Regulation of monocyte subset proinflammatory responses within the lung microvasculature by the p38 MAPK/MK2 pathway

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O’Dea KP, Dokpesi JO, Tatham KC, Wilson MR, Takata M. Regulation of monocyte subset proinflammatory responses within the lung microvasculature by the p38 MAPK/MK2 pathway. Am J Physiol Lung Cell Mol Physiol 301:L812–L821, 2011. First published August 26, 2011; doi:10.1152/ajplung.00092.2011.—Margination and activation of monocytes within the pulmonary microcirculation contribute substantially to the development of acute lung injury in mice. The enhanced LPS-induced TNF expression exhibited by Gr-1high compared with Gr-1low monocytes within the lung microvasculature suggests differential roles for these subsets. We investigated the mechanisms responsible for such heterogeneity of lung-marginated monocyte proinflammatory response using a combined in vitro and in vivo approach. The monocyte subset inflammatory response was studied in vitro in mouse peripheral blood mononuclear cell-lung endothelial cell coculture and in vivo in a two-hit model of intravenous LPS-induced monocyte margination and lung inflammation in mice, by flow cytometry-based quantification of proinflammatory genes and intracellular phospho-kinases. With LPS stimulation in vitro, TNF expression was consistently higher in Gr-1high than Gr-1low monocytes, markedly enhanced by coculture with endothelial cells, and abrogated by p38 MAPK inhibitors. Expression of IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) was only detectable under coculture conditions, was substantially higher in Gr-1high monocytes, and was attenuated by p38 inhibition. Consistent with these differential responses, phosphorylation of p38 and its substrate MAPK-activated protein kinase 2 (MK2) was significantly higher in the Gr-1high subset. In vivo, p38 inhibitor treatment significantly attenuated LPS-induced TNF expression in “lung-marginated” Gr-1high monocytes. LPS-induced p38/MK2 phosphorylation was higher in lung-marginated Gr-1high than Gr-1low monocytes and neutrophils, mirroring TNF expression. These results indicate that the p38/MK2 pathway is a critical determinant of elevated Gr-1high subset responsiveness within the lung microvasculature, producing a coordinated proinflammatory response that places Gr-1high monocytes as key orchestrators of pulmonary microvascular inflammation and injury.

acute lung injury; endotoxia; tumor necrosis factor; margination

Margination of inflammatory leukocytes in the narrow capillaries of the lung, with their in situ activation and production of proinflammatory mediators, is central to the development of sepsis-related pulmonary microvascular inflammation and acute lung injury (ALI) (1, 53, 57). In contrast to extensive research on the importance of neutrophils to these processes, the roles of monocytes have not been elaborated. We have previously shown in mice that monocyte margination to the lungs during acute endotoxia produces a direct activation of the pulmonary endothelium via contact- and TNF-dependent mechanisms (45). We have also found that with a subclinical endotoxia there is substantial mobilization of monocytes of the “Gr-1high” (Ly-6G/Ly-6C) or “Ly-6C<sup>high</sup>” subset from the bone marrow and their accumulation within the pulmonary circulation (44). These lung-marginated Gr-1<sup>high</sup> monocytes were found to be primed, responding more vigorously in situ to further LPS challenge than their Gr-1<sup>low</sup> subset counterparts, as well as contributing to the evolution of ALI after secondary zymosan challenge (44) or injurious mechanical ventilation (58). Margination to the lungs of the Gr-1<sup>high</sup> monocyte subset and their activation in situ is now an emerging feature in various models of ALI, induced by transfusion (48), pneumococcal infection (28), LPS (2, 44), and ventilation (41, 58). Thus defining the nature and regulation of their proinflammatory responses within the pulmonary microvascular environment is of considerable physiological importance.

Gr-1<sup>high</sup> and Gr-1<sup>low</sup> monocytes are the two principal monocyte subsets in mice (24, 46), defined phenotypically on the basis of their respective CCR2<sup>+</sup>CX<sub>3</sub>C<sub>R1</sub><sup>low</sup>CD62L<sup>+</sup> and CCR2<sup>−</sup> CX<sub>3</sub>C<sub>R1</sub><sup>high</sup>CD62L<sup>−</sup> expression profiles. The preferential recruitment of the less mature Gr-1<sup>low</sup> subset to inflamed sites via CCR2-dependent mechanisms (31, 50, 54) is well established, leading to the term “inflammatory monocytes” (24). A specialized role for Gr-1<sup>high</sup> monocytes in inflammation is further supported by their enhanced expression of proinflammatory cytokines in vivo compared with Gr-1<sup>low</sup> monocytes, in the microvasculature as indicated by the higher LPS-induced TNF expression in lung-marginated Gr-1<sup>high</sup> monocytes in our previous study (44), and at local sites of inflammation where they exhibit a greater capacity to express TNF and IL-1β (3, 42). Although it has been suggested that similar differential human monocyte subset responses to viral agonists are determined by alternate upstream signal-pathway usage (13), the central in vivo mechanisms regulating the heterogeneity of monocyte subset responses to septic stimuli have not been elucidated. Moreover, modulation of such cell type-specific responses by the immediate cellular environment during monocyte margination (as opposed to migration), in particular by the interactions between monocytes and pulmonary endothelium that take place in vivo within the microvasculature during ALI, remains to be investigated.

In this study, we investigated the mechanisms that regulate the proinflammatory response of lung-marginated monocyte subsets, using flow cytometry-based quantification of key proinflammatory genes and intracellular phospho-kinase levels of p38 MAPK and MAPK-activated protein kinase 2 (MK2). To evaluate the modulatory effects of lung margination in isolation from systemic factors, we used in vitro mouse peripheral
blood mononuclear cell (PBMC)-lung endothelial cell coculture to simulate cell-cell interactions within the pulmonary microvasculature. Verification of the mechanisms elucidated in vitro was performed in an in vivo model of LPS-induced lung inflammation in mice, thereby determining regulation of the actual in situ responses. The results demonstrated an enhanced proinflammatory capacity of Gr-1<sup>high</sup> monocytes marginated to the pulmonary microvasculature that is coordinated by differential activation of the p38 MAPK/MK2 pathway, indicating its central role in orchestrating monocyte-mediated pulmonary microvascular inflammation during ALI.

**MATERIALS AND METHODS**

**Animals.** All experimental protocols involving animals were approved by the Ethical Review Board of Imperial College London and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 (project license number: 70/6854). Experiments were performed using male C57BL6 mice (Charles River, Margate, UK) aged 8–12 wk (22–26 g).

**Isolation and culture of mouse LEC.** Mice were euthanized and 0.8 ml of dispase (1 mg/ml; Invitrogen, Paisley, UK) was instilled (eBioscience, Hatfield, UK) followed by LEC enrichment with bioselvines in microfuge tubes (4×10<sup>3</sup>/H11003) and 0.4 ml of Ca<sup>2+</sup>-free Dulbecco’s phosphate buffered saline 15 min before secondary LPS. Lungs were excised, blotted to remove surface blood, and placed directly into 2 ml of Cytofix/Cytoperm buffer (Cytofix/Cytoperm buffer, BD) for 5 min at 37°C and washed with permeabilizing medium (PBS, 0.2% saponin, 2% FCS, 0.1% sodium azide). Cells were then incubated with the appropriate antibodies in permeabilizing medium for 30 min at room temperature. For measurement of IL-6, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS), PBMC were treated with Cytofix/Cytperm followed by washing in permeabilizing medium and incubation with appropriate MAb.

**Measurement of in vivo responses.** Mice received two intravenous (iv; via tail vein) injections of LPS 2 h apart (primary 20 ng, secondary 100 ng) and were euthanized at predetermined time points after secondary LPS. For in vivo p38 inhibition experiments, mice were injected iv with SB203580-HCl (10 mg/kg) (Tocris, Bristol, UK) in saline 15 min before secondary LPS. Lungs were excised, blotted to remove surface blood, and placed directly into 2 ml of Cytofix/Cytperm buffer at 37°C and homogenized with a gentleMACS Dissociator (Miltenyi Biotech) for 1 min. After incubation at 37°C for 10 min, lung cell suspensions were filtered through 40-μm sieves and centrifuged. Cells were washed and resuspended in permeabilizing medium and then stained with antibodies at room temperature for 30 min.

**Flow cytometry.** The following fluorophore-conjugated rat anti-mouse MAb (unless stated otherwise) were used: CD11b (clone M1/70), Gr-1 (RB6-8C5), Ly-6G (1A8), Ly-6c (AL-21), NK-1.1 (PK136), TNF (MP6-XT22), mouse anti-phospho-p38 MAPK (pT180/pY182) (in vitro measurements), TLR4-MD2 (MTS510), CD34 (RAM34) E-selectin (10E9.6), ICAM-1 (3E2), ICAM-2 (3C4), PECAM-1 (MEC13.3), VCAM-1 (429) (BD); F4/80 (Cl:A3-1) (AbD Serotec, Kidlington, UK), anti-p38α/β (A 12), iNOS (C-11), and COX-2 (29) (Santa Cruz Biotechnology, Santa Cruz, CA); endoglin (M7/18), VE-cadherin (eBioBV13) (eBioscience); IL-6 (MP-5/20F3) (Biologic); rabbit polyclonal anti-phospho-MK2 (Thr383/Thr385) (27B7), anti-phospho-p38 (Thr180/Thr182, 28B10) (in vivo measurements), and control rabbit polyclonal (Cell Signaling Technology, Danvers, MA). In vitro samples were acquired by using a FACSCalibur flow cytometer and Cell Quest software (BD). In vivo samples were acquired by use of a Cyan flow cytometer (Beckman Coulter, High Wycombe, UK). Analysis of data was performed with Flowjo software (Tree Star, Ashland, OR).

**Statistical analysis.** Data are expressed as means ± SD. Statistical comparisons were made by ANOVA with Bonferroni tests or t-tests. A value of P < 0.05 was regarded as significant.

**RESULTS**

**Regulation of LPS-induced monocyte subset TNF expression during coculture with LEC.** We previously evaluated the monocyte subset response in vivo, quantifying memTNF expression on monocytes marginated within the pulmonary circulation after intravascular LPS challenge (44, 45). To further investigate regulatory mechanisms of this monocyte subset inflammatory response, we developed an in vitro coculture model of PBMC and primary LEC to simulate monocyte-endothelial cell contact within the pulmonary vasculature. Primary LEC cultures were generated from the lungs of C57BL6 mice by magnetic affinity cell sorting and their endothelial phenotype confirmed by constitutive markers as previously reported (7) and inducible surface markers (Fig. 1, A and B).

LPS-induced TNF production in the different cell populations in this coculture model was determined by measurement of memTNF levels by flow cytometry. Cultures were stimulated with LPS for up to 4 h, and a metalloprotease inhibitor (BB94) was added 1 h before the cell harvest to inhibit memTNF cleavage mediated by TNF-α converting enzyme, a
A method to enhance the flow cytometry signal as well as enabling measurement of TNF production by individual cells during each 1-h period throughout the time course (9, 45). Monocyte subsets were identified as CD11b$$^+$$, F4/80$$^+$$, Gr-1$$^{\text{high}}$$, or Gr-1$$^{\text{low}}$$ cells as described previously (44). In untreated monocytes, levels of memTNF were negligible. At 1 h after LPS treatment (100 ng/ml), monocyte memTNF production increased and was substantially higher in Gr-1$$^{\text{high}}$$ monocytes than Gr-1$$^{\text{low}}$$ monocytes (Fig. 2A, P $$<$$ 0.001). Levels of memTNF expressed during each 1-h time period (in presence of BB94) decreased over time (1–4 h) in both subsets, with no significant difference seen between Gr-1$$^{\text{high}}$$ and Gr-1$$^{\text{low}}$$ monocytes after 1 h. This early peak of the response was in accordance with the kinetics of memTNF expression we observed previously in vivo (45).

For comparison with coculture, control PBMC-only incubations were performed in microcentrifuge tubes that were rotated slowly to maintain cells in a suspension state and minimize contact with plastic surfaces. This procedure approximated conditions for circulating (but not marginated within the...

Fig. 1. Characterization of cells in primary lung endothelial cell (LEC) cultures by flow cytometry. A: representative histograms showing staining of endothelial cell surface markers on cells in primary LEC culture (black line), compared with isotype-matched control antibody binding (gray fill). B: representative histograms showing staining of E-selection and VCAM-1 (black line), compared with isotype-matched controls (gray fill), on untreated or LPS-stimulated (100 ng/ml, 4 h) LEC (n = 3 each group), P $$<$$ 0.01 E-selectin, P $$<$$ 0.001 VCAM-1; untreated vs. LPS-stimulated.

Fig. 2. LPS-induced membrane TNF (memTNF) expression by monocyte subsets in vitro. A: peripheral blood mononuclear cells (PBMCs) were added to confluent LEC monolayers and after 2 h were stimulated with LPS (100 ng/ml). BB94 (10 $$\mu$$M) was added to each culture 1 h prior to cell harvest to inhibit memTNF release from the cell surface, to determine memTNF expression during the preceding 1-h period before the time of cell harvest. Levels of memTNF are expressed as the geometric mean of fluorescent intensity (MFI) with values of isotype control subtracted (n = 4 each group). B: PBMC-only stimulations were performed as with cocultures but under rotating, non-sedimenting conditions in Eppendorf microfuge tubes (n = 4 each time point). *P $$<$$ 0.05, **P $$<$$ 0.001 Gr-1$$^{\text{high}}$$ vs. Gr-1$$^{\text{low}}$$. Representative histograms are shown in each panel for subset memTNF expression at 1 h post-LPS treatment (black line), with isotype control antibody (gray fill) for comparison.
microcirculation) cells in vivo. A similar pattern of enhanced Gr-1\textsuperscript{high} memTNF expression was observed in PBMC-only incubations (Fig. 2B); however, the response in both subsets was considerably lower (>15- to 20-fold). Moreover, Gr-1\textsuperscript{high} memTNF expression in PBMC-only culture was not uniform, i.e., not all of the Gr-1\textsuperscript{high} monocytes were responding to LPS (Fig. 2B). This pattern was replaced by a markedly higher and uniformly positive memTNF expression pattern in PBMC-LEC coculture (Fig. 2A), similar to previous in vivo findings in lung-marginated Gr-1\textsuperscript{high} monocytes responding to a secondary LPS challenge (44). Thus coculture with LEC is optimal for TNF expression under in vitro conditions and better simulates the conditions and consequences of prolonged monocyte-endothelial cell contact that exist in vivo.

As one of the important regulators of the LPS-induced cellular TNF response (8, 36), we assessed the contribution of p38 MAPK signaling pathway to this “differential” TNF expression in monocyte subsets in the coculture setting. PBMC-LEC cocultures were pretreated with two selective inhibitors, SB203580 or SB202190 (18), followed by stimulation with LPS (100 ng/ml) and measurement of memTNF on subsets at 1 h. LPS-induced memTNF expression was completely inhibited by SB203580 treatment in both Gr-1\textsuperscript{high} (100 ± 0%) and Gr-1\textsuperscript{low} (92 ± 9%) monocytes (Fig. 3A, P < 0.01). Inhibition with SB202190 was marginally less effective (Fig. 3B) with memTNF levels reduced by 98 ± 2% in Gr-1\textsuperscript{high} monocytes and by 65 ± 14% in Gr-1\textsuperscript{low} monocytes (P < 0.05). This effective inhibition of TNF expression by different p38 inhibitors established that the p38 MAPK pathway is a principal factor controlling the proinflammatory response phenotype of both subsets under coculture conditions.

LPS-induced expression of p38-regulated genes in monocyte-LEC coculture. To determine whether expression of other cytokines is differentially regulated between monocyte subsets, we assessed production of IL-6, an important proinflammatory cytokine that is also induced by LPS and has been shown to be regulated by the p38 MAPK pathway (33, 55). PBMC-LEC cocultures were stimulated with LPS (100 ng/ml), with or without SB203580 for 4 h, in the presence of brefeldin A to prevent protein export and secretion. Intracellular accumulation of IL-6, determined by flow cytometry, was markedly higher in Gr-1\textsuperscript{high} than Gr-1\textsuperscript{low} monocytes (Fig. 4A, P < 0.01). The presence of the p38 inhibitor SB203580 resulted in a 58 ± 18% and 72 ± 1% reduction in LPS-induced IL-6 expression in Gr-1\textsuperscript{high} (P < 0.05) and Gr-1\textsuperscript{low} monocytes, respectively.

We also assessed expression of the LPS-inducible intracellular enzymes, COX-2 and iNOS, both of which have been shown to be posttranscriptionally regulated by the p38 MAPK pathway (15, 19). Cumulative expression of COX-2 and iNOS was compared in monocyte subsets after LPS (100 ng/ml) stimulation of PBMC-LEC cocultures for 4 h, with or without SB203580. Intracellular COX-2 levels were enhanced substantially by LPS treatment in Gr-1\textsuperscript{high} monocytes, but only modest or negligible increases were seen in Gr-1\textsuperscript{low} monocytes (Fig. 4B). A similar pattern for iNOS levels was observed with its upregulation much more marked in the Gr-1\textsuperscript{high} subset than the Gr-1\textsuperscript{low} subset (Fig. 4C, P < 0.05). Inhibition of p38 activation significantly reduced upregulation of both mediators in Gr-1\textsuperscript{high} monocytes (P < 0.05), but, as with IL-6, the attenuation was not complete, 64 ± 6% in the case of COX-2 and 86 ± 14% with iNOS. The incomplete inhibition observed could indicate a partial role for alternative signal transduction pathways. However, the “p38-independent” component of protein expression still remained higher on LPS-stimulated Gr-1\textsuperscript{high} than Gr-1\textsuperscript{low} monocytes, suggesting that if other pathways are involved, they are also preferentially activated in the Gr-1\textsuperscript{high} subset.

In contrast to substantial induction of IL-6, COX-2, and iNOS in Gr-1\textsuperscript{high} monocytes in coculture, increases were undetectable or negligible in PBMC-only incubations (data not shown), suggesting that interaction with endothelium is essential to produce optimal p38 pathway activation and proinflammatory responses to LPS in monocytes in vitro.

LPS-induced p38/MK-2 pathway activation in monocyte subsets. We investigated whether differential activation of p38 and its downstream substrate, MK2, was responsible for the observed monocyte subset response heterogeneity in PBMC-LEC coculture. To measure responses in coculture, we used quantitative flow cytometric analysis of protein phosphorylation at the level of individual cells. We considered this approach essential for measuring kinase activation within mixed cell populations, where the effect of interactions between different cell populations is being determined and where isolation of primary cell subpopulations might by itself alter their phenotype (26, 35). Increased levels of phospho-p38 in Gr-1\textsuperscript{high} monocytes were evident at 15 min post-LPS (100 ng/ml) treatment and remained higher at 30 min (Fig. 5A, P < 0.001).
In comparison, phospho-p38 increases in Gr-1low monocytes were much lower, with almost negligible increases evident at 15 min. LPS-induced phosphorylation of MK2, which has been shown to play a major role in mediating mRNA stability and translation of genes (e.g., TNF and IL-6) (33, 43), was also assessed in monocyte subsets under the same conditions (Fig. 5, C). As with phospho-p38, levels of phospho-MK2 were higher at 15 and 30 min post-LPS in Gr-1high monocytes (P < 0.001), and although increases in phospho-MK2 were detectable in Gr-1low monocytes, these were modest compared with the Gr-1high subset. In PBMC-only incubations, LPS-induced increases in phospho-p38 and phospho-MK2 were higher in Gr-1high than Gr-1low monocytes (Fig. 5, B and D), but their overall levels were lower (~2-fold) than PBMC-LEC coculture models, consistent with the protein expression data. These results demonstrated that the p38/MK2 pathway was preferentially activated in the Gr-1high subset and, together with the p38 pharmacological inhibition data, indicated that it is responsible for the greater capacity of the Gr-1high subset monocytes to express key mediators of the inflammatory response.

Evaluation of the total intracellular p38α/β protein expression in monocytes by flow cytometry prior to LPS treatment indicated no marked difference between subsets (Gr-1high MFI: 154.9 ± 19.9; Gr-1low MFI: 137.9 ± 18.9, n = 3). Levels of the active membrane TLR4-MD2 complex on subsets also showed no difference (Gr-1high MFI: 13.8 ± 6.8; Gr-1low MFI: 13.4 ± 3.4, n = 4). Therefore, the enhanced activation of the p38 MAPK signaling pathway in Gr-1high monocytes did not appear to result from differences in total p38 available for phosphorylation or LPS recognition by the TLR4-MD2 receptor signaling apparatus.

In vivo regulation of the lung-marginated monocyte subset response to LPS challenge. To investigate the in vivo regulation of the monocyte subset proinflammatory phenotype in relation to the in vitro coculture findings, we used a two-hit model of LPS-induced lung inflammation. In this model, a low-dose LPS pretreatment produces prolonged monocyte margination to the lungs and induction of a primed state, which results in enhanced TNF expression on lung-marginated monocytes in response to secondary LPS challenge (44). After the secondary LPS, animals were euthanized and cell suspensions were prepared from the lungs by immediate tissue homogenization in a fixation/permeabilization buffer, for intracellular TNF and phospho-protein measurement. Because this sample preparation procedure induced a pronounced reduction in the reactivity of the dual-specificity Gr-1 (anti-Ly-6C/Ly-6G) MAb (16, 23) with monocytes (Ly-6Chigh/low, Ly-6G+), a modified staining and gating strategy was developed (Fig. 6A). Monocyte subsets were identified by using an alternative Ly-6C-reactive MAb (AL-21) that recognizes a formaldehyde-resistant epitope (34). Neutrophils (Ly-6Cmedium, Ly-6G+) were distinguished from monocytes by anti-Ly-6G staining or by Gr-1, which retained its reactivity with neutrophils presumably through Ly-6G binding.

Consistent with our previous in vivo findings of memTNF (44), Ly-6Chigh (Gr-1high) monocytes expressed higher levels of total TNF (membrane + intracellular) than Ly-6Clow (Gr-1low) monocytes at 30 min after secondary LPS challenge (Fig. 6B, P < 0.001). The TNF response was significantly inhibited (51 ± 15%, P < 0.01) by systemic administration of SB203580 prior to the secondary LPS in Ly-6Chigh (Gr-1high) monocytes. Phospho-p38 and phospho-MK2 levels were determined at 15 min postsecondary LPS challenge, the time of maximal expression based on in vivo pilot studies. Significantly higher levels of each phospho-kinase were evident in the Ly-6Cmedium (Gr-1high) than in Ly-6Clow (Gr-1low) monocytes (Fig. 6, C and D, P < 0.001).

There was an overall correspondence between LPS-induced expression of phospho-p38, phospho-MK2 and TNF, i.e., highest in Ly-6Cmedium (Gr-1high) monocytes, lower in Ly-6Clow (Gr-1low) monocytes, and lowest in neutrophils. We found that in vivo SB203580 treatment produced a substantial but incomplete inhibition of p38 “activity” in Ly-6Cmedium (Gr-1high) monocytes, as estimated by the reduction in phospho-MK2 levels in inhibitor-treated mice (79 ± 26%, n = 3). In view of such
incomplete nature of SB203580 inhibition under in vivo conditions, which is consistent with previous literature (5, 56), the reduction of TNF expression observed here confirms a major role for the p38 MAPK pathway in the in vivo primed response of lung-marginated Ly-6Chigh/Gr-1high subset monocytes.

**DISCUSSION**

Rapid margination of monocytes to the lungs is a fundamental feature of the early response to systemic inflammation. However, only recently has this process and the significance of monocyte proinflammatory response heterogeneity for the development of ALI been appreciated. In this study, we investigated the LPS-induced proinflammatory response of mouse monocyte subsets in relation to the lung margination process, i.e., modulation by pulmonary endothelial contact. We demonstrated that Gr-1high monocytes express higher levels of TNF and other key inflammatory mediators compared with their Gr-1low counterparts in vitro and that the monocyte response is substantially enhanced by the pulmonary endothelial cell interaction. Underlying this response enhancement and heterogeneous pattern of gene expression was differential activation of the p38/MK2 signaling pathway. In vivo data on the in situ response of lung-marginated monocytes supported these findings, providing further evidence that Gr-1high subset monocytes, regulated by the p38/MK2 pathway, have a central role in the development of pulmonary inflammation and ALI.

We previously observed that Gr-1high monocytes marginated to the pulmonary microcirculation during subclinical endotoxemia respond more vigorously to secondary iv LPS challenge and express much higher levels of memTNF than Gr-1low monocytes (44). Here we found that the pattern of higher TNF expression in Gr-1high compared with Gr-1low monocytes was maintained in vitro, irrespective of whether monocytes were stimulated in the PBMC-only or the PBMC-LEC culture settings. However, coculture of PBMC with LEC did produce a markedly enhanced expression of TNF, consistent with the

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**Fig. 5.** LPS-induced p38 and MK2 activation in monocytes subsets in vitro. PBMC-LEC cocultures (A and C) or PBMC-only cultures (B and D) were stimulated with LPS (100 ng/ml), and cells were fixed and permeabilized directly after recovery at the time points indicated. Activated p38 levels (A and B) were determined in monocyte subsets by using a PE-conjugated anti-phospho p38 MAb (pT180/pY182) and shown as MFI with values of isotype control subtracted (n = 4/each time point). Activated MK2 levels (C and D) were determined by using an Alexa Fluor 647-conjugated anti-phospho-MK2 polyclonal antibody with MFI values of a normal rabbit polyclonal control subtracted (n = 4/each time point). ***P<0.001 Gr-1high vs. Gr-1low. Representative histograms are shown in each panel for phospho-p38 and phospho-MK2 levels at 15 min post-LPS treatment (black line), with isotype control antibody (gray fill) for comparison.
previous evidence of enhanced TNF expression by human monocytes in contact with human umbilical vein endothelial cells (22). These in vitro findings suggest that prolonged contact of lung-marginated monocytes with the endothelium could be responsible for the priming effect we observed previously in endotoxemic mice following secondary LPS challenge (44) and support the emerging role of monocytes in other “two-hit” models where a priming step is essential for induction of ALI (11, 25, 52).

To assess the upstream mechanisms determining such differential monocyte responses, we examined the p38 signaling pathway because of its previously described important role in LPS-induced TNF expression (27, 36). Almost complete attenuation of early TNF expression in both subsets with two different p38 inhibitors indicated that response heterogeneity was unlikely to depend on alternative or differential signaling pathway usage by each subset. By contrast, such differential signaling pathway usage has been described in human monocyte subsets by Cros et al. (13), where stimulation with TLR7/8 agonists produced preferential p38 MAPK and p42 MEK1 pathway-dependent activation and cytokine expression in CD14+ and CD14dim subsets, respectively. We extended our observations on differential TNF expression to demonstrate that additional proinflammatory genes (IL-6, COX-2, and iNOS), which are central to the LPS-induced monocyte inflammatory response and have been shown to be regulated by the p38 pathway (14, 49), are also preferentially expressed in Gr-1high monocytes during coculture, and their expression was substantially reduced by p38 inhibition. The higher proinflammatory capacity of Gr-1high monocytes indicates that, in addition to their preferential margination in the lungs, they would also produce the bulk of key monocyte-derived mediators implicated in ALI. Although signaling by other MAPK contributes to LPS-induced expression of these genes, e.g., optimal COX-2 expression has been reported to require both p38 and ERK activation in a mouse macrophage cell line (29), a recent study demonstrated that p38 may play the dominant role in determining LPS-induced cytokine release by primary human monocytes, whereas combined p38 and ERK signaling assumes more importance in differentiated monocytes and mature tissue macrophages (55).

Fig. 6. LPS-induced TNF, phospho-p38, and phospho-MK-2 expression in lung-marginated monocyte subsets in vivo. A: to evaluate lung-marginated monocyte subset activation in situ, mice were injected with LPS (20 ng iv) to induce enhanced and prolonged margination and then rechallenged with LPS at 120 min (100 ng iv), followed by lung excision and immediate homogenization in the presence of fixation and permeabilization buffer. This procedure resulted in the loss of Gr-1high monocyte recognition by the Gr-1 antibody, presumably because of a complete denaturation of the anti-Gr-1 (Ly-6C/Ly-6G reactive) Ly-6C epitope. A: a new staining and gating strategy was developed in which monocytes and neutrophils were first gated in lung cell suspensions as CD11b+ NK1.1+ events (R1). Then neutrophils (Ly-6Cmedium, Ly-6G+−) were identified by using either anti-Ly-6G or Gr-1 (R4/R7), whereas monocyte subsets (Ly-6Chigh/Low, Ly-6G−) could be identified by the anti-Ly-6C MAb (R2/R5 and R3/R6) but were not recognized by the Gr-1 MAb. B: total TNF (membrane + intracellular) expression was determined at a 150-min time point after the initial LPS pretreatment; 1) with no further treatment (control 1-hit, monocyte margination only); 2) at 30 min after the secondary LPS challenge (2-hit); or 3) at 30 min after the secondary LPS challenge, with treatment with SB203580 iv 15 min before the secondary LPS (2 hit + p38 inhibition). C and D: phospho-p38 and phospho-MK2 were determined at a 135-min time point after the initial LPS pretreatment with no further treatment (control 1 hit), or at 15 min after secondary LPS challenge (2 hit). Binding of fluorophore-conjugated MAbs is expressed as MFI with values of isotype control subtracted (n = 3–6/each time point). *P < 0.05, **P < 0.01, ***P < 0.001.

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Quantification of phospho-kinase levels by flow cytometry is a relatively new analytic tool for investigating intracellular signaling and has been validated in numerous studies (12, 30, 34, 47, 59). By use of this technique to evaluate kinase activation kinetics in monocyte subsets, levels of phosphorylated, activated-form p38 were found to be markedly higher in the Gr-1\textsuperscript{high} than the Gr-1\textsuperscript{low} monocytes following LPS stimulation both in vitro and in vivo. Levels of MK2 mirrored those of p38, confirming that p38 activity was higher in Gr-1\textsuperscript{high} monocytes and linking the mechanism of enhanced gene expression to that of MK2-dependent posttranscriptional regulation, presumably through enhanced RNA stability and translation of genes (e.g., TNF and IL-6) that contain AU-rich elements in their 3′-untranslated region (33, 43). A recent flow cytometric study by Zhao et al. (59) showed no apparent heterogeneity of constitutive p38 expression or p38 and MK2 phosphorylation among CD11b\textsuperscript{+} monocytes in response to anisomycin stimulation. The difference observed in that study could reflect the requirement for more detailed monocyte subset markers (e.g., Gr-1 or Ly-6C) to provide a clearer resolution of subset phospho-protein levels or for a more physiological stimulus (e.g., LPS compared with anisomycin) to reveal response heterogeneity.

Heterogeneity of the human monocyte subset proinflammatory response is well established, but the data are restricted to in vitro experimentation and there is some inconsistency as to which subset may be considered more proinflammatory in the literature (6, 13, 40, 51, 60). Discrepancies in mouse subset phenotype are also apparent when comparing responses under different conditions, with in vitro whole blood assays (10) displaying a different subset response profile from those observed here (in vitro and in vivo), in purified monocyte subsets in vitro (13), or in tissue-migrated monocytes in vivo (3, 42). Thus, in addition to any intrinsic heterogeneity in their proinflammatory response, stimulus and environment-determined factors are likely to be critical in influencing monocyte responsiveness in vivo. For example, striking differences between the in vivo and ex vivo LPS-induced p38 phosphorylation levels and patterns were observed in splenic CD11b-positive subpopulations (35). Therefore, defining the mechanisms responsible for differential p38 pathway activation in monocyte subsets during systemic inflammation in vivo will require due attention to the role that environment-derived signals have, which may vary spatially both within the vasculature and after monocyte emigration, for instance within the alveolar space in contact with epithelial cells.

Enhancement of myeloid cell activation by culture with endothelial cells and matrix components has been widely reported and considered to be important in the process of migration and extravasation to local sites of inflammation. In the case of monocytes in coculture with endothelial cells, higher expression of mediators in addition to TNF has been found, including IL-8, monocyte chemoattractant protein-1, tissue factor, and COX-2 (32, 38, 39), but the comparative effects on subset responses have not been previously described. We observed a marked elevation of LPS-induced cumulative expression of IL-6, iNOS, and COX-2 over 4 h in mouse monocytes in coculture, compared with the undetectable or negligible levels in PBMC-only culture, with expression of these genes substantially higher in Gr-1\textsuperscript{high} than Gr-1\textsuperscript{low} subsets. In light of the high propensity of monocytes, in particular Gr-1\textsuperscript{high} subsets, to migrate to the pulmonary capillaries for prolonged periods when activated (20, 44, 45), regulation of monocyte responses by endothelial cell contact should be considered as an important factor to determine the intensity of systemic inflammatory responses and organ inflammation, in addition to its role in the process of extravasation.

In summary, our findings indicated that monocytes’ interactions with the pulmonary endothelium, promoted by the margination response to intravascular stimuli, could have a critical role in regulating the monocyte responsiveness and, in turn, the development of ALI. Our findings also indicate that differential activation of the p38/MK2 pathway in subsets is the major determinant of both response intensity and heterogeneity. Previous reports have demonstrated a role for the p38/MK2 pathway in mouse models of ALI (4, 17, 37), which, taken together with our findings here and previously (44, 58), points to the Gr-1\textsuperscript{high} monocytes as important effectors of ALI. The diverse substrate specificity of MK2, including target molecules implicated in cell migration and phagocytosis (21), suggests that preferential p38/MK2 activation in Gr-1\textsuperscript{high} monocytes may be a central coordinator of monocyte subset behavior during infection and inflammation. By pinpointing p38/MK2 as a central hub defining the monocyte subset proinflammatory response, further insights may be gained into the specific roles of lung-marginated Gr-1\textsuperscript{high} monocytes in the propagation and development of ALI.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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