Altered Neutrophil Trafficking During Sepsis


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Altered Neutrophil Trafficking During Sepsis


In sepsis, dysregulation of the inflammatory system is well known, as reflected in excessive inflammatory mediator production, complement activation, and appearance of defects in phagocytic cells. In the current study sepsis was induced in rats by cecal ligation/puncture. Early in sepsis the \( \beta_1 \) and \( \beta_2 \) integrin content on blood neutrophils increased in a nontranscriptional manner, and the increase in \( \beta_2 \), but not \( \beta_1 \), integrin content was C5a dependent. Similar changes could be induced in vitro on blood neutrophils following contact with phorbol ester or C5a. Direct injury of lungs of normal rats induced by deposition of IgG immune complexes (IgG-IC) caused 5-fold increases in the myeloperoxidase content that was \( \beta_2 \), but not \( \beta_1 \), dependent. In contrast, in cecal ligation/puncture lungs myeloperoxidase increased 10-fold after IgG immune complex deposition and was both \( \beta_1 \) and \( \beta_2 \) integrin dependent. These data suggest that sepsis causes enhanced neutrophil trafficking into the lung via mechanisms that are not engaged in the nonseptic state. The Journal of Immunology, 2002, 169: 307–314.

Neutrophil sequestration in lung is a common feature during acute lung inflammation and has been linked to requirements for adhesion molecules, including the integrins, selectins, and ICAMS (1). Integrins are a family of \( \alpha \beta \) heterodimeric transmembrane glycoproteins expressed on the cell surface and mediate cell-cell and cell-matrix interactions. Eighteen \( \alpha \)-chains and eight \( \beta \)-chains have been characterized at the molecular level (2). Among the large number of \( \alpha \beta \) heterodimers, \( \beta_1 \) and \( \beta_2 \) integrins appear to play important roles in cell migration. To date, 12 \( \beta_1 \) integrins and 4 \( \beta_2 \) integrins have been identified as heterodimers (2, 3). \( \beta_1 \) integrins are expressed by most cell types and predominantly mediate cell-matrix adhesion, while the \( \beta_2 \) integrins are primarily expressed on leukocytes and mediate cell-cell adhesion (2, 3). The firm adhesion of neutrophils to vascular endothelium and their subsequent transmigration are believed to be modulated by certain numbers of \( \beta_1 \) (CD18) integrins, such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (4, 5). Neutrophil recruitment into lung also involves \( \beta_2 \) integrin-independent pathways. For instance, \( \beta_2 \) blockade had no effect on neutrophil infiltration into lung induced by intrapulmonary deposition of C5a (6). Neutrophil emigration during Pseudomonas aeruginosa-induced acute pneumonia is CD11/CD18 dependent. However, in the recurrent pneumonia induced by P. aeruginosa, neutrophil emigration is involved in CD11/CD18-independent pathways (7). Recent studies have implicated \( \beta_1 \) integrins in neutrophil migration during lung injury. Treatment with Abs to \( \alpha_4 \) and/or \( \alpha_5 \) integrins had no effect on PMN accumulation in lungs induced by intratracheal administration of LPS in rats. However, blockade of VLA-4 or VLA-5 in the presence of \( \beta_2 \) mAbs treatment more effectively inhibited PMN recruitment in lung compared with blockade of \( \beta_2 \) alone, indicating that \( \alpha_4 \beta_1 \) and \( \alpha_5 \beta_1 \) function as major alternate cell adhesion molecules to the \( \beta_2 \) integrins in mediating PMN migration to lung (8).

The acute respiratory distress syndrome is a common complication of septic shock and is associated with a high mortality (9). Neutrophil sequestration into lung seems to play a pivotal role in the pathogenesis of acute lung injury during sepsis. Neutrophil accumulation in the lung and other organs may result in tissue damage by releasing excessive amounts of oxygen metabolites and proteases, which may contribute toorgan failure. \( \beta_2 \) integrin expression on circulating neutrophils is increased in animals and humans during sepsis under these conditions (10). Blood neutrophils from septic, but not control, patients expressed \( \alpha_4 \beta_1 \) integrin, which caused increased adhesiveness to immobilized VCAM-1 (11). This evidence suggests the important roles of \( \beta_2 \) integrins as well as \( \beta_1 \) integrins in neutrophil migration during sepsis. However, the mechanism and functional expression of \( \beta_1 \) and \( \beta_2 \) integrins in neutrophils under septic conditions are not fully understood. In this study we sought to determine the patterns of \( \beta_1 \) and \( \beta_2 \) integrin expression in neutrophils during cecal ligation/puncture (CLP)-induced sepsis and to study the effects of increased levels of \( \beta_1 \) and \( \beta_2 \) integrin on neutrophil accumulation in lung. We have obtained evidence that during sepsis \( \beta_1 \) integrins play a major role in neutrophil trafficking into lungs.

Materials and Methods

**Rat model of cecal ligation and puncture**

Male, Long-Evans, specific pathogen-free rats (275–300 mg; Harlan, Indianapolis, IN) were used in all studies. Anesthesia was induced by i.p. administration of ketamine (20 mg/100 mg body weight). After shaving the abdomen and application of a topical disinfectant, a 2-cm midline incision was made, and the cecum was identified and ligated below the ileocecal valve, with care being taken not to occlude the bowel. The cecum was then subjected to a single through-and-through perforation with a 21-gauge needle. After repositioning the bowel, the abdominal incision was closed in layers with plain gut surgical suture 4-0 (Ethicon, Somerville, NJ) and metallic clips. Sham animals underwent the same procedure in the absence of cecal ligation and puncture. Before and after surgery animals had unlimited access to food and water.
IgG-immune complex (IgG-IC)-induced lung injury

Lung injury was induced in normal or CLP rats by intrapulmonary deposition of IgG-IC as described previously (12). Briefly, 10 mg BSA (Sigma, St. Louis, MO) was given i.v. after intratracheal administration of 2.5 mg polyclonal rabbit anti-BSA IgG (ICN Pharmaceuticals, Basingstoke, U.K.) in a total volume of 300 μl. Animals were sacrificed 4 h after IgG-IC-induced alveolitis. This interval selected for sacrifice represent times of peak lung injury as determined in previous experiments (12). The pulmonary circulation was then flushed with 10 ml PBS, and lungs were harvested for myeloperoxidase (MPO) analysis or bronchoalveolar lavage (BAL).

BAL fluids were collected at the times indicated, using repetitive (three times) instillation and withdrawal of 10 ml saline via an intratracheal cannula. BAL samples were centrifuged at 1500 rpm for 10 min, and cell pellets were assayed for differential cell counts and for flow cytometric analysis.

Animals receiving anti-CSa Ab treatment were injected i.v. at the time of the CLP procedure with 500 μg rabbit anti-rat C5a Ab (purified and characterized as described previously (13)) or with 500 μg preimmune rabbit IgG. To study the effects of β1 and β2 integrin content on neutrophil migration into lung, 100 μg anti-β1 or anti-β2 was intratracheally administered together with anti-BSA at the time of induction of IgG-IC deposition. The intratracheal route of delivery was chosen for Ab intervention to administer together with anti-BSA at the time of induction of IgG-IC deposition. The intratracheal route of delivery was chosen for Ab intervention to

Flow cytometric analysis

β1 and β2 integrins were evaluated by direct immunofluorescence staining of whole blood using an established lyse/wash procedure (BD PharMingen). Flow cytometric analysis was conducted immediately after blood collection. FITC-conjugated hamster anti-β1, IgM (Ha2/5), normal hamster IgM, anti-β2, mouse IgGl1 (Wt.3), and mouse IgGl1 were purchased from BD PharMingen. One microgram of FITC-labeled Ab in 100 μl staining buffer (PBS with 0.1% sodium azide and 1% FBS) was incubated with 100 μl rat whole blood for 30 min at room temperature. Erythrocytes were lysed for 10 min by addition of 1X FACS lysis solution (BD PharMingen). After washing, the leukocytes were resuspended in a fixation solution (1% paraformaldehyde prepared in PBS with 0.1% sodium azide). Granulocytes were gated by the typical forward and side light scatter profiles. We identified the gated population as granulocytes by staining of whole blood with an FITC-labeled rat granulocyte marker, HIS48 (BD PharMingen), revealing that ≥90% of gated cells were granulocytes. The cells were analyzed using a flow cytometer (Coulter, Miami, FL).

Western blot analysis

Twenty-microliter BAL samples retrieved from rats undergoing IgG-IC deposition and/or CLP were electrophoresed in a denaturing 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with TBST (40 mM Tris (pH 7.6), 300 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 12 h at 4°C. Membranes were incubated with a polyclonal goat anti-rat fibrinectomy Ab (Santa Cruz Biotechnology). The membranes were developed by the ECL technique according to the manufacturer’s protocol (Amersham, Arlington Heights, IL).

MPO activity

Tissues were weighed and homogenized in a homogenate buffer, 0.5% hexadecyltrimethylammonium bromide, and 5 mM EDTA in 50 mM potassium phosphate buffer, pH 6.0. The samples were sonicated for 1 min, and then centrifuged at 20,000 x g for 15 min. Ten microliters of each sample was added to a 96-well plate, followed by addition of 250 μl assay buffer (0.1% H2O2, and 0.5 mM η-dianisidine dihydrochloride and 100 mM potassium phosphate, pH 6.0). The change in OD at 460 nm was measured over a period of 6 min at 15-s intervals, using a kinetics mode in a spectrophotometer (Molecular Devices, Sunnyvale, CA). The slope of the change in OD was calculated to reflect the rate of change in units per gram of lung per minute. All samples were diluted 1/5 to guarantee a linear response.

Statistical analysis

In groups with equal variances, datasets were analyzed using one-way ANOVA, and individual group means were then compared with the Student-Newman-Keuls multiple comparison test. In groups containing unequal variances, Kruskal-Wallis ANOVA was performed, followed by Dunnett’s method for multiple comparison. All values were expressed as the mean ± SEM. Significance was assigned where p < 0.05. For percent change between groups, values obtained from negative controls were subtracted from each data point.

Results

Changes in β1 and β2 content of blood neutrophils after CLP

The β1 and β2 content of blood neutrophils was quantitatively evaluated by flow cytometric analysis 0, 3, 6, 12, 24, and 36 h after the onset of CLP. Neutrophils from control rats (0 h) showed low levels of staining for β1 (mean fluorescence intensity (MFI) = 2.56 ± 0.54). The typical histogram for β1 integrin content contained two peaks (Fig. 1A, middle panel), suggesting that there were two population of neutrophils, one (~20% of cells) with high levels of β1 content and the other (~80%) containing low levels of β1 integrin. Twelve hours after CLP the β1 content of neutrophils was significantly increased (MFI = 6.77 ± 0.71; p < 0.01; Fig. 1B), but not before that time point (3 and 6 h). The β1 content remained constantly elevated at 24 and 36 h after CLP (Fig. 1B).

An isotype-matched Ab showed no positive staining of neutrophils at the various time points (Fig. 1, A and B).

Typical histograms for β2 staining of neutrophils are shown in Fig. 1C. The isotype control IgG showed no positive staining of cells. After CLP the β2 content quickly increased to 4.33 ± 0.35 from 2.23 ± 0.15 (p < 0.03) h after CLP (Fig. 1, C and D). The β2 integrin content remained at elevated levels at all times from 3 h onward after CLP (Fig. 1D). These data indicate that β1 and β2 integrin expression on blood neutrophils is elevated during sepsis. Increased levels of β1 integrin are delayed compared with changes in β2 integrin expression on blood neutrophils.

Effect of C5a on β1 and β2 integrin expression on neutrophils in vivo and in vitro

It is known that C5a plays an important role in the pathogenesis in CLP-induced sepsis in rats (13, 14). To investigate the possible role of C5a in β1 and β2 integrin expression on blood neutrophil during sepsis, C5a was blocked by an i.v. infusion of 500 μg anti-C5a rabbit IgG immediately after the onset of sepsis. A companion group of CLP rats was similarly treated with 500 μg preimmune IgG. β1 and β2 integrin expression was analyzed by flow cytometry 12 h after CLP. Systemic blockade of C5a had no effect on the elevated content of neutrophil β1 integrin in sepsis, but the β1 content of blood neutrophils was decreased in CLP rats by 40% in the presence of anti-C5a (p < 0.05; Fig. 2A). These data suggest that increased in vivo expression of β2 integrin on blood neutrophils, but not β1 integrin, is C5a dependent. To determine whether C5a directly affects β1 and β2 expression, whole blood from normal rats was incubated with rat recombinant C5a (over a range of 0–200 nM) for 1 h, and β1 and β2 integrin expression was determined by flow cytometry as described above. Preparation, purification, and characterization of rat recombinant C5a have been described previously (15). The β1 integrin content was moderately, but significantly, increased by exposure to C5a at a dose of 200 nM C5a (from 1.8 ± 0.42 to 2.98 ± 0.19; p < 0.05), whereas no effects were seen at doses of 0–100 nM (Fig. 2B). In contrast, 100 and 200 nM C5a significantly increased β2 expression from 2.89 ± 0.14 to 4.7 ± 0.49 and 4.99 ± 0.32, respectively (p < 0.05; Fig. 2B), suggesting that β2 integrin expression on blood neutrophils is more sensitive to C5a stimulation than is β1 integrin expression.
Effects of PMA on $\beta_1$ and $\beta_2$ content of neutrophils

To further investigate $\beta_1$ and $\beta_2$ integrin expression on blood neutrophils, whole blood from normal rats was incubated with PMA for 30–60 min at 37°C, and $\beta_1$ and $\beta_2$ integrin expression was determined by flow cytometry. When whole blood was incubated with 200 nM PMA, there was no elevation during the first 30 min, but a modest increase in $\beta_1$ content on blood neutrophils was found 60 min after stimulation (Fig. 2C, left). In contrast, $\beta_2$ integrin expression was drastically increased from 2.75 ± 0.09 to 11.69 ± 1.15 ($p < 0.01$) 30 min after stimulation and rose to 14.83 ± 1.79 ($p < 0.01$) at 60 min (Fig. 2C, right). $\beta_1$ and $\beta_2$ integrin expression in response to different doses of PMA (0–200 nM) was also evaluated during a 60-min exposure time (37°C). $\beta_1$ integrin expression significantly increased from 2.40 ± 0.37 to 6.11 ± 0.59 ($p < 0.05$) with 50 nM PMA. No further increase in $\beta_1$ integrin expression was found with increasing PMA concentrations (Fig. 2D). $\beta_2$ integrin expression was dramatically increased from 2.39 ± 0.08 to 10.69 ± 1.09 in response to 50 nM PMA ($p < 0.001$), with additional increases after exposure to 100 nM PMA (MFI = 14.20 ± 1.04), reaching a plateau thereafter (Fig. 2D).

Influence of CLP on $\beta_1$ and $\beta_2$ expression on neutrophils

To study the effects of $\beta_1$ and $\beta_2$ integrin expression on neutrophil trafficking into lung sepsis, we determined $\beta_1$ and $\beta_2$ integrin expression on blood and BAL neutrophils during CLP. No significant increase in BAL neutrophil numbers was found on 24 or 36 h after the onset of CLP (data not shown). In normal rats the $\beta_1$ content of blood neutrophils was low, as was the case in rats undergoing intrapulmonary deposition of IgG-IC, in which blood or BAL neutrophils were evaluated (Fig. 3A). In fact, in the latter case $\beta_1$ levels fell significantly compared with those in blood neutrophils. Acute lung injury was induced in rats 24 or 36 h after CLP by lung deposition of IgG-IC to induce neutrophil accumulation in the alveolar compartment. This lung injury is characterized by infiltration of large numbers of neutrophils into the lung interstitium and into the alveolar compartment (12), the latter being readily sampled by BAL, and the neutrophil content for the combined lung interstitial and alveolar compartments being readily measured by total lung content of MPO. In both CLP and non-CLP rats, >90% of cells obtained in BAL fluids 4 h after IgG-IC deposition were neutrophils as determined morphologically (data not shown). In confirmation of the data presented in Fig. 1, $\beta_1$ content significantly increased in blood neutrophils 24 and 36 h after the onset of CLP (Fig. 3, B and C, middle sets of black bars). Lung challenge of these septic rats with IgG-IC resulted in no significant changes in $\beta_1$ integrin content of blood neutrophils (third set of black bars). However, there was a significant decrease in the $\beta_1$ integrin content of BAL neutrophils compared with levels in blood neutrophils (2.89 ± 0.67 vs 7.24 ± 1.59; $p < 0.05$) in rats challenged by IgG-IC 24 h after CLP (Fig. 3B). Similar results were obtained in CLP rats 36 h after the onset of sepsis and 4 h after challenge with IgG-IC (Fig. 3C). The $\beta_1$ content of BAL neutrophils was much lower than that of blood neutrophils (2.73 ± 0.39 vs 9.23 ± 1.88; $p < 0.05$; Fig. 3C). A reduction of $\beta_1$ integrin content in BAL neutrophils was also shown by flow
suggesting that differences between the two sources of cells (data not shown), integrin in blood neutrophils and BAL neutrophils failed to show blood neutrophils (2.80 ± 0.45 vs 10.67 ± 1.59; p < 0.01; Fig. 3D). Similarly, in animals treated with IgG-IC 24 h after CLP, there was a significant increase in the β2 integrin content of BAL neutrophils compared with that of blood neutrophils (10.21 ± 0.65 vs 3.92 ± 0.48; p < 0.01; Fig. 3E). Nearly identical results were obtained when rats were injured by IgG-IC 36 h after the onset of CLP (Fig. 3F).

**Neutrophil content in blood and BAL fluids from CLP rats**

Blood and BAL neutrophil contents and lung MPO levels were assessed in otherwise normal (control) rats, in rats with acute lung injury following deposition of IgG-IC, in non-CLP rats, and in CLP (24 h) rats after intrapulmonary deposition of IgG-IC 4 h previously. The data are shown in Table I. As might be predicted, CLP induced a modest increase in blood neutrophils compared with non-CLP rats. In control (non-CLP) or CLP rats, the BAL content of neutrophils was <0.05 × 10⁶. In the presence of intrapulmonary deposition of IgG-IC in otherwise normal rats, 3.3 ± 0.39 × 10⁶ neutrophils were retrieved by BAL, whereas in CLP this number was reduced by 50% (to 1.36 ± 0.36 × 10⁶). Whole lung MPO was also measured, as reflected by the slope of the change in OD As would be expected, the MPO activity in control lungs was low, with an OD value of 16.60 ± 0.36. The presence of IgG-IC caused a 4.5-fold increase (OD = 74.85 ± 6.34), as was also the case in the lungs of CLP rats not undergoing IgG-IC deposition (5.0-fold increase; OD = 81.66 ± 7.37). In CLP rats

**FIGURE 2.** Effects of C5a or PMA on β1 and β2 integrin expression. A, Effect of in vivo blockade of C5a on β1 and β2 integrin contents of blood neutrophils during CLP. C5a was blocked by an i.v. infusion of 500 μg anti-C5a rabbit IgG immediately after the onset of CLP. β1 and β2 integrin expression was analyzed by flow cytometry 12 h after CLP. For each vertical bar, n = 6–8 animals. B, In vitro activation of β1 and β2 integrin on blood neutrophils after contact with C5a. One hundred microliters of whole blood from normal rats was incubated with rat recombinant rat C5a (0–200 nM) for 1 h at 37°C, and β1 and β2 integrin expression was determined by flow cytometry. All values are the mean ± SEM (n = 3 for each data point). C, Time-dependent activation of β1 and β2 integrin. β1 and β2 activation was achieved with 200 nM PMA. Whole blood was incubated with PMA for 30 or 60 min at 37°C; β1 and β2 integrin expression was determined by flow cytometry. For each vertical bar, n = 3. D, Dose-dependent activation of β1 and β2 integrin on blood neutrophils by a dose range of PMA. β1 and β2 integrin expression in response to PMA after an exposure time of 60 min (37°C). All values are the mean ± SEM (n = 3). Statistical comparisons are to the HBSS control group at each interval of time.
also subjected to IgG-IC deposition, lung MPO rose 8.3-fold (OD = 137.76 ± 7.51). These data suggest that in IgG-IC-inflamed lungs of CLP rats, whole lung MPO is elevated, but the number of neutrophils retrieved from BAL fluids is not correspondingly increased.

Effects of β1 and β2 integrin blockade on lung MPO content
To pursue further the data in Table I and to assess the role of β1 and β2 integrins in lung buildup of MPO, the studies described in Fig. 4 were performed. As indicated, rats received 100 μg normal hamster IgM, mouse IgG, hamster anti-β1 IgM, or anti-mouse β2 IgG intratracheally at the initiation of IgG-IC deposition, which was allowed to progress for 4 h. Where indicated, rats were used 24 h after the induction of CLP. As would be expected, the MPO content in normal lungs was low. This value was arbitrarily set at 1.0. In otherwise normal rats, IgG-IC deposition caused a nearly 5-fold increase in MPO content. Treatment with anti-β2 integrin reduced MPO buildup by 42%, whereas anti-β1 integrin had no such effect (Fig. 4, left side). In CLP rats at 24 h the lung MPO content was 5-fold greater than the lung MPO content in control rat lungs. In the presence of IgG-IC in CLP rats the MPO content rose nearly 10-fold above the level found in normal lungs and was twice that found in non-CLP rats receiving intrapulmonary deposition of IgG-IC. Instillation of anti-β1 integrin or anti-β2 integrin reduced MPO content by 76 and 58%, respectively (Fig. 4, right side). Thus, it would appear that sepsis alters the trafficking of neutrophils into the lung by engaging a β1 integrin-dependent pathway.

Morphological evaluation of lungs
In lungs from rats 24 h after CLP, the lungs appeared normal in appearance (Fig. 5A). At higher power, neutrophils could be seen

<table>
<thead>
<tr>
<th>Status</th>
<th>Blood PMNs (×10^6/ml)</th>
<th>Total BAL PMNs (×10^6)</th>
<th>Ratio of BAL PMNs to Blood PMNs</th>
<th>Lung MPO (fold increases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.64 ± 0.42</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>IC</td>
<td>4.24 ± 0.32</td>
<td>3.30 ± 0.39</td>
<td>0.79 ± 0.13</td>
<td>4.48 ± 0.31</td>
</tr>
<tr>
<td>CLP</td>
<td>5.87 ± 0.91</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>5.01 ± 0.52</td>
</tr>
<tr>
<td>CLP + IC</td>
<td>5.14 ± 0.72</td>
<td>1.36 ± 0.36^b</td>
<td>0.27 ± 0.08^c</td>
<td>8.30 ± 0.32^b</td>
</tr>
</tbody>
</table>

*a Rats were evaluated 24 h after CLP and 4 h after intrapulmonary deposition of IgG-IC.
^b p < 0.05 compared to the IC group.
^c p < 0.05 compared to the IC group.
in interstitial capillaries (data not shown). In lungs of otherwise normal rats 4 h after initiation of IgG-IC deposition, there was extensive intra-alveolar accumulation of neutrophils, hemorrhage, and some fibrin deposition (Fig. 5B). In CLP rats at 24 and 4 h after initiation of IgG-IC deposition, intra-alveolar hemorrhage and accumulation of neutrophils and fibrin deposits were prominently seen. In addition, there were prominent blue proteinaceous deposits within alveolar spaces in which neutrophils appeared to be embedded.

Influence of CLP on BAL content of fibronectin
BAL samples retrieved from rats (with or without CLP) undergoing IgG-IC deposition were evaluated for fibronectin levels by Western blot analysis. As shown in Fig. 5D, fibronectin levels increased modestly in BAL fluids obtained from rats 4 h after IgG-IC deposition, while there was no detectable fibronectin in BAL from rats 24 h after CLP. However, induction of IgG-IC deposition in CLP animals resulted in a sharp increase in detectable fibronectin.

Discussion
Sepsis involves widespread up-regulation of both neutrophil and endothelial adhesion molecules (10). In this study with a rat model of sepsis, it was observed that the content of $\beta_1$ and $\beta_2$ integrins on circulating neutrophils was elevated after CLP. Consistent with the original definition of $\beta_1$ integrins arising late after stimulation, in CLP the increased expression of $\beta_1$ integrin on blood neutrophils was not detectable until at least 12 h after CLP, while $\beta_2$ integrin was elevated as early as 3 h. Rapid elevation of $\beta_2$ integrin may be an important role in host defense and tissue repair by directing neutrophils into inflamed organs. Several lines of evidence support such a role for $\beta_2$ integrins in the mobilization of neutrophils into tissues. Patients with inherited deficiencies of $\beta_2$ integrins are much more susceptible to bacterial infection (16). In a canine model of lung inflammation (i.v. infusion of TNF-$\alpha$), anti-CD11b treatment reduced neutrophil accumulation early (within the first 24 h), but not later (>24 h after TNF-$\alpha$ infusion), and subsequent lung injury and mortality were also reduced (10). These data suggest that leukocyte trafficking may differ between the early and late stages of inflammation. As shown in Fig. 1, $\beta_1$ integrin up-regulation was delayed after CLP, in striking contrast to that of $\beta_2$ integrin. It is possible that delayed $\beta_1$ integrin expression may play an important role in neutrophil migration in the later stage of sepsis.

It is well known that human or animals with sepsis not only display high blood levels of pro-inflammatory cytokines but also produce high levels of anti-inflammatory cytokines to counterbalance inflammatory responses. The loss of the balance between pro- and anti-inflammatory mediators may result in exaggerated pro-inflammatory responses, immunosuppression, apoptosis, and organ dysfunction (17, 18). Activation of the alternative pathway of complement is considered to be an effective defense mechanism against invading micro-organisms. However, hyperactivation may lead to compromised host defenses. For instance, an excessive amount of complement activation products, such as C5a, exerts harmful effects in sepsis. In vivo blockade of C5a is highly protective and dramatically reduces thymocyte apoptosis (13, 14). In vitro experiments showed that recombinant C5a increases CD11b/CD18 expression on neutrophils and enhances adhesive interactions of both neutrophils and eosinophils to unstimulated HUVECs or to human bronchial epithelial cells (19, 20). In the current studies systemic blockade of C5a had no effect on $\beta_1$ up-regulation of blood neutrophils from CLP rats, but the same treatment reduced by 40% the $\beta_2$ content of neutrophils during sepsis. The in vitro study showed that $\beta_2$ integrin up-regulation on blood neutrophils
was more responsive to C5a or PMA than was β1 integrin expression (Fig. 2). These data imply that β1 and β2 may be activated in sepsis via different mechanisms. There was no evidence for transcriptional up-regulation of β1 and β2 integrin gene expression in neutrophils isolated from CLP animals (data not shown). Not surprisingly, β2 integrin was not fully activated after CLP, given the fact that β2 integrin could be further activated by PMA. Thus, neutrophils may contain sufficient integrin proteins in their granule reservoirs, allowing responsiveness via granule fusion to the cell membrane in the absence of transcriptional regulation.

Integrin up-regulation by chemoattractant receptor signaling is believed to be a protein kinase A-related event, while PMA, as a membrane-soluble diacylglycerol analog, enhances adhesion due to activation of protein kinase C and phosphorylation of the β-chain (21). Conformational changes may also lead to integrin activation and an increase in the avidity of neutrophils to ligands (3). It is of note that increased surface expression of β2 integrin on human neutrophils promotes β2 integrin-mediated adhesion of neutrophils to fibronectin (22). Conversely, β2 integrins may potentially alter β1 integrin avidity (21). These data suggest that cross-talk signaling may exist between β1 and β2 integrins on neutrophils. The mechanism underlying disparate activation of β1 and β2 integrins in the CLP model remains to be determined.

Cell-cell and cell-matrix interaction are essential for leukocyte trafficking both in uninjured tissues and in the setting of inflammation. Neutrophil trafficking into the lung involves transendothelial migration, migration through the tissue interstitium (primarily composed of fibroblasts and extracellular matrix), and transepithelial migration into alveolar space. Transendothelial migration is primarily mediated by members of the β2 integrins and α-containing integrins, such as α5β1. In vitro experiments have shown that neutrophils from septic, but not from normal, human donors express αβ1 integrin and can functionally bind to immobilized VCAM-1 (11). It has been demonstrated that neutrophil migration through connective tissue is partially mediated by CD11b/CD18, α4β1, α5β1, and α6β1 integrins on neutrophils (23). Transepithelial migration might be exclusively mediated by β1 integrin, since CD18-blocking Ab almost completely inhibited the transmigration across epithelial monolayers (24).

There was no significant presence of neutrophils in BAL fluids 24 or 36 h after CLP (data not shown), although MPO activity in lung increased by 5-fold 24 h after CLP (Table I), implying that the transmigratory migration of neutrophils did not occur, and that most of any infiltrated neutrophils accumulated in the lung interstitium. In the acute lung injury model induced by deposition of IgG-IC, the three compartments of neutrophil transmigration mentioned above are known to be involved (25, 26). In the otherwise uncomplicated single-hit model of IgG immune complex injury, >90% of cells present in BAL fluids 4 h after the onset of injury are neutrophils, and BAL neutrophils can be readily retrieved by the BAL procedure. In addition, the β1 and β2 contents on blood neutrophils were not altered (Fig. 3). Thus, deposition of IgG-IC in the CLP lung is a useful approach to study the contents of β1 and β2 integrins on neutrophils and the effects of blockade of either integrin on neutrophil transmigration. The β1 integrin content of BAL neutrophils was significantly lower than that of blood neutrophils in non-CLP rats challenged with IgG-IC (Fig. 3A). As might be expected, lung challenge of CLP rats with IgG-IC resulted in no significant changes in the β1 content of blood neutrophils. However, BAL neutrophils, compared with the levels in blood neutrophils, showed much lower levels of β1 integrin. Histograms from flow cytometric analysis clearly showed that the proportion of cells with a high level β1 integrin content markedly decreased when examined in BAL neutrophils (Fig. 3). Following deposition of IgG-IC, the number of neutrophils in BAL fluids retrieved from CLP rats was only half the BAL neutrophil counts in non-CLP rats, although MPO activity in CLP lungs was greatly increased following deposition of IgG-IC (Table I and Fig. 4). Initially, we thought that these data suggested that β1 integrin-enriched neutrophils had sequestered in the lung interstitium during sepsis, and that their transmigration has been interrupted before reaching the alveolar compartment, perhaps due to interactions between β1 integrins and their ligands in the connective tissue matrix. However, histological analysis of IgG-IC-induced lung injury in CLP rats indicated that neutrophils had transmigrated into the alveolar compartment, and a number of neutrophils appeared to be embedded in blue proteinaceous deposits within alveolar spaces (Fig. 5), perhaps because of binding to alveolar pools of protein to which neutrophils are adhesive via their β1 integrin content. Thus, the lower yield of neutrophils from the BAL procedure may be due to the inability to retrieve all alveolar neutrophils. Therefore, the number of BAL neutrophils retrieved from the two-hit lungs appears to be an underestimate. Fibronectin and collagen levels have been shown to be increased in BAL fluids and lung tissues from human and animals with acute lung inflammation (27). Increased fibronectin present in BAL fluids arises from plasma fluid leakage as well as from local production from lung cells (28). Induction of acute lung injury in CLP rats resulted in a greatly increased content of fibronectin in BAL fluids (Fig. 5D). Elevated levels of fibronectin at the site of injury may provide a pool of protein that can be recognized by β1 integrins, such as ααβ1, ααβ1, and ααβ1, affecting the directional migration of cells (27). β2 integrins on BAL neutrophils seemed to be fully activated by IgG-IC deposition in either non-CLP or CLP animals, suggesting that β2 may play a critical role in neutrophil migration across the epithelial wall, as has been documented by earlier β2 integrin blocking experiments (24).

In vitro experiments have shown that neutrophil migration through fibroblast barriers was suppressed by a combination of anti-β1 and anti-β2 Abs, and no inhibitory effects were observed when the Abs were used separately (29, 30). Treatment with Ab to α and/or α had no effect on neutrophil accumulation in lungs induced by intratracheal administration of LPS. However, blockade of α and α in the presence of anti-β treatment more effectively inhibited neutrophil recruitment in lung compared with β mAb treatment alone (8). A recent publication showed that anti-β1 treatment significantly inhibited both LPS and KC-induced neutrophil migration in mouse lung; this study suggests that α and α may mediate CD18-independent neutrophil accumulation (31). These data suggest that β1 and β2 integrins collectively coordinate neutrophil trafficking.

The role of β1 integrin in neutrophil migration appears to be amplified in sepsis. During sepsis, β1 integrin on neutrophils seems to be significantly up-regulated at the later stage of sepsis, reaching the high levels that can be induced in vitro by PMA. As mentioned previously, elevated levels of β2 integrin in blood neutrophils may play a critical role in orchestrating neutrophil migration during the early phase of sepsis, because the β1 integrin content is not changed. However, fully activated β integrin at the later stage of sepsis may alter the balance of integrin cooperativity. In IC-injured lung occurring in non-CLP rats, blockade of β2 integrin reduced MPO buildup by 42%, whereas anti-β1 integrin had no effect. Thus, this model may serve as a useful tool to assess the role of β1 integrin in neutrophil accumulation in lung at later stages of sepsis. In addition, we have previously shown that LPS as a second hit in CLP rats caused similar effects on neutrophil accumulation as seen with IgG-IC deposition (32). After IgG-IC deposition in rats 24 h after CLP, both anti-β integrin and anti-β2
integrin reduced MPO content by 76 and 58%, respectively. Anti-\(\beta_1\) integrin may suppress neutrophil transendothelial migration by inhibiting the adhesion between leukocyte \(\alpha_\beta\) and endothelial VCAM-1. It may also be that anti-\(\beta_1\) Ab interferes with cell motility by affecting the adhesive interactions of neutrophils to matrix, although the exact mechanism is not understood. Nevertheless, it is clear that sepsis alters the trafficking of neutrophils into the lung by engaging a \(\beta_1\) integrin-dependent pathway.

Acknowledgments

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References