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Blood Monocyte Migration to Acute Lung Inflammation Involves Both CD11/CD18 and Very Late Activation Antigen-4-Dependent and Independent Pathways

Xian Chang Li,* Masayuki Miyasaka,† and Thomas B. Issekutz2*

In acute lung inflammation, blood neutrophils and monocytes migrate into the lung parenchyma and bronchoalveolar space. The infiltration of the inflamed lung by monocytes is poorly understood because of difficulties in quantifying these cells in the presence of resident macrophages. Radiolabeled monocytes were used to study monocyte migration into the inflamed rat lung. Monocytes and neutrophils were purified from blood, labeled with 51Cr and 111In, respectively, and injected i.v. into rats given an intratracheal injection of LPS. The accumulation of 51Cr-labeled monocytes increased >10-fold in the lung parenchyma and 170-fold in the bronchoalveolar lavage (BAL) 18 h after LPS. 111In-labeled neutrophils increased >30-fold in the lung tissue and 500-fold in the BAL. Treatment of rats with a blocking anti-CD18 mAb inhibited monocyte accumulation in the lung and BAL by about 30%, whereas blocking very late activation Ag-4 (VLA-4) had no effect. Combined blockade of VLA-4 and CD18 inhibited approximately 30% of the migration to the lung parenchyma, but decreased the BAL by 80%. Monocyte migration to cutaneous inflammation was completely abolished by the combined mAb treatment. Neutrophil accumulation in the lung and BAL was not decreased by blocking either CD18 or VLA-4 and was only partially reduced by blocking CD18 plus VLA-4. Thus, monocyte migration to the LPS inflamed lung is substantially CD11/CD18 and VLA-4 independent, but accumulation in BAL is mediated by CD18 and VLA-4. Monocytes as well as neutrophils may use a previously unrecognized endothelial adhesion and migration pathway in the lung.

neutrophils and monocytes (21). Monocytes probably play an important role in acute inflammatory lung injury given their capacity to elaborate inflammatory cytokines, growth factors, oxygen free radicals, and proteolytic enzymes (22–24). However, the possible adhesion pathways used by monocytes to migrate into lung inflammation has not been examined. In the present study we used a recently developed quantitative technique to study blood monocyte migration into the lung parenchyma and the bronchoalveolar space after i.t. LPS and compared the migration of monocytes to that of neutrophils. Our results demonstrate that the majority of blood monocytes appear to use a CD11/CD18- and VLA-4-independent adhesion pathway to migrate into the LPS-inflamed lung.

Materials and Methods

Animals

Inbred male Lewis rats, weighing 200–225 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and used in all experiments.

Reagents

LPS (E. coli 0111) was obtained from List Biologics (Campbell, CA). Hesperan, consisting of 6% hetastarch in 0.9% sodium chloride, was obtained from DuPont Canada (Scarborough, Canada). Percoll was obtained from Pharmacia (Uppsala, Sweden). Zymosan-activated serum (ZAS), a source of the chemotactic factor C5a, was generated by activating complement in normal rat serum with zymosan A (Sigma, St. Louis, MO).


mAbs and Ab treatment

The mAbs WT.3 (IgG1) binds to the common β-chain of the CD11/CD18 integrins (27). TA-2 mAb (IgG2a), which reacts with the αL chain of VLA-4, and an isotype control mAb B9 (IgG1) that recognizes pertussis toxin were generated in our laboratory (28, 29). WT.3 and TA-2 mAbs have been shown to block leukocyte adhesion and in vivo cell migration (15, 28, 30). F(ab’)2 were generated from TA-2 by pepsin digestion.

The effects of these mAbs on monocyte and neutrophil migration to the lung were studied by giving each rat 2 mg of mAbs WT.3, TA-2 F(ab’)2, a combination of WT.3 and TA-2 F(ab’)2, or the B9 control mAb i.v. immediately before the i.v. injection of radiolabeled cells. A second injection of TA-2 F(ab’)2 was given 8 h later. Measurement of the concentration of mAbs in the blood of the rats demonstrated that the dose of mAbs used produced plasma levels at least 5–10 times that required to saturate the receptors on the leukocytes in the blood. Treatment with higher doses of WT.3 and TA-2 F(ab’)2 were no more effective. None of the mAb treatments caused neutropenia, monocytopenia, lymphopenia, or clearance of radiolabeled cells from the circulation.

Lung and dermal inflammation

Rats were anesthetized with ketamine and xylazine as described above. Endotracheal intubation was performed through the mouth with a 60-cm polyethylene tube (PE-240), and a catheter was inserted into the trachea through the endotracheal tube so the tip of the catheter reached the carina. The rats then received 100 µg of LPS in 0.2 ml of saline through the i.t. catheter at the time of inspiration. Control animals were given 0.2 ml of saline intratracheally. Dermal inflammatory reactions in recipient rats were induced by intradermal injection of LPS and ZAS. Briefly, the skin on the back of the animals was shaved and injected with LPS (10 ng), ZAS (50%), and 0.05 ml of diluent as a control intradermally in triplicate sites.

Lung perfusion and bronchoalveolar lavage (BAL)

Rats were anesthetized, and a 21-gauge butterfly needle was inserted into the right ventricle of the heart. The abdominal aorta was incised to allow the perfusate to drain, and the lung vasculature was perfused with 50 ml of saline. BAL was performed immediately after the vascular perfusion to harvest cells from the bronchoalveolar space. Briefly, a polyethylene catheter attached to a three-way stopcock was inserted into the trachea through an incision, and the lavage was performed with 30 ml of saline. After recovering 26–28 ml of lavage fluid, the cell concentration was determined using a Coulter counter (Coulter Electronics, Luton, U.K.). The cells in the lavage fluid were harvested by centrifugation, and the radioactivity was measured using a 1480 Wizard gamma counter (Wallac, Turku, Finland).

In vivo cell migration assay

The in vivo cell migration assay was performed as previously described (8). Briefly, rats were injected i.v. with radiolabeled cells, followed by i.t. injection of LPS or saline as a control. In the same experiments, animals also received an i.v. injection of blocking or isotype control mAbs 10 min after the injection of radiolabeled cells. Intradermal injection of LPS, ZAS, and diluent controls on the back of the rats was performed after the i.t. injection. The rats were anesthetized 18 h later, and blood samples were collected by cardiac puncture. The lung vasculature was then flushed with saline, and the lungs were lavaged to collect cells that had accumulated in the bronchoalveolar space as described above. The lung tissue was harvested immediately after the perfusion and lavage, dissected into right and left lobes, and weighed, and the radioactivity in each lung was determined by gamma counting. In addition, in some experiments leukocyte migration was measured 18–36 h after i.t. LPS injection.

Pathology

Tissue samples were obtained from the lung at sacrifice, fixed in 10% buffered formaldehyde, and embedded in paraffin. The tissue samples were cut at 3 µm, stained with hematoxylin-eosin, and examined microscopically for evidence of cellular infiltrates and tissue damage.

Statistics

Data are expressed as the mean ± SEM, and statistical significance was determined using Student’s t test.
Results

Effect of i.t. LPS on monocyte and neutrophil accumulation in the lung parenchyma

Radiolabeled monocytes and neutrophils were used to quantify leukocyte migration from the blood into the lung in response to i.t. LPS. Blood monocytes were labeled with Na$_2^{51}$CrO$_4$, and blood neutrophils were labeled with $^{111}$InCl$_3$, then cells were injected i.v. into recipient rats given an i.t. injection of 100 µg of LPS or saline as a control. The accumulation of radiolabeled cells in the lung parenchyma was assessed 18 h later, since in pilot experiments this was the time of maximum leukocyte accumulation in the LPS-injected lung. The left lung in rats was almost twice the size of the right lung as demonstrated by its wet weight. As shown in Fig. 1A, the saline-injected control animals had about 3,500 and 6,500 cpm of $^{51}$Cr-labeled monocytes in the right and left lungs, respectively, which is equivalent to about $6.5 \times 10^5$ cpm/g (right) and $6.9 \times 10^5$ cpm/g (left) in each lung. The i.t. injection of LPS increased the accumulation of $^{51}$Cr-labeled monocytes in the lungs by 10- to 12-fold to about 55,000 cpm (6.7 by 10$^3$ cpm/g) and 74,000 cpm (5.3 by 10$^3$ cpm/g) in the right and left lungs, respectively ($p < 0.001$).

Control rats had about 2,500 cpm (4.4 by 10$^3$ cpm/g) and 5,000 cpm (4.7 by 10$^3$ cpm/g) of $^{111}$In-labeled neutrophils in the right and left lungs, respectively. The accumulation of $^{111}$In-labeled neutrophils in the lungs of LPS-injected animals increased 30- to 40-fold to about 99,000 cpm in the right lung (1.2 by 10$^5$ cpm/g) and to 136,000 cpm in the left lung (1.0 by 10$^5$ cpm/g; $p < 0.001$; Fig. 1B).

Effect of i.t. LPS on monocyte and neutrophil accumulation in the bronchoalveolar space

The accumulation of blood monocytes and neutrophils in the BAL was also determined in animals given i.t. LPS or saline. The total number of cells recovered from the BAL of saline-injected control animals was about $7 \times 10^6$ cells/rat, and there were about 40 cpm of $^{51}$Cr-labeled monocytes and 100 cpm of $^{111}$In-labeled neutrophils, suggesting that almost all the cells in the BAL of control animals are resident cells. An i.t. injection of LPS increased the total number of cells in the BAL by about 9-fold to $6.8 \times 10^7$ cells/rat. However, the accumulation of $^{51}$Cr-labeled monocytes in the BAL increased >170-fold to about 7,000 cpm ($p < 0.001$). The accumulation of $^{111}$In-labeled neutrophils in the BAL of LPS-injected rats increased >500-fold to about 59,000 cpm compared with that in the saline-injected controls ($p < 0.001$).

Blood monocytes and neutrophils also demonstrated different patterns of accumulation in the LPS-inflamed lung parenchyma and the bronchoalveolar space. More than 94% of the $^{51}$Cr-labeled monocytes recovered from the lung 18 h after i.t. LPS were in the parenchymal tissue, and only about 6% were in the BAL, suggesting that only a small proportion of the large number of monocytes entering the lung could be recovered in the BAL. In contrast, 20% of the $^{111}$In-labeled neutrophils accumulated in the BAL and 80% accumulated in the lung parenchyma.

Effects of anti-CD18 and anti-VLA-4 on monocyte and neutrophil migration to the lung

The roles of CD18 and VLA-4 in the migration of blood monocytes and neutrophils into the LPS-inflamed lung were studied using specific blocking mAbs against CD18 and VLA-4. As shown in Fig. 2A, treatment of animals with anti-CD18 mAb reduced blood monocyte accumulation in the LPS-injected lung by about 30% compared with that in control Ab-treated animals ($p < 0.05$). In contrast, the accumulation of blood monocytes in the LPS-inflamed lung was not inhibited by treatment of animals with anti-VLA-4. Treatment with a combination of anti-CD18 and anti-VLA-4 mAbs inhibited monocyte accumulation in the LPS-inflamed lung about 30% compared with that in control Ab-treated animals ($p < 0.05$) and did not result in further inhibition compared with that produced by anti-CD18 treatment alone.

Treatment of animals with neither anti-CD18 nor anti-VLA-4 mAb inhibited blood neutrophil accumulation in the LPS-injected lung compared with that in control Ab-treated animals. However, treatment with both anti-VLA-4 and anti-CD18 mAb inhibited the accumulation of blood neutrophils in the LPS-inflamed lung by about 30% ($p < 0.05$; Fig. 2B).

Effects of anti-CD18 and anti-VLA-4 mAbs on monocyte and neutrophil recovery in BAL

The effects of anti-CD18 and anti-VLA-4 mAb on the recovery of monocytes and neutrophils in the BAL of LPS-injected rats were also determined. As shown in Fig. 3A, accumulation of blood monocytes in the BAL of LPS-injected rats was inhibited 30% by blocking CD18 compared with that in control Ab-treated animals, but this inhibition was not significant ($p > 0.05$). Blocking VLA-4 failed to inhibit the accumulation of blood monocytes in the BAL of LPS-injected rats. In contrast, treatment with a combination of anti-CD18 and anti-VLA-4 mAbs blocked the accumulation of blood monocytes in the BAL by about 80% ($p < 0.001$).

The accumulation of blood neutrophils in the BAL of LPS-injected animals was not inhibited by blocking either CD18 or VLA-4 compared with that in the controls. However, blocking

![FIGURE 1. Accumulation of $^{51}$Cr-labeled monocytes (A) and $^{111}$In-labeled neutrophils (B) in the lung parenchyma. Rats were injected i.v. with radiolabeled cells, and 100 µg of LPS or saline as a control was given by i.t. injection. After 18 h the animals were euthanized, and the accumulation of radioactivity in the lung tissue was determined. Each bar shows the mean ± SEM of seven or eight rats in each group. *p < 0.001.](image-url)
both CD18 and VLA-4 inhibited the accumulation of blood neutrophils in the BAL of LPS-injected animals by about 75% ($p < 0.001$; Fig. 3B).

**Effects of CD18 and VLA-4 blockade on lung histology**

Histologic examination of the lung tissue after i.t. saline was unremarkable (data not shown). However, after the i.t. injection of LPS there was an intense cellular infiltrate in the lung (Fig. 4). This infiltrate consisted predominantly of neutrophils and monocytes. In animals that received i.t. LPS and were treated with both anti-CD18 and anti-VLA-4, the infiltration of the lung and the bronchoalveolar space by neutrophils and monocytes was still present. These findings suggest that a large proportion of blood monocytes and neutrophils infiltrate the LPS-inflamed lung using a CD18- and VLA-4-independent adhesion pathway.

**Effect of blocking CD18 and VLA-4 on monocyte and neutrophil migration to dermal inflammation**

The migration of monocytes and neutrophils from the blood into dermal inflammatory sites was also determined in the same animals given i.t. LPS or saline. As shown in Fig. 5, blood monocytes and neutrophils actively accumulated at dermal inflammatory sites in response to LPS and ZAS, a source of the chemotactic factor, C5a desArg. Animals given i.t. LPS showed a decreased accumulation of monocytes and neutrophils in response to intradermally injected LPS compared with animals receiving i.t. saline ($p < 0.05$).

Fig. 6A shows that migration of blood monocytes to dermal inflammatory sites induced by LPS and ZAS in animals receiving i.t. LPS was significantly (60–70%) inhibited by treatment with anti-CD18, but not by blocking VLA-4. Moreover, monocyte accumulation at both the LPS- and ZAS-injected dermal sites was completely abolished (>98%) by anti-CD18 plus anti-VLA-4, even though in these same animals, monocyte accumulation in the LPS-injected lung was decreased by only 30%.

Neutrophil migration to the dermal inflammatory sites induced by LPS and ZAS in the animals receiving i.t. LPS was strongly inhibited (>97%) by treatment with anti-CD18 mAb ($p < 0.001$; Fig. 6B) even though neutrophil migration to the LPS-inflamed lung was not inhibited by this treatment in the same animals (Fig. 2B). Treatment of animals with anti-VLA-4 mAb did not block neutrophil migration to LPS- and ZAS-injected dermal sites. However, blocking both CD18 and VLA-4 tended to be more effective than blocking CD18 alone and completely abolished neutrophil migration to the skin sites.

**Discussion**

In acute lung inflammation blood leukocytes infiltrate the lung parenchyma and the bronchoalveolar space. Although most investigations have focused on the neutrophils in the lung, monocytes also accumulate in the lung and contribute to the lung injury. By using radiolabeled blood monocytes the migration of monocytes from the blood into the lung and across the epithelium into the
bronchoalveolar space could be readily quantified after the i.t. injection of LPS. The data reported here are the first quantitative measurements of blood monocyte migration into the lung parenchyma and into the bronchoalveolar space in response to i.t. LPS and the first analysis of the contributions of the CD11/CD18 and VLA-4 integrins to this migration.

Monocyte accumulation in the lung after i.t. LPS injection was surprisingly large. Thirteen percent of the labeled monocytes injected i.v. were found in the lung after LPS injection. Neutrophil accumulation was also dramatically increased, with 24% of the injected neutrophils in the lung. These results suggest that there is substantial monocyte migration from the blood into the lung, which is not readily appreciated by histology, possibly because of the rapid differentiation of monocytes into macrophages.

There was a 170-fold increase in labeled monocytes and a 500-fold increase in labeled neutrophils in the BAL after LPS injection. The large increase in monocytes recovered in the BAL demonstrates the substantial contribution that monocytes make to these inflammatory exudates. The BAL fluid contained 6% of the labeled monocytes and 20% of the labeled neutrophils accumulating in the lungs. This suggests that the transit time of monocytes from the lung parenchyma into the bronchoalveolar space may be longer for monocytes than for neutrophils, although it cannot be excluded that the monocytes entered the lung sometime after the neutrophils. However, it does demonstrate that BAL may provide a selective sample of the cells infiltrating the lung, since BAL measures only cells that migrated into the bronchoalveolar space. Transmigration of the lung epithelium may also affect the composition of the BAL.

A key finding from our studies is the contribution of the CD18 and VLA-4 integrins to leukocyte accumulation in the LPS-treated lung. Previous studies showed that blood neutrophil migration in lung inflammation was partially dependent upon CD18 integrins but could also be CD18 independent (17–20). Blockade of CD18 inhibited the accumulation of neutrophils in response to i.t. E. coli, phorbol esters, and, significantly, but not completely to LPS, IL-1α, and IgG immune complexes (17–19). Neutrophil accumulation in response to C5a, S. pneumoniae, and hydrochloric acid was not reduced by CD18 blockade in rabbits (17, 19). The mechanisms of the CD18-independent migration to these stimuli is unknown. Our studies of neutrophil migration into the LPS-challenged lung show no reduction in neutrophil migration after anti-CD18, even though in the same animals CD18 blockade inhibited >90% of the neutrophil migration to the skin in response to LPS and ZAS (Fig. 6). The lack of an effect of CD18 blockade in our studies compared with studies in rabbits may be explained by the higher dose of LPS (100 vs 10 μg), the difference in timing (18 vs 4 h), or species differences between rats and rabbits (18). Higher doses of an inflammatory stimulus or repeated administration of bacteria have been shown to increase the CD18-independent component of neutrophil migration, suggesting that the dose of LPS may be the major reason for the small CD18-dependent component in our experiments (20).

Our laboratory previously reported that neutrophils in the rat express low levels of VLA-4, and this integrin can mediate in vivo neutrophil migration (15). Here we show that VLA-4 blockade alone does not affect neutrophil migration to the LPS-inflamed lung, and in combination with anti-CD18 inhibits neutrophil accumulation by only about 30% in the lung parenchyma. Interestingly, anti-CD18 or anti-VLA-4 alone did not reduce neutrophil migration into the bronchoalveolar space, as indicated by the recovery of cells in the BAL, but dual blockade of CD18 and VLA-4 decreased the recovery of neutrophils in the BAL by 80%. This suggests that migration of neutrophils from the lung parenchyma across the pulmonary epithelium into the bronchoalveolar space is mediated by both CD18 and VLA-4 integrins. Pulmonary epithelium in the LPS-inflamed lung expresses high levels of ICAM-1, an important ligand for the CD18 integrins (31, 32). In addition, VLA-4, which can bind to both VCAM-1 and fibronectin, may contribute to neutrophil transepithelial migration by binding to fibronectin in the interstitium. Thus, these studies show that neutrophil migration to LPS-induced lung inflammation in the rat is CD18 independent and that the CD18-independent component is only partially mediated by VLA-4, but that migration into the bronchoalveolar space by neutrophils depends to a large extent upon both CD18 and VLA-4.

**FIGURE 4.** Lung histology from animals that received i.t. LPS and i.v. a control mAb (A) or both anti-CD18 and anti-VLA-4 mAbs (B).
Unlike neutrophils, which have been previously shown to use a CD18-independent pathway for migration to the lung and to inflamed joints, the recruitment of monocytes to cutaneous inflammation and to arthritic joints was shown to be completely dependent on CD18 and VLA-4 (6, 8). Monocyte accumulation in LPS-induced lung inflammation was inhibited 30% by anti-CD18 treatment and was not affected by anti-VLA-4, and the effect of the combination was similar to that of anti-CD18 alone. Thus, in contrast to other tissues previously studied, monocytes use a CD18- and VLA-4-independent pathway for most of the migration to LPS-induced lung inflammation. In addition, VLA-4 appears to play no role in monocyte migration into the lung parenchyma, even though VLA-4 mediates monocyte recruitment to cutaneous inflammation, including LPS inflammation, and to inflamed joints (6, 8). The lack of a greater effect on monocyte accumulation in the lung parenchyma was not due to inadequate Ab treatment, since treatment with higher doses of these mAbs did not have a greater effect, the rats had circulating levels of the mAbs well above those required to saturate the receptors on the blood leukocytes, and the anti-CD18 plus anti-VLA-4 treatment abolished neutrophil and monocyte accumulation in the skin in response to LPS and ZAS.

Monocyte recovery in the BAL was reduced, although not significantly (p = 0.2), by anti-CD18 treatment, was unaffected by treatment with anti-VLA-4, and was inhibited by 80% when CD18 and VLA-4 were both blocked. This pattern is similar to that of neutrophils and suggests that CD18 and VLA-4 together mediate much of the migration of monocytes across the epithelium into the bronchoalveolar space. As discussed above, the mechanism of this migration may well depend upon the interaction of CD18 with ICAM-1 on epithelial cells and of VLA-4 with VCAM-1 or fibronectin.

The reason for the lack of inhibition of monocyte and neutrophil accumulation in the lung parenchyma by CD18 and VLA-4 blockade is unclear. Anti-CD18 plus anti-VLA-4 treatment increases the blood leukocyte concentration and thereby might increase the leukocyte content in the vasculature of the lung. However, the lungs were exhaustively perfused to remove intravascular leukocytes before dissection to minimize this effect. Furthermore, compared with the huge increase in labeled leukocytes in the lung parenchyma, the increase in the blood leukocyte count could account for only a very small component (<10%) of the labeled cells in the lungs. In addition, the lung histology clearly demonstrated that in rats treated with both anti-CD18 and anti-VLA-4 large numbers of leukocytes were in the lung parenchyma.

Thus, there appears to be a CD18- and VLA-4-independent pathway for migration of both monocytes and neutrophils into the lung. The mechanism behind this migration may lie in the highly specialized vasculature of the lung. Leukocytes entering the lung migrate through the capillaries rather than the postcapillary venules as in the systemic circulation (33). In the capillaries, the requirement for strong adhesion mediated by the integrins may be
less than that for migration out of the postcapillary venules in the systemic circulation. This may allow leukocytes to migrate out of the blood in the presence of integrin blockade. E-selectin, P-selectin, and platelet endothelial cell adhesion molecule-1 (PECAM-1) have been shown to contribute to migration in inflammatory tissues, including the lung (34–39). Selectin-mediated adhesion or adhesion directly mediated by PECAM-1 in the tight junctions between adjacent endothelial cells may be sufficient to allow leukocyte transendothelial migration. Further studies using strategies to block multiple selectins and/or PECAM-1 may help identify the CD18- and VLA-4-independent pathway for monocyte and neutrophil migration.

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