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Neutrophil depletion inhibits early and late monocyte/macrophage increase in lung inflammation

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1. ABSTRACT

Monocytes/macrophages have critical impact on outcomes of lung inflammation. Kinetics and mechanisms for the increase of monocytes/macrophages in lungs are not completely understood. To better understand these mechanisms, *E. coli*-LPS (250 micro grams; N = 35) or endotoxin-free saline (N = 5) were instilled intratracheally in Sprague-Dawley rats and the increase in monocytes/macrophages, neutrophils and monocyte chemotactic protein-1 (MCP-1) was quantified at various time points after LPS treatment. In contrast to typical pattern of neutrophil influx between 6 and 24 hours, monocytes/macrophages increased in two distinct phases, very early at 3 hours and late at 24 hours. The role of neutrophils in monocyte/macrophage increase was addressed in LPS-challenged neutropenic rats (N=8). Neutrophil depletion before instillation of LPS abrogated the early as well as late monocyte/macrophage increases in the lung. Quantification of MCP-1, which is one of the major chemoattractants for monocytes, in lung homogenates showed similar concentrations in neutropenic and non-neutropenic LPS-challenged rats. These findings show that increase in monocytes/macrophages in lung occurs in two, early and late phases, both being dependent on neutrophils but not on MCP-1.

2. INTRODUCTION

Acute inflammation in the lung and other organs is characterized by early influx of neutrophils followed by monocytes and macrophages (1, 2). It is well established that increase in monocyte numbers occurs in late, post-neutrophilic, phase of inflammation in the lung (3, 4, 5, 6, 7). However, in animal models of inflammation in other organs, such as peritonitis and dermatitis there is also a documentation of very early increase in monocyte/macrophage numbers either before or along with increase in neutrophil numbers (8, 9, 10). Such observations on the early increase in the monocyte/macrophage numbers have not been made in the context of lung inflammation. This could be either due to the primary focus of previous studies on monocyte/macrophage increase in the late phase of inflammation (3, 5, 11) or because most of the investigators have used broncho-alveolar lavage (BAL) to assess migration of inflammatory cells into inflamed lungs (4, 6, 7, 11). Although BAL is a highly valuable tool to assess migration of inflammatory cells into the lungs, it does not account for inflammatory cells that are present in the septum which includes cells in the microvasculature and interstitial space (5). Since monocytes/macrophages in inflamed lungs, irrespective of their location, can

significantly contribute to inflammation by producing cytokines and free oxygen radicals (5, 12, 13, 14), it is important to assess the total number of monocytes/macrophages accumulated in the lung instead of examining only those which migrate into the airspace. Therefore, we decided to examine the pattern of total monocyte/macrophage increase up to 36 hours post-LPS treatment in the lung.

MCP-1, a CC chemokine, is a major chemoattractant for monocytes and is produced by various cells including neutrophils (15, 16, 17). There is evidence that neutrophils regulate the monocyte increase in the late phase of C5a and LPS induced lung inflammation in rabbits and mice, respectively (3, 6). It is also suggested that MCP-1 release by neutrophils could possibly play a role in late monocyte/macrophage increase in inflamed lungs (11). But there is no direct evidence to show that neutrophils are the major source of MCP-1 in the lung. Therefore, we wanted to assess the role of neutrophils on MCP-1 concentration and its effect on monocyte/macrophage numbers in inflamed lungs. To address our questions, we conducted an *in vivo* quantitative study in rats and evaluated increase in total monocytes/macrophage numbers, without making a distinction between alveolar, interstitial and microvascular, in inflamed lungs. Then, we investigated the role of neutrophils in relation to MCP-1 expression in monocytes/macrophage increase in acute lung inflammation. The data show an early, in addition to previously established late, increase in monocytes/macrophage numbers. The data also shows that neutrophil depletion inhibits both early as well as the late increase in monocyte/macrophage numbers without affecting concentrations of MCP-1 in the lung.

3. MATERIALS AND METHODS

3.1. Rats and treatment groups

The experimental protocols were approved by the University of Saskatchewan Committee on Animal Care Assurance and experiments were conducted according to the Canadian Council on Animal Care Guidelines. Specific pathogen-free, ten-week-old, male Sprague-Dawley rats were procured from Charles River laboratories, Canada. Rats were maintained in the animal care unit and were acclimatized for a period of one week. Rats were randomly divided into eight groups of five each.

3.2. Acute lung inflammation

Rats were anaesthetized by intraperitoneal administration of xylazine (20 mg/Kg) and ketamine (100 mg/Kg). Trachea was exposed surgically and endotoxin-free saline (Sigma, St.Louis MO, USA) or *E. coli* LPS (250µg; serotype 0128:B12; Sigma, St.Louis MO, USA) was instilled intratracheally. Animals were euthanized at 1, 3, 6, 12, 24, 30 and 36 hours (n = 5 each) post-treatment. Control animals (n = 5) were euthanized at 6 hours post saline treatment.

3.3. Tissue collection and processing

From each rat, six pieces of lung were collected for light microscopy. Tissues for ELISA were snap frozen and stored at -80ºC till further use. Lung pieces for histology and immunohistology were fixed in 4% paraformaldehyde for 16 hours. Lungs were processed through ascending grades of alcohol and embedded in paraffin. Five micron sections were cut from all the 6 pieces from each rat. Lung samples for immuno-electron microscopy were fixed in 2% paraformaldehyde containing 0.1% glutaraldehyde for 3 hours at 4°C. These samples were dehydrated in ascending grades of alcohol and embedded in LR-white resin (London resin company, USA) followed by polymerization under UV light at -1^{o}C for 48 hours.

3.4. Immunohistology

Tissue sections were deparaffinized in xylene and rehydrated in descending grades of alcohol followed by treatment with 5% hydrogen peroxide in methanol to quench endogenous peroxidase. Sections were treated with pepsin (2 mg/ml in 0.01N hydrochloric acid; Sigma, St. Louis MO, USA) for 45 minutes to unmask the antigens and with 1% bovine serum albumin (Sigma, St. Louis MO, USA) to block non-specific binding. Sections were incubated with primary antibodies against rat monocyte/macrophage (1:75; ED-1, Serotec Inc**.** NC, USA) or rat MCP-1 (1:300; Torrey Pines Biolabs, Inc. TX, USA) or *E. coli*-LPS (1:300, Cedarlane Laboratories Limited, ON, Canada), followed by appropriate horseradish peroxidase(HRP) -conjugated secondary antibodies (1:100; Dako cytomation, ON, Canada). The antigen-antibody complex was visualized using a color development kit (Vector laboratories, ON, Canada). Controls consisted of staining without primary antibody or with isotype matched immunoglobulin instead of primary antibody. Proper quenching of endogenous peroxidase was confirmed by omitting both primary and secondary antibodies.

3.5. Quantification of monocytes/macrophages and neutrophils

Neutrophils were counted in hematoxylin and eosin (H&E) stained lung sections while monocytes/macrophages were counted in ED-1 immunostained lung sections. We did not use immunohistochemistry for identifying neutrophils as these cells are easily identifiable by their morphology. Cells were counted in 10 high power fields/section from each of the six lung pieces from every rat. Area of the field was calculated using a stage micrometer (Tissue area: 0.025mm^2/field, 0.25mm^2/section, 1.5mm^2/rat). The fields for counting the cells were randomly selected and those fields containing larger blood vessels, bronchioles and larger airways were excluded (18).

3.6. Immuno-electron microscopy

Thin sections (80-100 nanometer) were incubated with 1% bovine serum albumin to block non-specific sites. This was followed by incubations with primary (ED-1-1:50; MCP-1-1:250; 60 min) antibodies and appropriate 15 nm gold-conjugated secondary antibodies (1:100; British Bio Cell International, UK; for 30 min). Sections were stained with uranyl acetate and lead citrate and examined in Philips 410LS transmission electron microscope. Controls consisted of labeling with isotype matched immunoglobulin or without primary antibody.

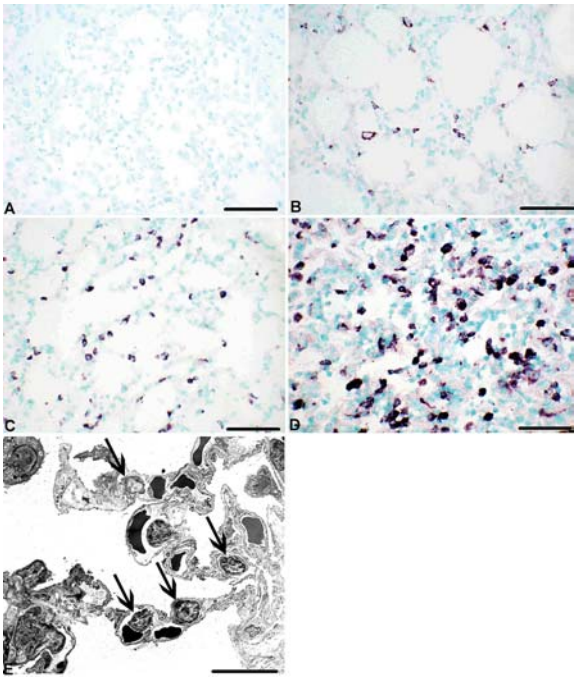

Figure 1. Monocyte/macrophage increase in acute lung inflammation. Monocytes/macrophages were identified using a monoclonal antibody, ED1. Staining with an isotype matched immunoglobulin did not show any reaction (A). Compared to saline treated controls (B), monocyte numbers appeared to increase at 3h post-LPS-treatment (C). The increase in monocyte numbers was much higher at 24h post-LPS-treatment (D). E). Electron microscopic observations showed that the early increase in the monocyte/macrophage numbers were mainly due to the presence of monocytes (arrows) in the lung microvasculature. Scale bar: A-D = 50 micron; E = 10 micron.

3.7. Induction of neutropenia and lung inflammation

Eight rats were given anti-neutrophil antibody (0.3ml/100g; intra-peritoneal; Catalogue number AIA51140; Accurate Chemicals, NY, USA) (19, 20). Differential counts were performed on peripheral blood samples before and after the treatment to confirm the induction of neutropenia (19). Before antibody treatment the differential count of neutrophil in peripheral blood was 12.65±2.3 % (mean ± SD). After 24 hours of the antibody treatment the neutrophil counts declined to 0% in six rats and 1% in two rats. After 24 hours of anti-neutrophil antibody treatment, rats were anaesthetized to instill 250µg of *E. coli*-LPS intratracheally followed by euthanasia at 3 hours (n=4) or 24 hours (n=4) after the treatment. Tissues were collected and processed for light microscopy, immunohistochemistry and ELISA.

3.8. Quantification of MCP-1 in lung homogenates

Purified anti-rat MCP-1 (clone C4) and biotinylated anti-rat MCP-1 (clone B4) and recombinant rat MCP-1 were purchased from BD Biosciences, ON, Canada. Lung samples were homogenized in HBSS (0.1g/ml) containing protease inhibitor cocktail (100µl/10ml; Sigma-Aldrich Co, MO, USA). Microtiter plates (Immulon 4 HBX, VWR CAN LAB, AB, Canada) were coated with 50µl of purified anti-rat MCP-1 antibody (10µg/ml) and incubated at 4°C overnight. After 12 hours, plates were washed with PBS containing 0.05%-Tween (PBST) before incubating with 200µl of blocking buffer (1% BSA in PBS) for one hour at 37°C. Plates were washed 5 times with PBST and incubated with 100µl standard or samples in duplicates for two hours at 37°C. After adding 100µl of biotinylated anti-rat MCP-1 antibody (2µg/ml) diluted in blocking buffer with Tween, plate was incubated at 37°C for an hour. This was followed by incubation with streptavidin-HRP (1:2500 in PBS; Dako cytomation, ON, Canada) for 30 min at 37°C. The reaction was visualized using TMB substrate (Mandel Scientific, ON, Canada) and reaction was stopped using 50µl of 1M sulfuric acid, followed by reading at 450nm.

3.9. Statistical analyses

All values are presented as mean ±SE, unless otherwise mentioned. Differences between two groups were tested using independent-samples *t*-test and more than two groups were compared using one-way analysis of variance with Fisher's LSD for post hoc comparisons. Statistical significance was accepted at $P<0.05$.

4. RESULTS

4.1. Monocyte/macrophage and neutrophil kinetics in acute lung inflammation

Monocyte/macrophage and neutrophil counts were performed in sections stained with ED-1 antibody, which recognizes both monocytes and macrophages (Figure 1) and H&E, respectively. ED-1 positive cells in control animals consisted of alveolar macrophages and few monocytes in the septum (Figure 1B). Inflamed lungs, however, showed increased numbers of monocytes/macrophages in the lungs (Figures 1C and D). Electron microscopy confirmed that at 1 and 3 hours most of the septal cells were monocytes and were in the septal microvessels (Figure 1E).

Quantitative analyses showed elevated numbers of monocytes/macrophages at 1 hour (20.16±1.01; P=0.053), 3 hours (22.66±2.32; P=0.019), 6 hours (23.77±1.48; P=0.012) and 12 hours (23.9±2.19; P=0.011) post-LPS challenge compared to the controls (9.25±0.89; Figure 2); however, there were no differences between these post-LPS treatment time points. Second increase in monocyte/macrophage numbers occurred at 24 hours (38.23±6.97; P=0.000) 30 hours (38.72±4.21; P=0.000) and 36 hours (46.21±6.79; P=0.000) compared to controls (Figure 2). In contrast to monocytes/macrophages, neutrophils in the lungs increased steadily till 24 hours compared to the control (6 hours: 26.30±5.33, P=0.027; 12 hours: 38.00±7.42, P=0.001; and 24 hours: 57.14, P=0.000) followed by a decline at 30 hours (33.36±5.08; P=0.019) and 36 hours (35.33±4.24; P=0.031) compared to 24 hours (Figure 2).

4.2. Effect of neutropenia on number of monocytes/macrophages in lungs

We determined the role of neutrophils in early as well as late increase of monocytes/macrophages by

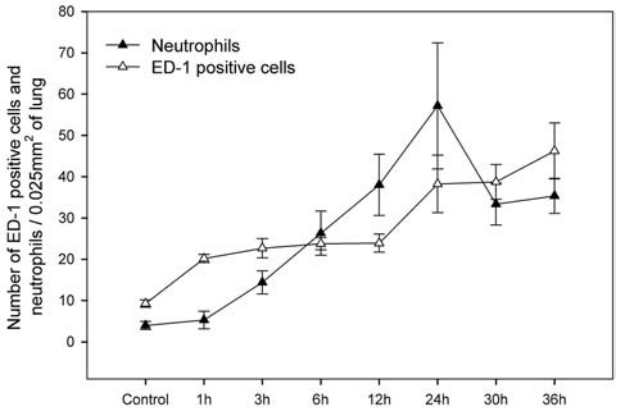

Figure 2. Monocyte/macrophage and neutrophil kinetics in acute lung inflammation. Numbers of monocytes/macrophages and neutrophils in lungs at various time points after LPS treatment are shown. Increase in monocyte/macrophage numbers was biphasic; the early increase was observed at 3h post-LPS-treatment and the late increase occurred at 24h post-LPS-treatment. Neutrophils increased from 6h, till 24h and followed by a decline at 30 and 36h post-LPS treatment. For monocytes/macrophages, time points from 3h to 36h are different from control; 1h, 3h, 6h and 12h are not different; 24h, 30h and 36h are different from 12h. For neutrophils, 6h to 36h time points are different from controls; 30h and 36h are different from 24h.

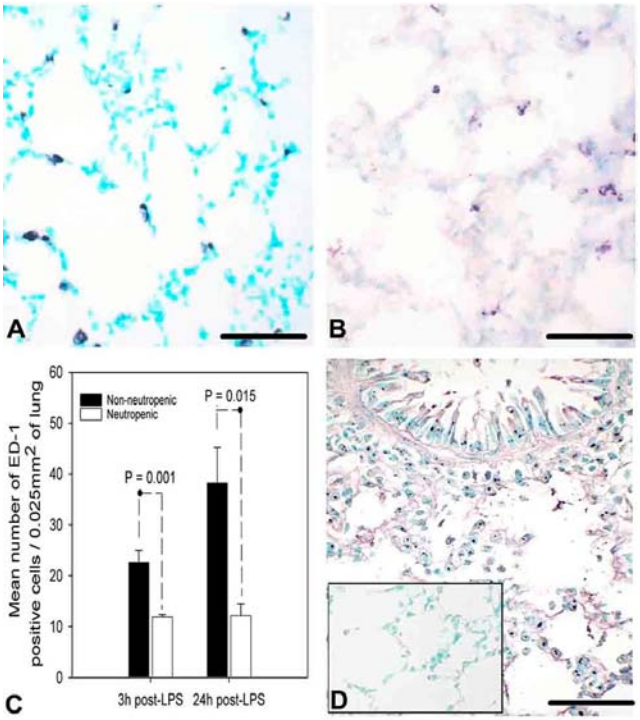

Figure 3. Monocyte/macrophage kinetics in non-neutropenic and neutropenic rat lungs. Monocyte/macrophage numbers in the neutropenic rat lungs at 3h (A) and 24h post-LPS-treatment (B) appeared to be less compared to non-neutropenic rat lungs (compare with Figures 1C and D). Quantification of monocyte/macrophage numbers showed a significantly lower number of monocytes in neutropenic rat lungs (C). D: The LPS localization using anti-LPS antibody in the bronchial epithelium, smooth muscle cells and alveolar epithelium confirmed proper instillation of LPS. Inset: staining with an isotype-matched immunoglobulin showed no reaction. Scale bar = 50 micron.

provoking acute lung inflammation in neutropenic rats. The antibody reduced the differential count of neutrophil in peripheral blood from 12.65±2.3 % (mean ± SD) before the treatment to 0% in six rats and 1% in two rats at 24 hours after the treatment. The antibody used in our experiment has been shown not to affect the monocyte and alveolar macrophage numbers in rats (21). Lungs from neutropenic rats demonstrated a reduction in monocyte/macrophage numbers at 3 hours and 24 hours post-LPS administration compared to non-neutropenic time-matched LPS-challenged rats (Figures 3 A-C; 22.66±2.32 v/s 11.92 ± 0.46, P= 0.001 and 38.23±6.97 v/s 12.19 ± 2.25, P= 0.015,

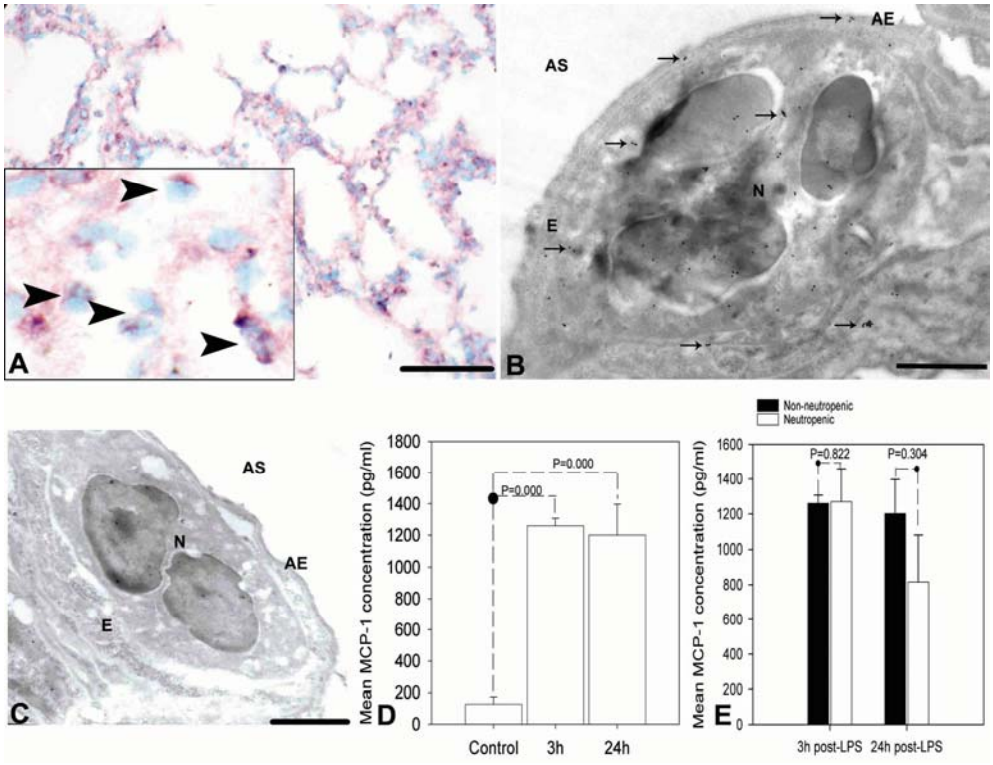

Figure 4. MCP-1 expression and concentrations in lung. A. At 3 hours post-treatment, MCP-1 expression was present in the neutrophils (arrowheads). B. Immuno-electron microscopy confirmed the presence of MCP-1 in neutrophils while lack of labeling with secondary antibody alone (C), ruled out non-specific labeling (N: neutrophil; AE: alveolar epithelium; E: microvascular endothelium; AS: alveolar space; Arrows indicate gold particles labeled for MCP-1). D and E show concentration of MCP-1 in lung homogenates. Compared to controls, the concentrations of MCP-1 in lung homogenates were increased at 3 and 24 hours post-treatment. But there was no difference between 3 and 24 hours time points (D). E). Similar concentrations of MCP-1 in neutropenic rats compared to non-neutropenic rats at both 3- and 24h after LPS treatment. Scale bar: A= 50 micron; B=1 micron. C=10 micron

respectively). Immunohistology for the LPS showed staining in the alveolar epithelium, macrophages, bronchiolar epithelium and smooth muscle cells (Figure 3G) and ruled out the possibility that reduction in monocyte/macrophage numbers is due to improper instillation of LPS.

4.3. MCP-1 expression and concentrations in lung

To obtain an insight into implications of MCP-1 as a downstream signal in neutrophil-dependent monocyte/macrophage increase, we first confirmed expression of MCP-1 in neutrophils using immunohistochemistry (Figure 4A) and immuno-electronmicroscopy (Figure 4B). MCP-1 staining was observed in alveolar septa as well as neutrophils in the septum. Lung sections stained as the controls for immunohistology (data not shown) or immuno-electron microscopy (Figure 4C) showed no labeling. Next, we determined MCP-1 concentrations in lung homogenates from non-neutropenic and neutropenic rats at 3 and 24 hours post-LPS treatment (Figure 4D and 4E). We chose 3 and 24 hours post-LPS intervals because significant increases in monocyte/macrophage numbers occurred at these times points (Figures 1 and 2). Compared to saline treated controls (126.19 ± 46.97 pg/ml), MCP-1 concentrations increased at 3 hours (1260 ± 46.08 pg/ml; P=0.000) and 24 hours (1201 ± 195.16 pg/ml; P=0.000) post-LPS treatment in non-neutropenic LPS-treated rat lungs (Figure 4D). However, there were no differences in MCP-1 concentrations at 3 hours and 24 hours time points (P = 0.717). MCP-1 concentrations were also similar between LPS-challenged non-neutropenic and neutropenic rats at 3 hours (1260.18 ± 46.08 pg/ml v/s 1269.31± 190.35 pg/ml; P = 0.822) and 24 hours (1201.86 ± 195.16 pg/ml v/s 815 ± 263.11 pg/ml; P= 0.304) post-treatment (Figure 4E).

5. DISCUSSION

In this manuscript, we report an early increase in monocyte/macrophage numbers in acute lung inflammation. Our data further demonstrate that neutrophil depletion inhibits both early as well as the late increase of monocytes/macrophages without affecting MCP-1 concentrations in inflamed lungs.

First, we wanted to know if there is an early increase in monocyte/macrophage numbers in inflamed

lungs, as observed in peritonitis and dermatitis (8, 9, 10). For this we undertook a detailed quantification of monocyte/macrophage numbers in normal and inflamed lungs. This became necessary because of existing lack of data on *in situ* quantification of monocytes/macrophages in inflamed lungs. Most of the previous studies have used only BAL to quantify cells such as mononuclear phagocytes and neutrophils in normal and inflamed lungs (4, 6, 7, 11). Despite critical usefulness of BAL analyses in evaluation of cells that have migrated into the air spaces, it does not provide information on the inflammatory cells present in the septa (5). For example, recent data convincingly showed that although bromo-deoxyuridine labeled monocytes disappeared from the peripheral blood into alveolar septa within 1 hour of their infusion, an increase in their numbers in BAL was observed after 48 hours (22). Therefore, BAL may not capture early increase in monocyte/macrophage numbers in the septum. Because intravascular monocytes/macrophages can influence course of lung inflammation, it is important to undertake direct *in situ* quantification of all the monocytes/macrophages in unlavaged lungs.

Our effort resulted in an observation of a very early increase in ED-1 positive monocyte/macrophage numbers in inflamed lungs. Previously, the early increase of monocytes/macrophages may have been missed due to reliance on BAL analyses or the studies' primary focus on the late post-neutrophilic increase of monocytes/macrophages (3, 4, 5, 6, 7, 11).The early increase in the cell numbers could be either due to increased recruitment of monocytes or due to local proliferation of macrophages (23). Local proliferation of macrophages could be one of the pathways mainly in chronic lung inflammation (24) and such an event is very unlikely at 3h after the LPS stimulation (23), time at which we observed an increase in monocyte/macrophage numbers. Also, our electron microscopic observations showed that the early increase in ED-1 cells was largely due to the monocytes present in septal microvessels, which may be a prelude to their migration into the interstitium and alveolar spaces. We counted ED-1 cells without making a distinction between the alveolar, microvascular and the interstitial because both macrophages and monocytes, whether present in the alveolar space or interstitium or microvasculature can influence the inflammatory process by producing free oxygen radicals and cytokines (14, 13, 5, 12). Furthermore, monocytes are the source of the renewal of pulmonary macrophages (23) and produce more cytokines, such as IL-1beta, than alveolar macrophages (14). Therefore, early increase in monocytes/macrophages in inflamed lungs may significantly impact the course of inflammation.

Next, we addressed the role of neutrophils in relation to MCP-1 expression in the early as well as the late phase of monocyte/macrophage recruitment by inducing lung inflammation in neutropenic rats. Our data confirms the previously addressed role of neutrophils in the late phase of monocyte/macrophage increase in lung (3, 6). Because early recruitment of monocytes/macrophages in lung inflammation has not been reported so far, the importance of neutrophils in this early monocyte/macrophage increase in the lung remains unexplored. Now, our experiments provide the data to show that neutrophil depletion blocks early increase in monocyte/macrophage numbers in inflamed lungs. Although neutropenia inhibited early increase in monocyte/macrophage in inflamed lungs, at 3 hours, the non-neutropenic LPS-challenged rats had very few neutrophils in their lungs. Therefore, the data suggest that even fewer numbers of neutrophils may mediate the early increase of monocyte/macrophage numbers and underscores the complexity of cellular and molecular interactions in the recruitment of monocytes/macrophages in lung inflammation. Interestingly, inhibition of monocyte/macrophage recruitment in neutropenic animals was not accompanied by expected suppression of MCP-1 expression. Similar to previous observations, our light and electron immunocytochemistry showed MCP-1 in neutrophils (15, 16, 17). Because neutrophil depletion did not alter MCP-1 concentrations in inflamed lungs, these cells may not be major contributors of MCP-1. An intriguing fact remains that elevated concentrations of MCP-1 were not accompanied by an increase in numbers of monocytes/macrophages in inflamed lungs of neutropenic rats. At this stage, we do not have an explanation for this apparent discord between MCP-1 expression and lack of monocyte/macrophage recruitment in inflamed lungs. One of the possibilities may be that neutrophil depletion alters the production of other mediators of inflammation such as IL-1beta, IL-8, fibronectin and elastin, which participate in monocyte/macrophage sequestration (7, 11, 25, 26, 27, 28, 29). Another explanation could be that an interaction between neutrophils and MCP-1 is required to signal monocyte/macrophage increases in inflamed lungs. Nevertheless, our data shows significance of neutrophils as well as complexity of cellular and molecular interactions in the recruitment of monocytes/macrophages in inflamed lungs.

Although, our data shows an early increase of monocyte/macrophage increase in acute lung inflammation, there are certain limitations to this study. First, we did not study the functional implication of early monocyte/macrophage increase. Next, we observed that even with increased MCP-1 concentration, monocyte/macrophage increase does not occur in the absence of neutrophils. Neither our study nor existing literatures offer any explanation about the possible mechanisms. Since monocyte/macrophages are capable of influencing the inflammatory process, further studies including MCP-1 blocking in neutropenic animals are warranted to establish the functional significance of early increase in monocyte/macrophage numbers and to identify the complex interaction between neutrophils and MCP-1. Lastly, it will be important to explore, if neutrophil depletion alters expression of any other monocyte/macrophage chemoattractants.

6. ACKNOWLEDGEMENTS

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