Effect of Acute Lung Injury on VLA-4 and CXCR4 Expression in Resident and Circulating Hematopoietic Stem/Progenitor Cells

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Key Words

Acute lung injury • Chemotaxis • Hematopoietic stem/progenitor cells • Integrins • L-selectin

Abstract

Background: The effect of acute lung injury on adhesion molecule expression in hematopoietic stem/progenitor cells (HSPCs) is poorly understood. **Objectives:** The aim of this study was to determine whether there is a relationship between pulmonary inflammation, expression of VLA-4 (CD49d), LFA-1 (CD11a), L-selectin (CD62L), CXCR4, and chemotaxis in resident HSPCs, as well as the level of circulating HSPCs. **Methods:** Following intratracheal administration of a single LPS bolus in C57Bl/6 mice, the number of inflammatory cells, differential counts, and amounts of cytokines/chemokines were studied in cytopspins and bronchoalveolar lavage fluid (BALF) specimens. Expressions of adhesion molecules and CXCR4 were analyzed in HSPCs by flow cytometry, as well as SDF-1-directed chemotaxis. Levels of HSPCs in the blood were studied in ungated and circulating subpopulations. **Results:** In coincidence with a peak of airway neutrophils, cytokine (IL-1 β , TNF- α , and IL-6), chemokine (KC, MIP-2, and SDF-1) levels in BALF and the number of

marrow HSPCs expressing CD49d and CXCR4 significantly increased at 48 h. The number of CD49d- and CXCR4-positive HSPCs dropped at 72 h. The HSPC subset comprising bigger cells behaved the same for CD49d. Chemotaxis of the marrow HSPC subset of bigger cells was higher in LPS-treated animals than in controls at 72 h. Finally, we could detect a significant decrease in circulating Sca-1⁺ cells in the mononuclear population at 72 h in LPS-treated mice. **Conclusions:** Our data provide evidence for a temporal relationship between pulmonary inflammation, CD49d and CXCR4 expression fluctuation in resident HSPCs, and the level of circulating HSPCs.

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Introduction

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome, is a clinical syndrome whose pathological features are: increased endothelial and epithelial permeability, alveolar edema, elevat-

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ed increase in cytokine concentrations in the lung, and neutrophilic alveolar infiltrates [1]. One of the best models reproducing the human disease is the administration of lipopolysaccharide (LPS) in the mouse lung [2, 3]. In the airways, inflammatory stimuli, such as a microbes or LPS, induce a rapid and massive release of neutrophils from the bone marrow [4]. Later, monocytes, lymphocytes, and platelets are also released. The pulmonary recruitment of these cells is important for host defense and resolution of an inflammatory response. Moreover, inflammatory stimuli, such as LPS, a component of Gram-negative bacterial cell walls, in the lung airways induce a rapid mobilization of bone marrow-derived endothelial progenitor cells (EPCs) into the circulation of mice. EPCs then accumulate within the inflammatory site and differentiate to become endothelial and epithelial cells [5]. Human studies have also indicated that EPCs are important and required for lung repair after bacteria-induced lung injury [6]. Although the critical role of hematopoietic stem/progenitor cells (HSPCs) in initiating LPS-induced neutrophil recruitment from the vascular space to the airspace has been documented [7], likely contributing to the macrophage population, the role of adhesion molecules in their egress from the bone marrow is not understood in this model of pulmonary inflammation. The current view regarding the retention of HSPCs within the bone marrow stem cell niche is that it depends mainly on two molecular pathways: the adhesion molecules $\alpha_4\beta_1$ -integrin [very late activation antigen-4 (VLA-4) or CD49d/CD29] and vascular cell adhesion molecule (VCAM)-1, and the CXCR4-stromal derived factor-1 (SDF-1 or CXCL12) chemokine pathways [8, 9]. Disruption of the VLA-4-VCAM-1 and CXCR4-CXCL12 axes by blocking antibodies [10] and functional antagonists [11] and in genetically deficient mice [12–14] has resulted in HSPC mobilization. Additional pathways probably participate in a cooperative or synergistic fashion [reviewed in 15]. The role of the so-called leukocyte integrins that share the common β_2 (CD18) chain, $\alpha_L\beta_2$ [lymphocyte function-associated antigen-1 (LFA-1) or CD11a/CD18] and $\alpha_M\beta_2$ (macrophage-1 antigen, CD11b/CD18), is still under debate. Although some studies have reported their involvement in HSPC retention [16, 17], others have indicated that the effect of β_2 -integrins becomes apparent only in synergy with $\alpha_4\beta_1$ [18]. HSPCs have also been shown to express L-selectin [19, 20]; however, the role of L-selectin in HSPC mobilization is still controversial [reviewed in 8, 21].

We have correlated the expression of adhesion molecules on resident and circulating HSPCs with pulmonary inflammation determined by LPS administration. This

study provides evidence of a temporal relationship between pulmonary inflammation, expression of VLA-4 and CXCR4, chemotaxis of resident marrow HSPCs, and the number of HSPCs in the blood.

Materials and Methods

Model of ALI

All animal procedures were approved and controlled by the Institutional Review Board of the University of Foggia/Istituto Zooprofilattico di Puglia and Basilicata and by the Italian Ministry of Health and carried out according to the guidelines of the Italian law on protection of animal life and the Helsinki Declaration. The experiments were performed using 6- to 8-week old male C57Bl/6 mice (Charles River Laboratories, Calco, Italy) with a body weight of 23–27 g. They were housed and handled according to institutional guidelines which comply with Italian legislation and were given food and water ad libitum.

ALI was induced by intratracheal administration of LPS from *Pseudomonas aeruginosa* (serotype 10; Sigma). Mice were anesthetized intraperitoneally with 2,2,2-tribromoethanol (Avertin, prepared 2.5% weight/volume in PBS and used 0.015 ml/g body weight; Sigma) and restrained in a vertical position with their necks tilted back so as to result in an accessible position of the trachea for intubation. Using two pairs of tweezers, the mouth was carefully opened and the tongue was gently pulled out. Then, each animal received a single 50- μ l bolus of LPS in saline (20 μ g) or 50 μ l of saline (control mice) introduced into the trachea using a 22-gauge intravascular cannula sleeve with the needle removed (25 mm, 0.9 mm OD, 0.6 mm ID; Baxter, Germany). To avoid false intubation into the esophagus, the trachea was illuminated via an otoscope held perpendicularly to the animal's throat, producing a well-illuminated trachea through dispersed light.

To obtain bronchoalveolar lavage fluid (BALF) specimens, the trachea was exposed through a midline incision and cannulated with a sterile 23-gauge needle. BAL was performed by instilling 0.9% NaCl in two separate 1-ml aliquots. The fluid was recovered by gentle suction and placed on ice for immediate processing. An aliquot of the BALF was processed immediately for total and differential cell counts. Total cell counts were performed with a hemocytometer, whereas differential cell counts were performed on cytopspin preparations stained with the May-Grunwald Giemsa stain. The remainder of the lavage fluid was spun at 200 g for 10 min, and supernatant was removed aseptically and stored in individual aliquots at -80°C .

After lavage, lungs were removed and fixed in formaldehyde. The fixed samples were embedded in paraffin, thin-sectioned, and mounted on glass microscope slides using standard histopathological techniques. Serum was obtained from blood drawn by cardiac puncture of anesthetized mice.

The amounts of cytokines and chemokines were determined in BALF supernatants using commercially available multiplex sandwich ELISA according to the protocol provided by the manufacturer (Searchlight Mouse Chemokine/Cytokine Array; Thermo Fisher Scientific, Waltham, Mass., USA). SDF-1 was measured in serum samples by commercial ELISA (Cusabio Biotech, Wuhan, PR China).

Bone Marrow Cell Isolation

Both ends of the femur and tibia of the sacrificed mice, as described above, were removed and the bone marrow was flushed out with PBS using a needle. Recovered total bone marrow cells were filtered (Cell trics 100 mm; Partec, Münster, Germany), centrifuged, washed, and resuspended in PBS supplemented with 2% FBS and 1 mM EDTA. The total number of cells was determined with a Burkert cytometer. Positive selection of bone marrow stem/progenitor cells by EasySep (Mouse Sca1 selection kit; StemCell Technologies) was done accordingly to the manufacturer's instructions. Briefly, recovered total bone marrow cells, resuspended in the appropriate medium, were incubated with the anti-Sca-1; PE-conjugated antibody was added to isolated bone marrow cells. Then, tetrameric antibody complex, which recognizes both PE and dextran, was added, followed by the addition of dextran-coated magnetic nanoparticles. Magnetically labeled target cells were then separated from unlabeled unwanted cells using the EasySep magnet by pouring the unlabeled cells off.

Immunostaining and Cytofluorimetric Analysis of Sca-1⁺ HSPCs

For each time point, 3–4 mice in the saline- and LPS-treated experimental groups were used to obtain a pool of HSPCs. Aliquots of 2×10^5 Sca-1⁺ HSPCs were pelleted by centrifugation at 337 g for 10 min at +4°C, resuspended in 100 µl of PBS, and then incubated with FITC-conjugated monoclonal antibodies (used at 1:200) for 30 min at +4°C. The monoclonal antibodies tested were: rat IgG2b κ anti-mouse CD49d, rat IgG2a κ anti-mouse CD11a, rat IgG2a κ anti-mouse CD62L, and rat IgG2b κ anti-mouse CXCR4. Isotype control antibodies were also used. All antibodies were purchased from BioLegend (San Diego, Calif., USA), except for anti-CXCR4 antibody which was from eBioscience (San Diego, Calif., USA). After incubation, the cells were pelleted, washed twice with PBS, and analyzed by fluorescence-activated cell sorting (FACS) with an EPICS XL MCL flow cytometer (Beckman Coulter, Fullerton, Calif., USA).

Ki67 Cytofluorimetric Assay

For each time point, 3–4 mice in the saline- and LPS-treated experimental groups were used to obtain a pool of HSPCs. Sca-1⁺ HSPCs (2×10^5) were fixed in PBS containing 3% paraformaldehyde and 2% sucrose for 5 min at room temperature, permeabilized in PBS containing 20 mM Hepes, 300 mM sucrose, 5 mM NaCl, 3 mM MgCl₂, and 0.5% Triton-X100 for 5 min at room temperature, incubated with rabbit monoclonal anti-Ki67 antibody (FITC-conjugated) (used at 1:100; Abcam, Cambridge, UK) for 30 min at +4°C, and then analyzed by flow cytofluorimetry. An isotype control antibody was also used.

Immunostaining and FACS Analysis of Circulating Sca-1⁺ Cells

For each group per time point, 3–4 mice in the saline- and LPS-treated experimental groups were analyzed. A blood aliquot of 100 µl, obtained from each mouse, was incubated with rat IgG2a κ anti-Sca-1 PE-conjugated antibody (used at 1:100; StemCell Technologies) and either a cocktail of antibodies against B and T lymphocytes and NK cells (mouse IgG2a κ anti-mouse NK-1.1, rat IgG2a κ anti-mouse CD19, and Armenian hamster IgG anti-mouse CD3ε), or rat IgG2b κ anti-mouse

CD11b for monocytes/macrophages, or rat IgG2b κ anti-mouse Ly-6G/Ly-6C (Gr-1) for granulocytes. In parallel, other blood aliquots were incubated with isotype control antibodies. All monoclonal antibodies identifying blood cell populations were FITC-conjugated (BioLegend) and used at 1:200 for 30 min at +4°C. Then, erythrocytes were lysed using the IOTest 3 lysing solution (Immunotech SAS, Beckman Coulter Company, Marseille, France) according to the manufacturer's instructions, and washed and resuspended in PBS for flow cytometer analysis. Dot plots for lineage marker and side scatter were obtained in order to gate lymphocytes, monocytes, and granulocytes. Identified populations were analyzed for Sca-1 expression. The final analysis of double positive cells for Sca-1 and each lineage marker was carried out on ungated lymphomonocytes and granulocytes. The number of granulocytes was obtained by studying the cell population stained with anti-Ly-6G/Ly-6C antibody and with high side scatter.

To identify circulating Sca-1⁺ cells expressing adhesion molecules, blood samples were incubated with anti-Sca-1 antibody and either FITC-conjugated anti-mouse CD49d, CD11a or CD62L at a final dilution of 1:200 for 30 min at +4°C. Blood cell populations were identified by forward scatter and side scatter, gated, and used for analysis of double positive cells for Sca-1 and each adhesion molecule.

Transendothelial Chemotaxis Assay

To induce polarization, bovine aortic endothelial cells (bAEC; Lonza Walkersville, Inc., Walkersville, Md., USA) were seeded into the upper chamber of Transwells with a diameter of 6.5 mm and pores of 3 µm (Costar; Corning Incorporated, USA) and grown for 5 days. Under these conditions, cells grow as a polarized sheet of cells and develop a transendothelial resistance of 50 Ω × cm² on average, as measured by a volt ohm meter (Millicell-ERS; Millipore, Vimodrone, Italy).

For each time point, 3–4 mice in the saline- and LPS-treated experimental groups were used to obtain a pool of HSPCs. Sca-1⁺ HSPCs were resuspended in endothelial cell basal medium (EBM; Lonza) and a volume of 300 µl containing 5×10^5 cells was loaded into the upper chamber of the Transwell. EBM (600 µl) with or without recombinant mouse CXCL12/SDF-1 (200 ng/ml; R&D Systems) was added to the bottom chamber. The assays were performed at 37°C for 4 h. Transmigrating cells (bottom chamber) were counted for 120 s using a flow cytometer.

Statistical Analysis

Data were evaluated using Prism 4 (GraphPad Software, Inc., La Jolla, Calif., USA). All results are expressed as means ± SD. The statistical significance of the differences between the mean values of the groups was analyzed by the unpaired t test and ANOVA (Tukey's multiple comparison test as post hoc). The Spearman rank test was used for correlation of pulmonary inflammatory parameters with antigen expression by HSPCs, number of circulating cells in blood, and serum SDF-1, and the Spearman correlation coefficient is reported (r_s). Correlation analysis was performed among experimental groups which presented all parameters. $p < 0.05$ was considered statistically significant.

Fig. 1. Inflammatory cells in the BALF of saline-treated mice (controls) and LPS-treated mice. Total (**a**) and differential (**b**, **c**) counts of inflammatory cells were analyzed on cytospins. LPS-treated mice showed a higher total number of inflammatory cells than saline-treated mice at 24 h ($p < 0.05$) and 48 h ($p < 0.001$). Neutrophils made up the prevalent cell type in LPS-treated mice (**c**) at 48 and 72 h, whereas macrophages were the only cell type found in saline-treated mice at all time points (**b**). $n = 11$ –16 per time point. * $p < 0.05$; ** $p < 0.001$, treated vs. controls.

Results

LPS Induces a Sustained Pulmonary Inflammation

In the first part of the study, we sought to characterize the model of pulmonary inflammation obtained by intratracheal instillation of LPS. Histopathologic examination of hematoxylin and eosin-stained lung tissue sections of mice treated with LPS showed massive bronchopneumonia with peribronchial accumulation of inflammatory cells at 24 h posttreatment. The inflammation persisted at 48 and 72 h. No apparent increase in inflammatory cells was detected in the lungs of saline-treated mice (not shown).

The type and time course changes of the inflammatory cells in BALF after intratracheal instillation with LPS and saline are shown in figure 1. The total number of inflammatory cells in the BALF of LPS-treated mice was significantly higher than those of saline-treated mice at 24 and 48 h posttreatment (fig. 1a). The number of inflammatory cells in BALF at 72 h posttreatment declined in LPS-treated mice. Neutrophils made up the majority of the inflammatory cells in the BALF of LPS-treated

mice at 48 and 72 h (70% of total number of cells; fig. 1c), whereas macrophages were the only represented cell type in the saline-treated group (fig. 1b).

A multi-parametric chemiluminescent ELISA was used to study inflammatory cytokines (IL-1 β , TNF- α , and IL-6) and chemokines (KC, MIP-2, and SDF-1) in BALF. IL-1 β , TNF- α , and IL-6 levels reached a peak at 48 h in LPS-treated mice (fig. 2a–c). This behavior was also observed for KC and MIP-2, two neutrophil chemokines (fig. 2d, e). Interestingly, SDF-1 levels remained high even at 72 h (fig. 2f).

Altogether, these results showed a sustained inflammatory condition for up to 72 h in the mouse lung, which was reflected by increased levels of cytokines and chemokines at the same time point.

VLA-4 Expression in HSPCs Fluctuates during Pulmonary Inflammation

We next determined the effect of sustained pulmonary inflammation on the expression of adhesion molecules in HSPCs isolated from total bone marrow by means of Sca-1 positive selection. Cytofluorimetric analysis revealed

Fig. 2. Cytokine and chemokine levels in BALF. BALF was obtained from LPS-treated and saline-treated mice (controls) at 24, 48, and 72 h. Cytokine (IL-1 β , TNF- α , and IL-6) and chemokine (KC, MIP-2, and SDF-1) levels were evaluated by a multiplex ELISA. All cytokines and chemokines showed a peak level at 48 h. $n = 4$ per time point. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$, treated vs. controls.

that the number of CD49d (VLA-4), CD11a (LFA-1), and CD62L (L-selectin)-positive cells peaked at 48 h. However, only CD49d-positive cells were significantly higher in LPS-treated mice than in saline-treated mice at 48 h ($p < 0.05$; fig. 3a), whereas CD11a- and CD62L-positive cells showed a trend toward increased levels but it was not significant (fig. 3b, c). At 72 h, the number of CD49d-positive cells decreased in LPS-treated mice and was not significant compared to control mice ($p > 0.05$). Since changes in adhesion receptor expression in HSPCs have been associated with the cell cycle [22], to investigate whether the increase in adhesion molecules is due to the proliferation of HSPCs, we studied Ki67 expression. The expression of this mitogenic marker did not vary among the different time points (fig. 3d). Thus, the inflammatory process in the lung seems to perturb the expression of adhesion molecules in bone marrow HSPCs without affecting mitosis.

We have previously shown that the Sca-1⁺ population in naïve mice is comprised of two subpopulations, one

smaller and one bigger, according to their physical parameters [23]. These two subsets were further analyzed for CD49d expression in the context of LPS-induced inflammation. The biggest population (R1) was comprised of cells always with a higher expression of CD49d than the smallest population (R2) both in controls and in LPS-treated mice (fig. 4). R1 cells showed higher levels of CD49d than R2 cells at 48 h post-LPS administration ($p < 0.05$), resembling the behavior of the total marrow population. At 72 h, the percentage of CD49d-positive cells decreased in LPS-treated mice and was not significant compared to control mice ($p > 0.05$). Although R2 cells also showed an increase at 48 h post-LPS administration compared to the control mice, this difference was not significant ($p > 0.05$).

Since CD49d was the adhesion molecule which presented significant fluctuation during the pulmonary inflammatory process, we sought to find any correlation with lung parameters. There was a positive correlation between expression of CD49d by resident HSPCs and

Fig. 3. Expression of adhesion molecules and Ki67 in HSPCs. CD49d (**a**), CD11a (**b**), and CD62L (**c**) expression was studied by cytofluorimetry in naïve mice and in mice treated either with saline (controls) or LPS (treated). Ki67 expression (**d**) was evaluated

in controls and treated mice. Data are expressed as numbers of events (**a–c**) or percentages of positive cells (**d**) and shown as the mean $\pm$ SD of 3 experiments (cell pools from 3–4 mice were used for each time point and each group). * $p < 0.05$; ** $p < 0.001$.

some pulmonary cytokine/chemokine levels in BALF: at 48 h with IL-1 β and MIP-2, and at 72 h with KC (all $r_s = 1$ and $p < 0.001$). Of note, CD49d expression in the R1 subpopulation of HSPCs correlated with SDF-1 at 48 h and with KC at 72 h (all $r_s = 1$ and $p < 0.001$).

CXCR4 Expression in HSPCs and SDF-1 Levels in Serum

We next addressed the question of whether the SDF-1 receptor CXCR4 expression was modified by LPS-induced pulmonary inflammation. Figure 5a shows that in LPS-treated mice the number of CXCR4-positive cells significantly increased at 48 h compared to control mice. The CXCR4 number of positive cells at 48 h was also significantly higher than at 24 h and compared to naïve

mice. No correlation was found between CXCR4 expression by HSPCs and lung inflammatory parameters.

To investigate the role of SDF-1 in the fluctuation of CD49d and CXCR4 in resident HSPCs, we next examined the serum levels of SDF-1, the ligand of CXCR4. The serum concentration of SDF-1 was higher in LPS-treated mice at all time points compared to control mice, reflecting BALF data (fig. 5b). SDF-1 in serum correlated positively with BALF SDF-1 at 72 h ($r_s = 1$ and $p < 0.001$).

SDF-1-Directed Chemotaxis of HSPCs

Previous studies have shown that the disruption of the CXCL12/CXCR4 axis in C57Bl/6 mice determines HSPCs mobilization [24]. To evaluate the function of CXCR4 on HSPCs during the development of LPS-induced pneumo-

Fig. 4. Expression of CD49d in HSPC subpopulations. HSPC subpopulations were studied for CD49d levels by cytofluorimetry at the indicated time points after saline (controls) or LPS (treated) intratracheal administration. Whole Sca-1⁺ cells were gated according to their forward and side scatter in two subsets: R1 and R2 (a). Anal-

yses of CD49d-positive cells in R1 and R2 gates at different time points and for each experimental group were then performed (b). Data are expressed as percentages of positive cells and shown as the mean $\pm$ SD of 3 experiments (cell pools from 3–4 mice were used for each time point and each group). * $p < 0.05$.

Fig. 5. SDF-1/CXCR4 axis during LPS-induced pulmonary inflammation. **a** CXCR4 expression was studied by cytofluorimetry in naïve mice and in mice treated either with saline (controls) or LPS (treated). Data are expressed as numbers of events and shown as the mean $\pm$ SD of 3 experiments (cell pools from 3–4 mice were used

for each time point and each group). * $p < 0.005$. **b** Serum levels of SDF-1 protein were obtained from naïve mice and mice treated either with saline (controls) or LPS (treated). Data are expressed as picograms per milliliter and shown as the mean $\pm$ SD of 3 experiments ($n = 6$ mice per each time point). * $p < 0.05$; ** $p < 0.001$.

nia, chemotaxis versus a CXCL12/SDF-1 gradient was investigated. To mimic the bone marrow vascular niche, Sca-1⁺ cells were allowed to migrate through an endothelial monolayer. SDF-1 increased the migration of Sca-1⁺ cells of naïve mice almost twelve-fold, although this dif-

ference was not statistically significant due to the big SD ($p > 0.05$; fig. 6a). There was a trend toward an increase in cell migration with time in both controls and treated mice; however, SDF-1-induced chemotaxis was significantly higher (6.5-fold) in treated animals compared to

Fig. 6. Effect of LPS administration on chemotaxis of HSPCs. **a** HSPCs were allowed to migrate through the bAEC monolayer against an SDF-1 gradient for 4 h and cells that migrated to the bottom of the Transwells were quantified by cytofluorimetry (cell pools from 3–4 mice were used for each time point and each group). Migrated cells were analyzed for R1 and R2 subpopulations in control wells without SDF-1 (**b**) and in SDF-1-containing wells (**c**). * $p < 0.05$; ** $p < 0.001$.

controls at 72 h ($p < 0.05$). We sought to understand whether the two HSPC subpopulations were differently able to migrate against the SDF-1 gradient. It was the case since only R1 bigger cells were found in the lower well in SDF-1-stimulated chemotaxis (fig. 6c), while this different behavior was not observed in unstimulated HSPCs (fig. 6b).

Levels of Circulating Sca-1⁺ HSPCs and Adhesion Molecule Expression

To study the level of circulating HSPCs during the inflammatory response in the lung, the number of HSPCs, as identified by the expression of the Sca-1 antigen, was studied in the blood. LPS administration determined significant neutrophilia at 48 h compared to controls, which persisted at 72 h (fig. 7a). Naïve mice presented $6.6 \pm$

2.5% , $7.0 \pm 3.0\%$, and $0.4 \pm 0.2\%$ (mean $\pm$ SD) of Sca-1⁺ in the ungated, lymphomonocyte, and granulocyte populations, respectively, showing that almost all circulating HSPCs were in the mononuclear population. We could detect a significant decrease in Sca-1⁺ cells in the ungated population and mononuclear cells at 72 h following LPS administration compared to controls (fig. 7b, c). Although, for unknown reasons, the number of Sca-1⁺ cells was high when studied in the ungated and lymphomonocyte populations of control mice at 72 h, this was not statistically significant compared to all of the other time points. Interestingly, the number of Sca-1⁺ cells in the granulocyte population significantly increased at 48 h (fig. 7d), in parallel with the increase in absolute numbers of blood granulocytes. The levels of adhesion molecules were studied on Sca-1⁺ cells. Although there was a trend towards a decrease

Fig. 7. Effect of LPS administration on circulating Sca-1⁺ cells. Numbers of granulocytes (**a**), Sca-1⁺ in ungated blood cells (**b**), LMN (**c**), and granulocytes (**d**) were evaluated in naïve mice and in saline- (controls) and LPS-treated mice at different time points. The number of granulocytes was obtained on the whole blood cell population stained with anti-Ly-6G/Ly-6C antibody and with

high side scatter. The analysis of double positive cells for Sca-1 and lineage markers for LMN (CD19, CD3 ϵ , NK-1.1, and CD11b) and granulocytes was carried out as described in Materials and Methods. Data are shown as the mean $\pm$ SD of 5 experiments ($n = 5$ for naïve mice and $n = 3-4$ for each time point and each group). * $p < 0.05$; ** $p < 0.001$.

at 48 h in the number of Sca-1⁺ cells positive for CD11a and CD49d, this effect did not yield significance in comparison with untreated mice (not shown).

We sought also to determine any correlation between circulating cells and pulmonary inflammatory markers. We found that there was a positive correlation between the number of granulocytes and cytokine/chemokine levels in BALF: at 48 h with TNF- α and KC, and at 72 h with KC and SDF-1 (all $r_s = 1$ and $p < 0.001$). A positive

correlation was found also between Sca-1⁺ cells in the ungated population and KC BALF levels at 72 h, and between Sca-1⁺ cells in the lymphomonocyte population and the total number of inflammatory cells and IL-6 BALF levels at 72 h (all $r_s = 1$ and $p < 0.001$). A significant positive correlation was found between SDF-1 serum levels and the number of Sca-1-positive cells in the ungated and lymphomonocyte blood population at 72 h (both with $r_s = 1$ and $p < 0.001$).

Discussion

In this report we document a temporal relationship between a pulmonary increase in inflammatory cytokines and chemokines and VLA-4 and CXCR4 on marrow HSPCs at 48 h and increased chemotaxis of HSPCs in conjunction with decreased levels of circulating stem/progenitor cells at 72 h in a model of acute pulmonary inflammation. So far, the role of neutrophils in this model has been investigated in terms of an acute inflammatory response (up to 24 h) and not as persistent inflammation [25]. Others have studied the mobilization of EPCs in the LPS model at 1 week and found that EPCs increased in the circulation and homed to the lung giving rise to alveolar epithelial and endothelial cells [5], but the role of HSPCs was not studied. However, the significance of circulating neutrophils was substantiated in the lung inflammatory model presented in this paper by the positive correlation we found with BAL levels of KC and TNF- α at 48 h and of KC and SDF-1 at 72 h.

The interaction between VLA-4 ($\alpha_4\beta_1$) on HSPCs and VCAM-1 on stromal cells has been implicated in the retention of HSPCs in the bone marrow [26–29]. Disruption of this interaction by neutralizing antibodies directed against VLA-4 or VCAM-1 [16] or bone marrow neutrophils [30] resulted in HSPC mobilization. In the present study, the elevation of VLA-4 expression in HSPCs (fig. 4) is coincident with the cytokine/chemokine increase in BALF (fig. 3), and precedes increased HSPC chemotaxis along an SDF-1 gradient at 72 h (fig. 6), when VLA-4 returns to basal levels. Overall, these results suggest that cytokines/chemokines are involved in the initial increase in VLA-4 levels on marrow stem/progenitor cells and that, in coincidence with the drop of VLA-4 at 72 h, this and other factors are involved in the mobilization of HSPCs. This hypothesis is strengthened by the finding of a positive correlation between the number of HSPCs positive for CD49d and chemokine levels in BALF, indicating that MIP-2 and KC are involved, besides neutrophil egress from the bone marrow, also in preparing HSPCs to exit from the bone marrow.

Although HSPC proliferation is associated with their mobilization from the bone marrow [15, 21, 31, 32], we did not find any change in Ki67⁺ HSPCs upon LPS challenge (fig. 3). Ki67 nuclear staining has been reported to agree with conventional proliferation assays (BrdU incorporation and dye dilution) in assaying lymphoproliferation [33]. Furthermore, the low percentages of Ki67⁺ HSPCs found in this study are in line with previous reports showing that the mouse HSPCs are largely quiescent with

a small percentage in the S/G2/M phase [34]. These observations are not unexpected since it has been previously shown that different mobilization protocols may be independent of cell cycle status [35].

In humans, the expression of β_2 -integrin LFA-1 ($\alpha_L\beta_2$; CD11a/CD18) is confined to the more mature HSPCs, and blocking LFA-1 with neutralizing antibodies completely prevented the IL-8-induced mobilization of HSPCs with colony-forming or radioprotective capacity in mice. However, the role of β_2 -integrins in the adhesion of HSPCs to stromal cells is controversial. Teixidó et al. [28] found that anti- β_2 antibodies partially blocked the adhesion of CD34^{high} cells to stromal cells, while antibodies directed against β_2 -integrins were unable to mobilize progenitors into the bloodstream [10, 36]. It has been hypothesized that abrogation of β_2 -integrin function contributes to significant mobilization when $\alpha_4\beta_1$ -integrin is concurrently inhibited [37]. Our data, showing that CD11a levels do not change during a sustained pulmonary inflammation, suggest that LFA-1 is not likely involved in HSPC mobilization in this model.

L-selectin expression is higher on the more primitive population of mobilized CD34⁺ cells [19], and the degree of hematopoietic recovery following chemotherapy correlates with the expression of L-selectin on CD34⁺ human HSPCs [20], although L-selectin does not appear to contribute to HSPC interactions in bone marrow microvessels [38] and in fucoidan-induced mobilization in mice [39]. To account for these differences, it should be considered that Sca-1 in mice is similar to c-kit in detecting very primitive hematopoietic stem cells (HSCs) whereas CD34 recognizes a pool of progenitor cells (including EPCs) able to repopulate the bone marrow upon transplantation in humans, although it does not necessarily identify true stem cells. Nevertheless, in the model of pulmonary inflammation used in this study, it seems that L-selectin does not play a significant role, like in CD11a, and these data indicate that a selective effect on VLA-4 has been obtained. It has been shown that mobilized CD34⁺ cells show a different pattern of expression for LFA-1, VLA-4, and L-selectin [19]. Overall, these data indicate that, like for the homing process, the mobilization of HSPCs from the bone marrow is dependent on a different set of adhesion molecules belonging to either selectin or integrin families.

HSPCs can be mobilized from the bone marrow parenchyma to the circulation by various agonists whose common downstream action leads to alteration of the expression or function of the chemokine CXCL12/SDF-1 and adhesion molecules mediating migration [8, 40]. At

least two different mechanisms disrupt the CXCL12-CXCR4 chemotactic axis during HSC egress. First, CXCL12 and CXCR4 reductions coincide with the induction of proteolytic activity in the bone marrow [41]. The second mechanism involves the desensitization of CXCR4 and consequent stem cell entry into the circulation [42]. Irrespective of the mechanism, our data indicate that CXCR4 expression fluctuates during LPS-induced pulmonary inflammation and that, after an initial increase at 48 h, its levels decrease, although not sharply, at 72 h. At this time point, a significant increase in HSPC chemotaxis was observed. It should be stressed that bovine and murine endothelial cell types allow adhesion of murine progenitor T cells in the same amount and with the same molecular mechanism, i.e. CD49d dependent [43]. So, bovine endothelial cells are an appropriate cell model to investigate murine HSPC chemotaxis.

On the other hand, CXCL12 is released from injured lungs to recruit CXCR4 bone marrow-derived HSPCs in murine models of pulmonary fibrosis [44, 45], indicating that the CXCL12/CXCR4 axis is critical in the bone marrow response to some kind of lung injury. Thus, it seems that a balance between bone marrow and peripheral CXCL12 levels is critical for HSPC mobilization from bone marrow to the injured tissues. In the model presented in this paper, serum SDF-1 levels significantly increased in LPS-treated mice at all time points compared to the control group. However, blocking SDF-1 with an intravenously administered blocking antibody did not exert any effect on CD49d expressed on whole HSPCs and R1/R2 subpopulations at 48 h following LPS treatment (data not shown), and these data suggest that the marrow SDF-1 gradient was not disrupted. Alternatively, other chemokines, such as KC and MIP-2 [46, 47], could be relevant for HSPC mobilization in this model (see discussion above). Indeed, the absolute number of granulocytes and the number of Sca-1⁺ cells in the granulocyte population were increased by the LPS treatment at 48 h. Overall, we can only suggest that HSPCs are induced to migrate from the bone marrow into the blood circulation along chemotactic gradients upon the induction of ALI and are likely endowed with different properties depending on which blood population they belong to. Indeed, Sca-1⁺ cells in the ungated population correlated with KC BALF levels at 72 h, and the number of Sca-1⁺ cells in the ungated and lymphomonocyte blood population correlated with SDF-1 serum levels at 72 h. Altogether, these data indicate a relevance of KC and SDF-1 for HSPC mobilization from the marrow to the blood at later time points.

The apparent discrepancy between the increase in resident HSPC chemotaxis and the decrease in circulating HSPCs at 72 h may reflect different *in vitro* and *in vivo* kinetics. Alternatively, HSPCs entered into the circulation migrate faster out of the blood, and their decrease may indicate their entry into the lung. Thus, we sought to determine whether these cells could be found at the lung level by investigating a specific marker. ALDH 9 family member A1 (ALDH9A1) is expressed by HSPCs [48] and at very low levels in the lung [49]. Thus, the influx of HSPCs into the lung was studied by analyzing the levels of ALDH9A1 in lung homogenates following LPS treatment. However, we could not detect any difference between control and treated mice at any time points. These data strongly suggest that if a migration of circulating Sca-1-positive cells happened in the time frame of the experiments presented in this paper, the level of engraftment was much too low to be detected by this assay. These results are in line with those presented by our and other groups demonstrating that the level of engraftment is not higher than 0.01–0.025% of the total lung cells [reviewed in 50].

Many studies point to intrinsic heterogeneity in HSCs [51–54], and even in functionally identified stem cell populations, cellular and molecular properties and behavior can be diverse [54, 55]. We have previously shown that resident HSPCs are comprised of two subsets as determined by physical parameters and mitochondrial content [23]. R1 (bigger) cells are less primitive in that they display higher levels of mitochondria, as also shown in human CD34⁺ cells [56]. The R1 subset of resident HSPCs showed the higher expression of CD49d at 48 h in LPS-injected mice compared to controls (fig. 5), similarly to the whole population, suggesting that R1 cells should preferentially migrate from the bone marrow. Indeed, the R1 subpopulation was found to transmigrate through the endothelium, a finding which is in agreement with the report of Mohle et al. [57] who found that transmigrated CD34⁺ cells expressed higher levels of CD38 compared with nonmigrating cells and may therefore represent predominantly committed progenitor cells. Interestingly, the number of R1 cells positive for CD49d correlated with SDF-1 and KC at 48 and 72 h, respectively, indicating a role for both of these chemokines in the mobilization of this subset of HSPCs; however, this hypothesis should be confirmed by further work.

In summary, our data are suggestive of a temporal relationship among elevation of inflammatory cytokines/chemokines in the lung, modification of CD49d and CXCR4 expression in resident marrow HSPCs, chemotax-

is of HSPCs, and a decrease in circulating Sca-1⁺ cells in the blood. We can only speculate that the HSPC subset with the bigger size is involved in the mobilization process. Their precise role in the early and late phases of the inflammatory process in the lung remains to be analyzed.

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