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Mobilization and Margination of Bone Marrow Gr-1\textsuperscript{high} Monocytes during Subclinical Endotoxemia Predisposes the Lungs toward Acute Injury\textsuperscript{1}

Kieran P. O’Dea,* Michael R. Wilson,* Justina O. Dokpesi,† Kenji Wakabayashi,* Louise Tatton,* Nico van Rooijen,‡ and Masao Takata\textsuperscript{2*}

The specialized role of mouse Gr-1\textsuperscript{high} monocytes in local inflammatory reactions has been well documented, but the trafficking and responsiveness of this subset during systemic inflammation and their contribution to sepsis-related organ injury has not been investigated. Using flow cytometry, we studied monocyte subset margination to the pulmonary microcirculation during subclinical endotoxemia in mice and investigated whether marginated monocytes contribute to lung injury in response to further septic stimuli. Subclinical low-dose i.v. LPS induced a rapid (within 2 h), large-scale mobilization of bone marrow Gr-1\textsuperscript{high} monocytes and their prolonged margination to the lungs. With secondary LPS challenge, membrane TNF expression on these premarginated monocytes substantially increased, indicating their functional priming in vivo. Zymosan challenge produced small increases in pulmonary vascular permeability, which were markedly enhanced by the preadministration of low-dose LPS. The LPS-zymosan-induced permeability increases were effectively abrogated by pretreatment (30 min before zymosan challenge) with the platelet-activating factor antagonist WEB 2086 in combination with the phosphatidylcholine-phospholipase C inhibitor D609, suggesting the involvement of platelet-activating factor/ceramide-mediated pathways in this model. Depletion of monocytes (at 18 h after clodronate-liposome treatment) significantly attenuated the LPS-zymosan-induced permeability increase. However, restoration of normal LPS-induced Gr-1\textsuperscript{high} monocyte margination to the lungs (at 48 h after clodronate-liposome treatment) resulted in the loss of this protective effect. These results demonstrate that mobilization and margination of Gr-1\textsuperscript{high} monocytes during subclinical endotoxemia primes the lungs toward further septic stimuli and suggest a central role for this monocyte subset in the development of sepsis-related acute lung injury. The Journal of Immunology, 2009, 182: 1155–1166.

\textsuperscript{1}Abbreviations used in this paper: ALI, acute lung injury; PIM, pulmonary intravascular macrophage; memTNF, membrane-bound TNF; PAF, platelet-activating factor; PC-PLC, phosphatidylcholine-phospholipase.

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\textsuperscript{3}Abbreviations used in this paper: ALI, acute lung injury; PIM, pulmonary intravascular macrophage; memTNF, membrane-bound TNF; PAF, platelet-activating factor; PC-PLC, phosphatidylcholine-phospholipase.
recruitment and participation in local inflammatory responses, little is known of the kinetic behavior of these monocyte subsets among the bone marrow, circulating, and marginated pools during acute systemic inflammation such as sepsis. ALI is a frequent complication of sepsis, but the margination profiles of the monocyte subsets to pulmonary vascular beds and their potential roles in the progression of ALI have not been investigated.

Certain species, including livestock animals, possess a resident lung macrophage population attached to the pulmonary endothelium known as pulmonary intravascular macrophages (PIM) (28, 29). The localized and vigorous production of vasoactive mediators and proinflammatory cytokines by PIM seems to be directly responsible for the unique susceptibility of sheep and pigs to experimental ALI of extrapulmonary origin induced by i.v. administration of LPS, particulate Ags, or bacteria (30–32). In species lacking PIM, which include humans and laboratory animals such as mice and rats, it has been suggested that monocyte accumulation within the lungs under sustained systemic inflammatory conditions could eventually give rise to a population with a PIM-like phenotype (10, 33, 34).

In this study, we hypothesized that monocytes marginated within the pulmonary microcirculation during subclinical endotoxemia, in particular those monocytes of the Gr-1<sup>hi</sup> inflammatory subset are responsive to secondary septic stimuli in situ and therefore contribute to the development of extrapulmonary ALI. We show that during subclinical endotoxemia, the rapid and substantial mobilization of Gr-1<sup>hi</sup> bone marrow monocytes and their prolonged margination within the pulmonary vasculature produce a state of latent “priming” in the lungs. By assessing the proinflammatory response of individual monocyte subsets to secondary LPS challenge and using a two-hit LPS-zymosan model of ALI, we provide direct in vivo evidence that suggests a central role for inflammatory monocytes in the evolution of ALI.

Materials and Methods

**Animals**

All protocols were reviewed and approved by the U.K. Home Office in accordance with the Animals (Scientific Procedures) Act 1986, U.K. Experiments were performed using male C57BL/6 mice (Charles River) ages 8–12 wk (22–26 g).

**Induction of endotoxemia**

Mice received a single i.v. (via tail vein) injection of 0.2 ng to 20 μg of LPS (Ultra Pure *Escherichia coli* O111:B4; Autogen Bioclear). At various times after challenge, mice were heparinized i.v. and sacrificed by isoflurane overdose. Blood was obtained by cardiac puncture and lungs were excised with care taken to avoid the hilum connective tissues. In some experiments, single femur and tibia were removed for analysis of bone marrow cells.

**Preparation of single-cell suspensions**

Lung cell suspensions were prepared from the excised lungs by mechanical disruption to release intravascular cells, as described previously (11). For quantitation and characterization of lung monocytes and neutrophils, finely minced lung tissue was homogenized on a 40-μm nylon mesh sieve (BD Falcon) with a syringe plunger and flushed through sieves with FACS medium (PBS with 2% FCS, 0.1% sodium azide, and 5 mM EDTA). Homogenization and flushing of cells was then repeated to ensure maximal recovery of intravascular cells. For determination of in situ membrane-bound TNF (memTNF) levels on lung monocytes, minced lung tissues were resuspended with 10 μM BB94 (British Biotech), a hydroxamate-based TNF-α-converting enzyme inhibitor, before tissue homogenization and all subsequent procedures were performed at 4°C or on ice to minimize changes in memTNF levels before flow cytometry analysis (11). Lavage of the lungs for recovery of intra-alveolar cells was performed using 750 μl of saline, as described previously (35). Bone marrow cells were obtained by insertion of a 23-gauge needle into dissected femurs and tibiae and flushing with 2 ml of FACS medium. Cells were then dispersed by pipetting and passed through a 40-μm nylon mesh sieve. After centrifugation, lung and bone marrow cells were resuspended in FACS medium supplemented with 20% goat serum (Invitrogen).

**Flow cytometric analysis**

Cells were stained in the dark at 4°C for 30 min with fluorophore-conjugated anti-mouse Abs for CD11b (clone M170), Gr-1 (RB6-8C5), Ly-6G (1A8), Ly-6C (AL-21), NK-1.1 (PK136), TNF (MP6-XT22) (BD Pharmingen), F4/80 (CL3-A3-1), CD115 (604B5 2E11), 7/4 (ASD Serotec), or appropriate isotype-matched control Abs. In experiments involving blood or bone marrow, RBC lysis and fixation were performed using FACS lysis solution (BD Pharmingen). Samples were acquired (a minimum of 1000 gated monocytes per sample) using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star). Absolute cell counts in samples were determined using microsphere beads (Caliga Medsystems) as previously described (11, 36).

**Immunohistochemistry**

Mice were heparinized i.v., exsanguinated via the brachial artery, and the lungs instilled with 750 μl of 4% paraformaldehyde. Lungs were then excised and fixed for 18 h before paraffin embedding and preparation of 5-μm-thick sections. Before Ab incubation, Ag retrieval was performed by treatment with proteinase K (Sigma-Aldrich) at 20 μg/ml in Tris-EDTA for 10 min at room temperature. Lungs were then incubated with the Gr-1 Ab or isotype-matched control (2 μg/ml, 30 min at room temperature) followed by detection with the Vectastain ABC rat IgG peroxidase kit (Vector Laboratories) according to the manufacturer’s instructions. After 3,3′–diaminobenzidine substrate (Vector Laboratories) development, sections were counterstained with methyl green.

** Localization of monocytes within lungs**

Localization of monocyte subsets within the lungs was determined by ex vivo perfusion of the pulmonary vessels and in vivo intravascular cell labeling. For washout and quantification of intravascular monocytes, we used a mouse-isolated perfused lung system (IL-1; Hugo-Sachs Elektronik) as previously described, (37, 38). Briefly, at 2 h after LPS challenge, mice were anesthetized, tracheostomized, and ventilated with air using a mouse ventilator (35). After midline thoracotomy and laparotomy, heparin (100 IU) was administered via the inferior vena cava, which was then cut for exsanguination. The pulmonary artery and left atrium were cannulated, and the lungs were perfused at a constant flow of 50 ml/kg and left atrial pressure of 2.5 mm Hg and ventilated with 21% O<sub>2</sub> and 5% CO<sub>2</sub> in N<sub>2</sub>. The perfusate used was RPMI 1640 without phenol red (Invitrogen) with pH adjusted to 7.35–7.45, supplemented with 4% BSA (Sigma-Aldrich) and 5 mM EDTA to facilitate release of marginated monocytes (39). After perfusion for 40 min at 37°C, the lungs were harvested and single-cell suspensions were prepared for analysis by flow cytometry. For in vivo staining of lung-marginated monocytes, we used a modified version of a labeling technique of intravascular cells previously reported in the literature (40). At 2 h after LPS challenge, mice received an i.v. injection of 2 μg of anti-CD45 PE-conjugated Ab (clone 30-F11; BD Pharmingen) just before they were sacrificed. A 10-min pulmonary arterial perfusion using PBS was performed to wash out residual Ab within the pulmonary vasculature, before flow analysis of lung single-cell suspensions by flow cytometry.

BrdU labeling of dividing monocytes in vivo

Mice received a single i.p. injection of 0.2 μl of BrdU (10 mg/ml in saline) and, after 6 h, were injected i.v. with saline or LPS. Margination of recently divided bone marrow cells to the lungs was determined by flow cytometry using a BrdU Flow Kit according to the manufacturer’s instructions (BD Pharmingen). Single-cell suspensions from lungs were stained with appropriate marker Abs to identify monocyte subsets and then washed, fixed, permeabilized, and treated with DNAse to reveal BrdU epitopes. BrdU incorporation was determined by staining with a FITC anti-BrdU mAb.

**Depletion of monocytes in vivo**

Clodronate (dichloromethylene bisphosphonate) was a gift from Roche Diagnostics and was incorporated into liposomes as previously described (41). Intravascular monocytes and macrophages were depleted by i.v. injection of 0.2 ml of clodronate as described previously (22, 23). Mice were used for LPS or LPS-zymosan challenge experiments at 18 or 48 h after clodronate-liposome treatment, before significant repopulation of splenic macrophages (43) and liver Kupffer cells (44).
**Zymosan challenge and measurement of pulmonary vascular permeability**

Zymosan-induced pulmonary vascular permeability changes were assessed using an adaptation of a previously reported fluorescence-based method for measuring vascular leak into the alveolar space (45, 46). Zymosan particles (Invitrogen) were suspended in saline and mixed with Alexa Fluor 594-conjugated BSA (0.1 mg/mouse; Invitrogen) and immediately injected i.v. in a total volume of 0.2 ml. To exclude the influence of intravascular BSA in the residual blood left in the lung samples, mice were injected i.v. with 0.2 ml of Alexa Fluor 488-conjugated BSA (0.2 mg/mouse; Invitrogen) along with heparin (100 U/ml) 5 min before sacrifice. Lungs were excised, rinsed briefly in saline, and swabbed gently with tissue to remove surplus blood. Lungs were then homogenized (Kinematica Polytron PTA 7; Philip Harris Scientific) and incubated with collagenase type IV (Sigma-Aldrich) for 30 min at 37°C to ensure release of tissue-sequestered BSA. After further homogenization, lung suspensions were centrifuged and supernatants were collected. Fluorescence levels were measured in lung homogenate supernatants and diluted whole blood samples (1/100) using a fluorescence plate reader (Flx-800; Bio-Tek Instruments). An index of vascular permeability was calculated by subtracting the lung:blood ratio of BSA-Alexa Fluor 488 nm (intravascular content) from the lung:blood ratio of BSA-Alexa Fluor 594 nm (total lung content).

**Pharmacological inhibition of LPS-zymosan-induced ALI**

Thirty minutes before zymosan challenge in the above LPS-zymosan model, mice were injected i.v. with a platelet-activating factor (PAF) receptor antagonist WEB 2086 (5 mg/kg in 1.25% DMSO in PBS) alone, or in combination with a nonselective cyclooxygenase inhibitor indomethacin (10 mg/kg in DMSO/PBS) or a phosphorylcholine-phospholipase C (PC-PLC) inhibitor D609 (40 mg/kg in PBS). DMSO in PBS solution served as a vehicle control.

**Statistical analysis**

Data are expressed as mean ± SD. Statistical comparisons were made by *t* tests or ANOVA with Bonferroni tests. Statistical significance was defined as *p* < 0.05.

**Results**

**Subclinical endotoxemia produces prolonged margination of Gr-1high inflammatory monocytes to the lungs**

Monocyte recruitment to the pulmonary circulation in mice during endotoxemia was evaluated by flow cytometric analysis of lung single-cell suspensions prepared from the excised lungs of mice as described previously (11). Monocytes were identified as CD11b<sup>F4/80<sup> events, and their subsets were defined as either Gr-1<sup>very high<sup> (R1) or Gr-1<sup>high<sup> (R2), differentiated from neutrophils as CD11b<sup>F4/80<sup>Gr-1<sup>very high<sup> events (R3). F4/80<sup> events did not express high levels of Ly-6G (R4), thereby ruling out contamination of R1 and R2 gates with neutrophils. Percentage frequencies among total CD11b<sup> events are depicted for each gate.

B. Staining with 7/4 or anti-NK-1.1 Abs (red line) compared with isotype-matched control Ab (blue fill) indicated that gate R2 did not contain eosinophils (7/4 low) or NK cells.
contrast to the increases seen in the lungs, circulating Gr-1\textsuperscript{high} monocyte numbers were not markedly altered compared with saline-treated mice, except with the lowest (0.2 ng) and highest (20 \mu g) LPS doses, where there were tendencies toward monocytopenia. Because the LPS dose of 20 ng produced maximal monocyte recruitment while being substantially lower than the clinical threshold dose of 2 \mu g, it was chosen for all subsequent subclinical LPS challenge experiments.

To determine whether the LPS-induced elevation of lung monocyte numbers is maintained for prolonged periods and therefore likely to have an influence on pulmonary physiology, time course experiments were performed (Fig. 3; \( n = 3–4 \) each time point). At 1 h after i.v. LPS challenge, numbers of lung-associated Gr-1\textsuperscript{high} monocytes were similar to baseline, suggesting that unlike the acute LPS challenge protocol with high bolus doses (20 – 200 \mu g) used in our previous study (11), subclinical LPS challenge did not induce an immediate relocation of circulating monocytes to the lungs. Numbers of lung-associated Gr-1\textsuperscript{high} monocytes then increased and were maximal at 2 h, but the decline thereafter was gradual, i.e., Gr-1\textsuperscript{high} monocytes remained elevated up to 8 h (\( p < 0.01 \)), with an 8.5-fold higher than baseline, albeit nonsignificant increase even at 18 h after LPS challenge. This gradual decrease in the lung Gr-1\textsuperscript{high} monocytes was accompanied by an increase in circulating Gr-1\textsuperscript{low} monocytes (\( p < 0.05 \)) and tendencies toward increases in the lung and circulating Gr-1\textsuperscript{low} monocytes. Significantly, the kinetics of the neutrophil response was quite distinct from that of monocytes, with increases in circulating and lung-associated cells as early as 1 h, but then a more rapid decline after 2 h, with numbers returning to normal by 18 h after LPS challenge.

Localization of these monocytes within the lungs, that is, whether they still reside in the capillaries (i.e., marginated) or have extravasated into the lung tissue, was assessed by both immunohistochemical and flow cytometric analysis. Gr-1\textsuperscript{staining of lung sections indicated that the density of positive cells (Gr-1\textsuperscript{high} monocytes and neutrophils) substantially increased in mice with i.v. LPS challenge (20 ng, 2 h) compared with the untreated mice (Fig. 4). These Gr-1\textsuperscript{+} cells were confined to the alveolar septa without significant infiltration into the alveolar space, although it was difficult to clearly differentiate whether they were within capillaries or interstitial spaces. To determine the proportion of monocytes residing within the vascular space after LPS challenge, ex vivo perfusion of the lungs was performed with three-color quantitative flow cytometric analysis of monocyte subsets as described above. Pulmonary arterial perfusion under stable physiological conditions for 40 min resulted in a marked reduction of both monocyte subsets (~70\% for Gr-1\textsuperscript{high} and ~85\% for Gr-1\textsuperscript{low}) recovered from the lungs, compared with the nonperfused lungs from control, surgery-only LPS-challenged mice (Fig. 5A). Consistent with these data from lung cell suspensions, substantial numbers of monocytes (0.59 ± 0.14 \times 10^6 cells for Gr-1\textsuperscript{low} and 1.61 ± 0.30 \times 10^6 cells for Gr-1\textsuperscript{high}) were found to be washed out and recovered in perfusates. Alveolar macrophages (high forward scatter, F4/80\textsuperscript{+}) were not detected in perfusates, indicating that the alveolar compartment cell barrier remained intact during the procedure. Further confirmation of monocyte subset location within the lungs was established by the in vivo intravascular cell-labeling technique using fluorescence-conjugated

FIGURE 2. Gr-1\textsuperscript{high} monocyte recruitment to the lungs during clinical and subclinical endotoxemia. Mice were injected i.v. with different doses of LPS (0.2 ng to 20 \mu g) and, after 2 h, the numbers of Gr-1\textsuperscript{high} monocytes, Gr-1\textsuperscript{low} monocytes, and neutrophils in lungs and blood were quantified by flow cytometry. Mice receiving less than the 2-\mu g dose of LPS appeared normal, displaying no clinical effects of the treatment. \( n = 3–4 \) for each LPS dose; \( *, p < 0.05; **, p < 0.01 \), different from saline-treated mice of the same cell type. The differences in dose-response curves among the cell types were confirmed by a significant interaction \( p \) value (\( p < 0.01 \)) by two-way ANOVA.

FIGURE 3. Time course of Gr-1\textsuperscript{high} monocyte recruitment to the lungs during subclinical endotoxemia. Mice were injected i.v. with the subclinical 20-ng LPS dose and sacrificed for quantification of the lung and blood cell subsets at the time points shown. \( n = 3–4 \) for each time point; \( *, p < 0.05; **, p < 0.01 \), different from saline-treated mice of the same cell type. The differences in the time course among the cell types were confirmed by a significant interaction \( p \) value (\( p < 0.01 \)) by two-way ANOVA.
confirm that these Ly-6Chigh (Gr-1high) monocytes released from bone marrow in response to subclinical endotoxemia were far in excess of those found in the lungs and blood. Distribution of Gr-1high monocytes among major body organs was therefore evaluated in control and LPS-treated mice and extrapolation of the above data using single femur and tibia to represent the whole-body bone marrow pool suggests that the numbers of Ly-6C high (Gr-1 high) monocytes released from bone marrow in response to subclinical endotoxemia were far in excess of those found in the lungs and blood. Distribution of Gr-1high monocytes among major body organs was therefore evaluated in control and

Anti-CD45 Ab injected i.v. just before sacrificing the mice. Following a short pulmonary arterial perfusion to recover loosely adhered cells as well as wash out residual Abs, the CD11b+ F4/80+ monocytes remaining in the lungs were found to be uniformly stained for CD45 (and therefore considered to be exposed in vivo to the intravascular space), and the level of this staining was similar to that of the washed out monocytes in the perfusates (Fig. 5B). Alveolar macrophages were negative for CD45 (data not shown), indicating that the in vivo labeling was restricted to cells within the intravascular compartment. Taken together, these results demonstrated that the majority, if not all, of the monocytes in lung cell suspensions harvested from LPS-challenged mice still reside within the pulmonary vasculature and therefore represent a marginated cell population.

Lung-marginated Gr-1high monocytes are derived from the bone marrow reservoir during subclinical endotoxemia

The normal size of the total monocyte circulating pool in mice has been estimated at 6 × 10⁵ (49). Given that the numbers of the Gr-1high subset in the lungs increased by ~1.0 × 10⁶ cells and that this increase was not accompanied by any marked change in circulating monocyte numbers, we investigated the possibility that the bone marrow is a major source of newly marginated monocytes in the lungs during subclinical endotoxemia. Mobilization of bone marrow monocytes was quantified by flow cytometry using anti-Ly-6C (the target of anti-Gr-1 binding to monocytes) and anti-CD115 Abs. This staining combination, despite reduced expression of CD115 on monocytes after LPS, produced clearer distinction of CD115 on monocytes after LPS challenge, mice were anesthetized and surgery/instrumentation was performed for isolated perfused lung preparations. Following exsanguination, the lungs were perfused at a constant flow using RPMI 1640 with 4% BSA and 5 mM EDTA for 40 min. Perfused lungs were analyzed by flow cytometry. n = 4 each group, **, p < 0.01 compared with nonperfused, surgery-only controls. B, Localization of monocyte subsets within the lungs after LPS challenge was confirmed by i.v. injection of PE-labeled anti-CD45 Ab just before sacrificing the mice, followed by a short (10 min) pulmonary artery perfusion step to remove excess Ab. Lung cell suspensions were stained in vitro for CD11b, F4/80, and Gr-1 and analyzed by flow cytometry. Panels on the right represent the in vivo CD45 staining of monocyte (CD11b+ F4/80+) subsets in the lungs after perfusion (top right) and the perfusate (bottom right), indicating that the majority of cells were exposed to intravascular space. For comparison, the left panel shows isotype control Ab staining of monocytes in nonperfused lungs of LPS-pretreated mice.

Pulse-label dividing cells in vivo. At 2 h after i.v. LPS challenge, BrdU-labeled Gr-1high monocytes in the lung cell suspensions showed a marked (~12-fold) increase compared with the control non-LPS-challenged mice (Fig. 6B; n = 3/each group, p < 0.01), indicating that subclinical endotoxemia induced substantial margination of recently divided bone marrow Gr-1high monocytes to the lungs.

Extrapolation of the above data using single femur and tibia to the whole-body bone marrow pool suggests that the numbers of Ly-6C high (Gr-1 high) monocytes released from bone marrow in response to subclinical endotoxemia were far in excess of those found in the lungs and blood. Distribution of Gr-1high monocytes among major body organs was therefore evaluated in control and
Bone marrow mobilization and margination of immature Ly-6C<sup>low</sup>-Gr-1<sup>high</sup> monocytes to the lungs during subclinical endotoxemia. A. Mice were injected i.v. with 20 ng of LPS, and, after 2 h, bone marrow (femur and tibia) and lungs were processed for flow cytometry. Bone marrow monocytes were identified using the combination of anti-Ly-6C and anti-CD115 (M-CSF receptor) Abs (top panel). Quantification of total immature monocytes (Ly-6C<sup>low</sup>-Gr-1<sup>high</sup>-CD115<sup>+</sup>) per single femur and tibia were performed in saline- and LPS-treated mice (bottom panel). n = 5/each group; **, p < 0.01 compared with control saline-treated mice. B. To determine the origin of lung-marginated monocytes, mice were injected i.p. with 2 mg of BrdU to label dividing bone marrow precursor cells in vivo. At 6 h after BrdU treatment, mice were injected with saline or LPS and, after 2 h, lungs were analyzed by staining of permeabilized cells with FITC-conjugated anti-BrdU Ab. Dots plots of Gr-1 vs anti-BrdU labeling in gated monocytes (CD11b<sup>+</sup>F4/80<sup>+</sup>, top panel) and the mean percentage of BrdU-positive cells among lung-marginated Gr-1<sup>high</sup> monocytes (bottom panel) are depicted. n = 3/each group; **, p < 0.01 compared with control saline-treated mice.

**FIGURE 6.** Bone marrow mobilization and margination of immature Ly-6C<sup>low</sup>-Gr-1<sup>high</sup> monocytes to the lungs during subclinical endotoxemia. A. Mice were injected i.v. with 20 ng of LPS, and, after 2 h, bone marrow (femur and tibia) and lungs were processed for flow cytometry. Bone marrow monocytes were identified using the combination of anti-Ly-6C and anti-CD115 (M-CSF receptor) Abs (top panel). Quantification of total immature monocytes (Ly-6C<sup>low</sup>-Gr-1<sup>high</sup>-CD115<sup>+</sup>) per single femur and tibia were performed in saline- and LPS-treated mice (bottom panel). n = 5/each group; **, p < 0.01 compared with control saline-treated mice. B. To determine the origin of lung-marginated monocytes, mice were injected i.p. with 2 mg of BrdU to label dividing bone marrow precursor cells in vivo. At 6 h after BrdU treatment, mice were injected with saline or LPS and, after 2 h, lungs were analyzed by staining of permeabilized cells with FITC-conjugated anti-BrdU Ab. Dots plots of Gr-1 vs anti-BrdU labeling in gated monocytes (CD11b<sup>+</sup>F4/80<sup>+</sup>, top panel) and the mean percentage of BrdU-positive cells among lung-marginated Gr-1<sup>high</sup> monocytes (bottom panel) are depicted. n = 3/each group; **, p < 0.01 compared with control saline-treated mice.

Lung-marginated Gr-1<sup>high</sup> monocytes are primed and respond vigorously to further intravascular LPS challenge

Accumulation of monocytes in the pulmonary vasculature in contact with endothelial cells provides the conditions necessary for cell-mediated and highly focused inflammatory signaling, potentially leading to active local monocyte-endothelial interactions in response to further intravascular septic stimuli. To evaluate the responsiveness of lung-marginated monocytes in such two-hit models of pulmonary microvascular inflammation, mice pretreated with low-dose i.v. LPS (to induce monocyte margination) were rechallenged at 2 h with low (20 ng)- or high-dose (20 μg) i.v. LPS, and the monocyte proinflammatory response was assessed by measuring levels of surface memTNF expression at 30 min after the secondary LPS challenge. Primary LPS followed by secondary low-dose LPS challenge produced a dramatic increase in memTNF expression on Gr-1<sup>high</sup> but not Gr-1<sup>low</sup> monocytes (n = 3–4/each group; p < 0.01), whereas memTNF levels after a single, low-dose LPS injection (at either the primary or secondary challenge time points) were negligible (Fig. 7). Thus, subclinical, low-dose LPS induced margination and priming (enhanced responsiveness to secondary septic stimuli) of Gr-1<sup>high</sup> monocytes in the lungs. When a high LPS dose was used for the secondary LPS challenge, this priming effect of low-dose LPS pretreatment on the Gr-1<sup>high</sup> subset was still evident despite the relatively high levels of memTNF expression on monocytes in nonprimed mice. Numbers of lung-marginated Gr-1<sup>high</sup> monocytes were unchanged at 30 min after secondary low-dose LPS challenge (1.0 ± 0.28 × 10<sup>6</sup> cells) when compared with mice receiving only the priming low dose of LPS (0.95 ± 0.11 × 10<sup>6</sup> cells), but increased after the high dose of LPS (1.7 ± 0.43 × 10<sup>6</sup> cells, n = 4/each group; p < 0.05), with a substantial reduction in circulating Gr-1<sup>high</sup> monocytes compared with the single LPS challenge (0.13 ± 0.088 vs 1.5 ± 0.58 × 10<sup>5</sup> cells/ml blood, n = 4/each group; p < 0.01). Thus, depending on the dose of the secondary LPS challenge, the lung-marginated monocyte population may expand further, enhancing the total cell-mediated pulmonary vascular inflammatory response. Only mice receiving the high secondary LPS dose developed symptoms over a 4-h observation period, with a tendency to increase the severity of symptoms in primed as compared with nonprimed mice.

**FIGURE 7.** Lung-marginated Gr-1<sup>high</sup> monocytes contribute directly to a pulmonary vascular leak in a model of zymosan-induced extrapulmonary ALI

To investigate whether monocyte margination and priming induced by subclinical LPS challenge would contribute to the progression of ALI of extrapulmonary origin, we developed a modification of the previously characterized LPS-zymosan two-hit model of ALI (50–52). In contrast to the model described in the literature in which mice are pretreated with a high clinical dose of LPS to enhance zymosan-induced ALI changes, we pretreated mice with the low subclinical dose of i.v. LPS (20 ng/mouse) to induce monocyte priming of the lungs in the absence of pronounced physiological effects. Secondary i.v. zymosan challenge (150 μg/mouse) at 2 h after LPS resulted in the rapid (within minutes) development of severe but transient shock-like symptoms, including crawling and prostration followed by a state of prolonged lethargy with piloerection. At a higher dose of zymosan, i.e., 250 μg/mouse similar to the highest dose used in previous studies (50–52), early mortality within 20–30 min was observed in more than half of LPS-primed mice, while the lower dose of zymosan produced overt clinical symptoms but no mortality. At the
lower dose of zymosan without LPS priming, clinical symptoms were completely absent, with mice appearing visibly unaffected. Zymosan-induced ALI was assessed by measuring changes in pulmonary vascular permeability, an early and direct cause of lung dysfunction in this model, at 1 h after zymosan challenge (Fig. 8A). Priming of mice with LPS, which on its own had no effect on lung permeability, led to a significant enhancement of zymosan-induced pulmonary vascular leak (n = 4–6/group, †, p < 0.01, different from control nontreated mice), i.e., 6-fold increases in the vascular permeability index as compared with control saline-treated mice, as assessed by accumulation of labeled BSA in lung tissue. At 1 h after zymosan in LPS-pretreated mice, neither monocytes nor neutrophils were present in appreciable numbers in lung lavage fluid (<100 gated cells, n = 3), indicating that migration of leukocytes across the endothelial-epithelial barrier into the alveolar space is not a feature of this model.

To gain an insight into the mechanisms responsible for increased vascular permeability in this model, we performed in vivo pharmacological inhibition studies. Contrasting with the response to the LPS-LPS two-hit model, memTNF expression on monocytes at 30 or 60 min after zymosan challenge were negligible (data not shown), suggesting that TNF signaling is unlikely to be a significant component in this LPS-zymosan model of ALI. Since PAF has been implicated as the major mediator in the previously described high-dose LPS-zymosan model (50), low-dose LPS (20 ng/mouse)-pretreated mice were administered (i.p.) the PAF receptor antagonist WEB 2086 at 30 min before zymosan challenge. Treatment with 5 mg/kg WEB 2086 resulted in a dramatic reduction in the severity of clinical symptoms described above and a partial reduction (30%) in the lung permeability index (Fig. 8B). Because higher doses of WEB 2086 did not produce any further decrease in permeability (data not shown), we then examined combined effects of WEB 2086 with inhibitors of two major downstream pathways implicated in PAF-induced ALI, i.e., cyclooxygenase-mediated pathway and PC-PLC-mediated activation of the sphingomyelinase/ceramide pathway (53). Treatment with WEB 2086 and indomethacin (cyclooxygenase inhibitor) did not result in any further attenuation, whereas treatment with WEB 2086 and D609 (PC-PLC inhibitor) produced a substantial decrease (~70%) in permeability. This treatment produced no significant effect on
the numbers of lung-marginated monocytes and neutrophils at 2 h after LPS (Gr-1low monocytes, $0.25 \pm 0.082 \times 10^6$ cells; Gr-1high monocytes, $1.6 \pm 0.35 \times 10^6$ cells; neutrophils, $1.3 \pm 0.20 \times 10^6$ cells; $n = 4$ for each group) compared with the data in Fig. 2, indicating that the protective effect was not due to a reversal of margination process.

The contribution of LPS-premarginated Gr-1high monocytes to a zymosan-induced pulmonary vascular leak was assessed by i.v. treatment with liposome-encapsulated clodronate (clodronate-liposomes), a standard method used for depletion of monocytes and resident intravascular macrophages (41). To confirm the ability of clodronate-liposome treatment to reduce monocyte margination to the lungs during subclinical endotoxia, mice were injected i.v. with clodronate-liposomes and then challenged with low-dose LPS (20 ng/mouse) 18 and 48 h later. At 18 h after clodronate-liposome treatment by injection of LPS and after an additional 2 h, determining numbers of lung-marginated monocytes and neutrophils ($n = 3/each group$). $B$, Attenuation of LPS-zymosan-induced pulmonary vascular leak by pretreatment with clodronate-liposomes 18 h before LPS challenge and its reversal at 48 h after clodronate-liposome pretreatment ($n = 4–6/each group$). †, $p < 0.01$, different from each other and **, $p < 0.01$, different from control nontreated mice (displayed in Fig. 8). Clod, Clodronate-liposomes.

**FIGURE 9.** Gr-1high monocytes mediate zymosan-induced pulmonary vascular leak in low-dose LPS-pretreated mice. $A$, To deplete monocytes, mice were injected i.v. with clodronate-liposomes (200 µl). The efficacy and specificity of depletion was assessed at 18 and 48 h after clodronate-liposome treatment by injection of LPS and after an additional 2 h, determining numbers of lung-marginated monocytes and neutrophils ($n = 3/each group$).

Discussion

Although monocyte accumulation within the pulmonary circulation in response to systemic septic stimuli is well documented, there is little evidence to suggest that lung-marginated monocytes are directly involved in the pathophysiology of sepsis-related ALI. In this study, we evaluated monocyte subset margination to the lungs during subclinical endotoxia, the responsiveness of the marginated monocytes to secondary stimuli, and their impact on pulmonary vascular permeability in the two-hit LPS-zymosan model of ALI in mice. Our results provide the first in vivo evidence that margination of bone marrow-derived Gr-1high monocytes during subclinical endotoxia transforms the lungs into a latent primed state, thereby playing a crucial role in the evolution of sepsis-related ALI.

We previously investigated margination of total monocytes to the lungs at early time points during acute overwhelming high-dose endotoxia and demonstrated that marginated monocytes can produce pulmonary endothelial activation through local and cell-contact-dependent signaling mechanisms (11). In this study, we extended our analysis to the more long-term consequences of monocyte margination during subacute endotoxia, in particular with subclinical low-dose LPS challenge. We studied monocyte margination at the level of individual monocyte subsets, namely, Gr-1high (inflammatory) and Gr-1low (resident) monocytes, in view of their recently described distinct migratory and inflammatory properties (13, 54). Contrasting with the previously described early margination event at 45 min after high-dose LPS challenge (11), subclinical endotoxia induced a delayed (apparent at 2 h) but long-lasting (up to 8 h) and very substantial ($>1.0 \times 10^6$ cells, exceeding neutrophil numbers) accumulation of monocytes within the pulmonary circulation, predominantly due to margination of the Gr-1high subset. This margination event was very sensitive to intravascular endotoxin occurring at LPS doses 3–4 orders of magnitude below the threshold dose that induces clinical septic symptoms. Based on the size of the normal circulating monocyte pool and the absence of monocytopenia, this margination could not be explained by subset-specific entrapment of circulating monocytes. Instead, it was shown to be a result of the large-scale mobilization of immature Gr-1high monocytes from the bone marrow reservoir,
and their preferential accumulation within the marginated, as opposed to circulating pool.

Mobilization of immature monocytes from bone marrow represents an important regulatory mechanism to expand the total monocyte pools within the vasculature during systemic or local infection and inflammation. Recent studies have identified CCR2 and its ligands, MCP-1 and MCP-3, as the key signaling molecules required for monocyte release from bone marrow to local sites under normal conditions, *Listeria* infection, and sterile peritonitis (18, 55). However, previous analyses of monocyte recruitment from bone marrow have focused mainly on the resultant blood mononcytosis, without paying much attention to the dynamic equilibrium that exists between the circulating and marginated pools. In the present study, during the first 2–3 h after LPS challenge, we did not detect mononcytosis in blood despite massive mobilization of bone marrow monocytes; instead, the newly released Gr-1high monocytes appeared to migrate directly to the peripheral organs including the lungs, liver, and spleen. In contrast, neutrophils displayed increases in both the circulating and marginated pools immediately after LPS challenge, but the increases did not last as long as those in monocytes. Thus, our findings indicate that monocyte trafficking profile from the bone marrow to the vasculature during endotoxemia is different from neutrophils, with a greater propensity of monocytes to reside within the microcirculation. Circulating blood monocytes may represent only the tip of the iceberg of total (circulating plus marginated) monocyte pools within the vasculature, highlighting potential limitations of monocyte analyses based on blood samples in sepsis or other inflammatory conditions.

The predominant margination of Gr-1high, as compared with Gr-1low, monocytes within the lungs could be attributed to the massive, LPS-induced influx of this subset from the bone marrow into the blood. The differences in cell adhesion and migration between the two subsets could also contribute to this Gr-1high predominance, i.e., L-selectin or CCR2 which are expressed on Gr-1high but not Gr-1low monocytes (13). The mechanism of margination of monocytes as well as neutrophils to the lungs during endotoxemia, as compared with other body organs, has been generally ascribed to the unique anatomical structure of the pulmonary microvascular bed. Leukocytes must deform to pass through the narrow pulmonary capillaries, and LPS-induced physical stiffening of leukocytes as well as recruitment of stiffer immature bone marrow cells (56, 57) would lead to enhanced physical entrapment by the capillaries (9, 58). It has been suggested that there are some differences in the lung margination mechanism between monocytes and neutrophils. Sustained margination of monocytes with LPS has been shown to be CD18 dependent (9), while neutrophil margination is not affected by CD18 blocking in vivo (58). Compared with neutrophils, much lower concentrations of LPS seem to be required for monocyte margination in vivo (9, 59) and adherence to endothelial cells in vitro (60, 61), which were not investigated here but could be relevant to the different margination behavior of monocytes and neutrophils during subclinical endotoxemia.

To assess the proinflammatory capacity of lung-marginated monocytes during subclinical endotoxemia, we evaluated their responsiveness to additional septic stimuli by measuring in vivo memTNF expression, which was previously shown to be involved in monocyte-primed pulmonary endothelial activation (11). The results demonstrated the priming effect of low-dose LPS on lung Gr-1high monocytes. Gr-1high monocytes in the lungs pretreated by low-dose LPS showed substantial enhancement of memTNF expression in response to both low- and high-dose secondary LPS, as compared with Gr-1high in the lungs of mice not pretreated with LPS. This implies that low-dose LPS pretreatment increased the sensitivity of these cells to secondary stimuli as well as the magnitude of their response to saturating stimuli. In contrast, lung Gr-1low monocytes displayed much less responsiveness than Gr-1high monocytes. This higher TNF-producing capacity of Gr-1high as compared with Gr-1low monocytes has recently been demonstrated in cells recruited to infarcted cardiac tissue (20). Thus, the increased Gr-1high monocyte margination and their enhanced responsiveness to secondary stimuli during subclinical endotoxemia produce a monocyte-mediated latent inflammatory priming of the lungs.

To explore the physiological effects of low-dose LPS-induced monocyte margination and priming on the development of ALI, we adapted a previously described two-hit ALI model comprised of high-dose i.v. LPS followed by i.v. zymosan challenge (51, 52). In our study, we modified the original model to use a much lower, subclinical primary LPS dose, but despite this, mice developed clear signs of ALI associated with a large increase in pulmonary vascular permeability following zymosan challenge. Conversely, mice administered the same doses of zymosan alone (without preceding low-dose LPS) appeared outwardly normal and showed only marginal permeability increases. Thus, the model enabled us to dissect out the impact of the LPS pretreatment on the development of ALI. We found a role for PAF signaling in induction of the pulmonary vascular leak in this model, but unlike the previous high-dose LPS-zymosan model (50), treatment with WEB 2086 at doses expected to ablate PAF signaling in vivo (62) resulted in only partial attenuation of vascular permeability. A recent report provided evidence of a role for the acid sphingomyelinase/ceramide pathway in PAF-induced pulmonary vascular leak (63), in addition to the previously described role for PGs (64). Consistent with this, we observed that WEB 2086 with D609 markedly attenuated permeability increases, but addition of indomethacin had no effect. These results indicate that lipid mediators including PAF and ceramide are at least in part responsible in the development of pulmonary edema in the low-dose LPS-zymosan model of ALI.

To directly address the role of marginated monocytes in this model, we chose a monocyte/macrophage depletion approach using i.v. clodronate-liposomes (65, 66). An adoptive transfer approach using purified bone marrow CD115+ Gr-1high monocytes was also attempted, but after transfer of cells with either prior ex vivo or in vivo low-dose LPS treatment, only a fraction of the cells injected remained detectable in the blood, lungs, or other tissues (data not shown) for prolonged periods (>30 min), suggesting differences in monocyte trafficking behavior between endogenously recruited cells from bone marrow vs exogenously purified and administered cells. Using the monocyte depletion strategy, we found a substantial attenuation of LPS-zymosan-induced symptoms and permeability increases at the 18-h time point after clodronate-liposome treatment. In contrast, no protective effects were observed at 48 h after clodronate-liposome treatment, the time point when bone marrow monocyte repopulation (27) and restoration of LPS-induced Gr-1high subset margination to the lungs had occurred, but before the recovery from any deleterious effects of i.v. clodronate-liposomes on tissue macrophages would have taken place (43, 67, 68). Taken together, these results strongly suggest that Gr-1high monocyte margination and priming within the lungs are important in the evolution of zymosan-induced ALI, presumably through a pathway involving both PAF and ceramide.

We speculate that the LPS-primed monocytes are likely to be the direct cell sources for PAF and ceramide production in response to zymosan in this model. In vitro studies have previously shown that PAF is produced by human and rabbit monocytes in response to zymosan stimulation (69, 70). Consistent with our in vivo findings of LPS priming toward zymosan-induced pulmonary
vascular leak, LPS pretreatment in vitro, although on its own it is a weak stimulus for lipid mediator production, enhances arachidonic acid metabolite and PAF production in response to stimuli such as zymosan (71–74). Furthermore, optimal PAF-mediated production of lipid mediators, including ceramide, is also dependent on LPS pretreatment, as shown in the mouse macrophage cell line P388D1 (75, 76). However, the precise molecular mechanisms whereby primed and primed Gr-1high monocytes contribute to pulmonary edema in response to septic stimuli such as zymosan remains to be further elucidated.

The critical role attributed to neutrophils in the evolution of ALI during systemic inflammation is based on a number of experimental observations, including neutrophil depletion studies using anti-neutrophil Abs or anticancer drugs (8, 77). However, caution should be taken when interpreting these depletion studies, as recently suggested by Rydström and Wick (78). The anti-neutrophil Ab RB6-C5, widely used to demonstrate neutrophil function in mice including models of ALI (79, 80), is essentially an anti-Gr-1 Ab, and relatively small doses of this Ab may deplete circulating Gr-1high monocytes in addition to neutrophils (81, 82). The tendency for cross-reactivity of neutrophil-depleting Abs with monocytes and the lack of specificity of pharmacological depletion methods using anticancer myelosuppressing drugs may lead to inappropriate overattribution of immunological and pathophysiological functions to neutrophils. Identification of a distinct inflammatory subset of mouse monocytes now allows measurements to be focused on the relevant population, and it has been described that the Gr-1high subset and neutrophils have a similar role in innate immunity in certain conditions. Gr-1high monocytes are required for the control of local Toxoplasma (83) and Listeria (84) infection, whereas during oral Salmonella infection, Gr-1high monocytes in the gut lymphoid tissues showed increased TNF and inducible NO synthase production and were capable of phagocytosis and killing bacteria in vitro (78). In addition to enhanced TNF-producing capacity, Gr-1high monocytes recruited to injured myocardium also possess higher protease activity (20). Evaluation of the mediator production by resting and primed Gr-1high monocytes in vivo and in vitro, particularly early, short-lived locally acting mediators such as reactive oxygen species and lipid mediators, will enhance our understanding of their role in microvascular pathophysiology during ALI.

The results of the present study confirm our previous notion (11) that the role of monocytes in acute systemic inflammation is concerned primarily with local cell-mediated effects within the microvasculature, as opposed to augmentation of systemic mediator release within the central circulation. The substantial accumulation of Gr-1high monocytes in the lungs, and their vigorous response to secondary septic stimuli, supports the hypothesis that without the need for extensive pretimulation or conditioning, these monocytes have the potential to fulfill a similar role to PIM observed in live-stock animals, replicating the functions of PIM in species lacking this population. This view is further supported by our findings that clodronate-liposome-mediated depletion of marginalized monocytes attenuates pulmonary vascular leak in the LPS-zymosan-induced ALI, analogous to the observation in the literature that immature monocyte subsets possess a specialized proinflammatory phenotype point to a complementary or independent role for monocytes in ALI and the potential for interactions between the two cell types during systemic inflammation. Further studies will be required to ascertain whether our results with subacute endothoxemia and the two-hit model of processes of ALI are applicable to other more complex pathophysiological processes of clinical ALI of extrapolummonary origin.

Disclosures
The authors have no financial conflict of interest.

References

MARGONOTIZED MONOCYTES PROMOTE ALI

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