during efficient leukocyte extravasation in vivo in nonhepatic tissues (25, 34) and in vitro across endothelial monolayers (35). The participation of integrins in neutrophil adhesion in liver was described, but only in postsinusoidal venules, and not the more abundant sinusoids where CD44 is dominant (6). In the fMLP-induced recruitment, the neutrophils not only crawled via Mac-1, but avidly emigrated. By contrast, in LPS-induced inflammation, neutrophils simply adhered and did not crawl, and only a few emigrated out of the blood vessels. No role for Mac-1 was noted in this systemic inflammation. It is reasonable to speculate that when LPS is detected in the vasculature, the system attempts to retain neutrophils in the vasculature, where they may trap bacteria perhaps using the recently described neutrophil extracellular traps (31, 36). Therefore, we propose a paradigm in which the CD44/HA pathway is induced to allow for retention of neutrophils by adhering in sinusoids, whereas down-modulation of Mac-1 may serve to retain neutrophils in the sinusoids, preventing them from emigrating out of the vasculature.

Recent reports have highlighted CD44-HA interaction as a dominant mechanism for neutrophil adhesion in sinusoids during endotoxemia (5) and ischemia-reperfusion (37). Interestingly,
anti-CD44 Ab-treated mice, and also CD44−/− mice (data not shown), displayed normal adhesive responses due to fMLP stimulus as a result of Mac-1 engagement. By contrast, LPS-induced systemic inflammation required CD44/HA interactions to sequester neutrophils in liver sinusoids (5). We observed no change in CD44 levels with LPS, entirely consistent with previous work (5). The prevailing view is that the CD44 ligand HA is modified by SHAP to change its quaternary conformation to enhance binding to CD44 (5). Indeed, we previously reported increase in levels of SHAP binding in sinusoids post-LPS treatment. Moreover, it has been shown that SHAP binding to HA greatly increases cellular adhesivity in vitro using flow chambers (38). Although the CD44/SHAP/HA pathway is induced with LPS, a simultaneous loss of Mac-1 adhesivity is observed through an IL-10-dependent mechanism.

IL-10 is known to play an important role as an anti-inflammatory molecule, by down-regulating NF-kB-mediated inflammatory processes and reducing production of cytokines, thereby inhibiting de novo synthesis of adhesion molecules (39). In our study, we report that IL-10 can down-modulate Mac-1 expression presumably via a protein synthesis-independent mechanism, most likely involving Mac-1 shedding, an inactivating process previously reported for other adhesion molecules (40). A recent report has shown that during endotoxemia, monocyte subpopulations can undergo apoptosis and, concomitantly, these cells down-regulate CD18 expression (41). We provide evidence that IL-10 is highly expressed in liver, and in the absence of this cytokine in IL-10−/− mice, Mac-1 expression in neutrophils is not low. Kupffer cells located in the vasculature release large amounts of IL-10 in response to LPS (22, 42), making them a likely candidate for this function. In fact, in the absence of IL-10, it was most interesting to see that LPS administration led to Mac-1-dependent neutrophil adhesion in sinusoids. Interestingly, IL-10 levels were high constitutively in liver, but fMLP was able to induce Mac-1-dependent adhesion. This may reflect a lack of release of IL-10 from cellular stores unless LPS is added.

The IL-10 effects were restricted to the liver as β2 integrin function was retained in the brain during endotoxemia. In contrast to the 10- to 20-fold higher levels of IL-10 relative to molecules like IL-6 and TNF-α in liver tissue, we found that serum levels of IL-10 were notably less (5-fold) than IL-6 and TNF-α levels. This 50- to 100-fold difference in anti-inflammatory vs proinflammatory levels in circulation can explain the absence of Mac-1 down-modulation in circulating neutrophils. Thus, we propose that the disproportionate expression of IL-10 in the liver overcomes the proinflammatory effects of circulating cytokines, driving a regulatory environment in the hepatic sinusoids. Whether there is a protein that retains IL-10 on the sinusoidal surface, thereby increasing local levels of IL-10 in the liver, is not known. Using a teleological argument, it may be that down-regulation of Mac-1 in liver during systemic infection would allow neutrophils to stop inside sinusoids, increasing the probability of bacterial encounter to execute intravascular bactericidal activity. Indeed, there is a growing body of evidence that the liver sinusoids are an important site of the immune system to prevent bacterial dissemination (43).

In Fig. 10, we summarize our major findings and propose a mechanism to explain the dynamics of neutrophil adhesion molecules in liver during local and systemic inflammation. Collectively, our data suggest that Mac-1 is necessary for neutrophil adhesion and crawling during local inflammatory stimuli in liver. During LPS-induced systemic inflammation, HA becomes adhesive for CD44, thereby mediating neutrophil adhesion in liver. Simultaneously, neutrophils become exposed to high concentrations of IL-10 in liver, which down-regulates Mac-1 in these cells, thereby reducing their capacity to crawl and emigrate, but perhaps increasing the intravascular filtering capacity of the liver for pathogens.

Acknowledgments
We thank Lori Zbytnuik for her assistance in experiments.

Disclosures
The authors have no financial conflict of interest.

References


