Role of alveolar macrophage and migrating neutrophils in hemorrhage-induced priming for ALI subsequent to septic challenge

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Lomas-Neira, Joanne, Chun-Shiang Chung, Mario Perl, Stephen Gregory, Walter Biffl, and Alfred Ayala. Role of alveolar macrophage and migrating neutrophils in hemorrhage-induced priming for ALI subsequent to septic challenge. Am J Physiol Lung Cell Mol Physiol 290: L51–L58, 2006. First published September 9, 2005; doi:10.1152/ajplung.00028.2005.—Acute lung injury (ALI) is identified with the targeting/sequestration of polymorphonuclear leukocytes (PMN) to the lung. Instrumental to PMN targeting are chemokines [e.g., macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), etc.] produced by macrophage, PMN, and other resident pulmonary cells. However, the relative contribution of resident pulmonary macrophages as opposed to PMN in inducing ALI is poorly understood. We therefore hypothesize that depletion of peripheral blood PMN and/or the obliteration of a macrophage-mediated PMN chemokine signal (via macrophage deficiency) will reduce the inflammation and ALI observed in mice following hemorrhage (Hem) and subsequent sepsis (CLP) in our murine model of ALI. To examine this we pretreated mice with either 500 μg anti-mouse Gr1 antibody/animal (to deplete PMN) or subjected mice deficient in mature macrophage (B6C3Fe-a/-CsF1op) to Hem (90 min at 35 ± 5 mmHg) followed by resuscitation. Twenty-four hours post-Hem, mice were subjected to CLP and killed 24 h later, and lung tissue samples were collected. Our data showed that in the absence of either peripheral blood PMN or mature tissue macrophages there was a suppression of IL-6, KC, and MIP-2 levels in lung tissue from Hem/CLP mice as well as a reduction in PMN influx to the lung and lung injury (bronchoalveolar lavage fluid protein). In contrast, lung tissue IL-10 and TNF-α levels were suppressed in the macrophage-deficient Hem/CLP mice compared with PMN-depleted Hem/CLP mice. Together, these data suggest that both the PMN and the macrophage are required to induce inflammation seen here, however, macrophage not PMN regulate the release of IL-10, independent of local changes in TNF.

acute lung injury; sepsis; mouse; neutropenic; macrophage deficient

ACUTE LUNG INJURY (ALI) is a progressive syndrome associated with significant mortality in trauma/sepsis patients (44). What has been observed is that for trauma patients there appears to be a period of time following their initial injury where a significant risk exists for the development of ALI upon exposure to a secondary infectious challenge. In this respect, the accumulation of activated neutrophils found in lung tissue from patients with severe inflammatory lung injury is suggested to play a significant role in the development of ALI. In a non-pathological immune response, neutrophils targeted to the lung are cleared once the invading pathogen has been eliminated. Our laboratory and others have found that in the pathogenesis of ALI, neutrophil function becomes dysregulated leading to their sequestration in the lung where their presence is associated with tissue injury (11, 28, 36).

Neutrophils are essential first responders in host defense. Upon recruitment to sites of infection and inflammation they release proteolytic enzymes and reactive oxygen species targeting/killing invading pathogens. Instrumental to neutrophil migration to the lung are chemokines produced both locally (by tissue macrophage and lung tissue endothelium) as well as systemically (distal inflammatory sites) (7, 15, 48). The relationship between systemic and local environmental responses involves a balance coordinated by cell-to-cell interactions as well as the production of specific pro- and anti-inflammatory cytokines (4, 15, 16, 32).

An example of this relationship in the lung is the production of a neutrophil chemoattractant, macrophage inflammatory protein-2 (MIP-2), by tissue macrophages and bronchoalveolar epithelial cells in response to inflammatory stimuli. Neutrophils, in response to migratory signaling, cross the endothelium and stimulate the release of IL-10 (a predominantly anti-inflammatory modulator of the immune response) (14) ostensibly from alveolar macrophages (19, 24, 37, 40). A hypothesis for neutrophil associated stimulation of IL-10 release from alveolar macrophage has been presented by Fadok et al. and Lucas et al (10, 29). Their studies have shown that phagocytosis of apoptotic neutrophils stimulates production of IL-10 by macrophage. The release of IL-10 is believed to play a role in suppressing cytokine production and the phagocytic activity of alveolar macrophages (14, 19, 24, 40). In this scenario, neutrophils serve as both a pro- and anti-inflammatory stimuli, interacting with both barrier (endothelial cells) and immune cell populations. Additionally, proinflammatory cytokines serve to up-regulate the expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, increasing neutrophil adhesion and migration from the circulation through the endothelium into the lung (7, 31, 33). These examples demonstrate the coordinated participation and interactions of resident cells in the immune response.

In previous experiments we have shown that antibody neutralization of MIP-2 abrogated the extent of inflammatory potential for donor neutrophil priming following hemorrhage in neutropenic recipients subjected to septic challenge [cecal
ligation and puncture (CLP)] (28). The reduction in MIP-2 levels coincided with a decrease in lung tissue levels of IL-6 and myeloperoxidase (MPO) activity (neutrophil influx) and increased IL-10 compared with isotype-treated controls. However, as both neutrophils and resident pulmonary macrophage can contribute to the release of MIP-2, it was unclear what significance these two cellular sources of MIP-2 individually played in the development of ALI.

On the basis of these data, observations from other laboratories mentioned above, and in continuity with our previous studies (26–28) we sought to further investigate the contributions of neutrophils as well as macrophage to the pathogenesis of ALI in our model of hemorrhage-induced neutrophil priming for ALI. We hypothesized not only that if mice were depleted of peripheral blood neutrophils and/or mice were deficient in chemokine-producing mature macrophages, they would exhibit reduced indexes of inflammation and lung injury following hemorrhage and subsequent septic challenge, but that these phagocytic lineages would make different contributions to this process.

MATERIALS AND METHODS

Reagents

Keratinocyte-derived chemokine (KC) and MIP-2 monoclonal capture antibody and secondary detection antibody for ELISA assays were purchased from R&D Systems (Minneapolis, MN). Mouse IL-6, IL-10, and TNF-α ELISA kits were purchased from BD Bioscience (San Diego, CA). Antibodies for immunohistochemistry were purchased from BD Pharmingen (San Jose, CA), and fluorescent streptavidin conjugates were purchased from Molecular Probes (Eugene, OR). All other chemicals were analytical reagent grade and purchased from Sigma Chemical (St. Louis, MO).

Mice

Male C3H/HeN mice (Charles River Laboratories, Wilmington, MA) 7–9 wk of age were used for neutrophil depletion experiments. Mice deficient in macrophage colony-stimulating factor-1 (M-CSF-1), 6–7 wk of age, colony-stimulating factor (CSF-1), B6C3Fe-a/a-CsxFr1+ (op–/–), and an untyped background control strain (op+/+) from The Jackson Laboratory (Bar Harbor, ME) were used for macrophage depletion experiments. Morphological assessment of lung macrophage deficiency via immunohistochemical staining for Mac-3 antigen (CSF-1R/CD115) is ~70% in op–/– vs. op+/+ control mice. The op–/– mice do not however, exhibit a significant decline in the number of circulating blood polymorphonuclear leukocytes (PMN). Experiments were performed in accordance with National Institutes of Health guidelines and approval from the Animal Use Committee of Rhode Island Hospital.

Neutrophil Depletion

Mice were depleted of resident neutrophils via intraperitoneal injection of 500 µg of rat anti-mouse neutrophil antibody, anti-Gr1 (clone RB6–8C5, rat IgG2b), per mouse 48 h before hemorrhage. Efficacy of antibody treatment was determined to be >95% based on reduction of the number of neutrophils in peripheral blood smears assessed 48 and 60 h posttreatment. The lack of circulating neutrophils at this time point, during the priming stimulus of hemorrhagic shock, prevents neutrophil activation by proinflammatory mediators released in response to hemorrhage (28). Alternatively, histological assessment of the number of CD115+ cells (macrophage) in the lungs indicated that antibody treatment did not reduce this cell population.

Mouse Hemorrhage/Sepsis Model for ALI

Hemorrhage. The hemorrhage model we have used for these experiments has been previously described (2). In brief, mice were anesthetized with methoxyflurane and restrained in supine position, and catheters were inserted into both femoral arteries. Anesthesia was discontinued, and blood pressure was continuously monitored through one catheter attached to a blood pressure analyzer (BPA; MicroMed, Louisville, KY). When fully awake, as determined by a mean blood pressure of ~95 mmHg, the mice were bled over a 5- to 10-min period to a mean blood pressure of 30 mmHg (~2.5 mmHg) and kept stable for 90 min. Immediately following hemorrhage, mice were resuscitated intravenously with Ringer lactate at four times drawn blood volume. After resuscitation, arteries were ligated, catheters removed, and catheter sites sutured closed. Sham hemorrhage (SHem), was performed as a control, these mice were anesthetized and restrained, and their femoral arteries were ligated, but no blood was drawn.

Polymicrobial sepsis. Twenty-four hours posthemorrhage (or sham hemorrhage), sepsis was induced as a secondary challenge via CLP as previously described (3). To summarize, mice were anesthetized with methoxyflurane and restrained in supine position. A 1-cm midline incision was made; the cecum was ligated with 5-0 silk thread and punctured twice with a 21-gauge needle. The cecum was then replaced, the incision was sutured, and lidocaine was applied, abdominal layer then skin. Mice were resuscitated with 1 ml of Ringer lactate subcutaneously and returned to their cages.

Sample Collection

Twenty-four hours post-CLP mice were killed with an overdose of methoxyflurane. Blood was collected via cardiac puncture into heparinized syringes. Blood samples were centrifuged, and plasma was collected and stored at ~70°C for later cytokine analysis.

Bronchoalveolar lavage (BAL) fluid was collected to assess protein concentration as an index of lung permeability (injury). The trachea was exposed via a midline incision and cannulated with a sterile polypropylene 18-gauge catheter. The lungs were gently lavaged with 0.6 ml of saline three times for an average of 1 ml of lavage fluid total per lung. Lavage fluid was centrifuged 1,000 g for 5 min at 4°C. Protein concentration in lavage fluid was assessed by Bradford assay.

Lung tissue was harvested for assessment of neutrophil influx (esterase+ cells), bronchial endothelial cell ICAM-1 expression, CSF-1R/CD115 expression (macrophage receptor), and tissue architecture. Due to the degradation of tissue architecture observed in lavaged mouse lungs, additional mice were used for histological assessment. Mice were killed with an overdose of methoxyflurane (Pitman-Moore, Mundelein, IL). Blood was then drawn from heart via cardiac puncture, the trachea was cannulated, and lungs were inflated to 25 cm of pressure with formalin. Lungs were excised and snap frozen in liquid nitrogen in embedding molds with optimal cutting temperature media (TissueTek, Elkhart, IN) for later processing of frozen sections.

Methods of Assessment

Cytokine and chemokine ELISAs. ELISAs for IL-6, IL-10, KC, MIP-2, and TNF-α were performed as per manufacturer’s protocol on lung tissue homogenates collected from experimental mice.

Lung MPO activity. MPO activity as an assessment of neutrophil influx was measured according to established protocols (28). In brief, lung tissue was homogenized in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.4, and centrifuged at 40,000 g at 4°C for 30 min. The supernatant was reserved for cytokine analysis. The remaining pellet was resuspended in 0.5 ml of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 12,000 g at 4°C for 10 min. Supernatants were then assayed at a 1:20 dilution in reaction buffer (530 nmol/l o-
dianisidine, 150 nmol/l H₂O₂ in 50 mM potassium phosphate buffer), and read at 490 nm.

Immunohistochemical staining for assessment of neutrophil influx and tissue architecture. Staining for leukocyte-specific esterase, Naphthol AS-D chloroacetate esterase (Sigma Diagnostics, St. Louis, MO), was performed on frozen tissue sections fixed in citrate-acetone-formaldehyde. Slides were incubated in a solution of sodium nitrate, Fast Red Violet BL base solution, TRIZMAL 6.3 buffer, and Naphthol AS-D chloroacetate solution in deionized water for 15 min at 37°C. After rinsing, slides were counterstained with Gills hematoxylin solution and coverslipped. Stained lung sections were examined microscopically for morphology and positively stained cells. To establish the total number (%) of cells (per field) that were neutrophils (esterase+) present in the sample, tissue sections were randomly screened (7–8 fields/slide) at 400 (25 μm²/field).

Bronchial epithelial cell ICAM-1 expression on endothelial cells in neutrophil-depleted and macrophage-deficient mouse lungs. Acetone-fixed frozen lung tissue sections from neutrophil-depleted and macrophage-deficient mice were cut and mounted on glass microscope slides. Immunohistochemistry for CD-144/vascular endothelial (VE)-cadherin (an endothelial cell marker) and CD-54/ICAM-1 was performed as per manufacturer’s protocol (BD Pharmingen). After rinsing to remove mounting media and blocking with PBS plus 5% serum for 30 min at 37°C, slides were incubated with purified hamster antibody against mouse CD-54/ICAM-1 for 1 h at room temperature in a humidity chamber. Slides were rinsed and incubated with a biotinylated mouse anti-hamster IgG2b antibody for 30 min at room temperature and then rinsed. Slides were then incubated with a streptavidin Alexa Fluor 594 conjugate (Molecular Probes, Eugene, OR) for 30 min, rinsed, and compared with negative control slides (without primary). Slides were then incubated with purified rat antibody against mouse CD-144/VE-cadherin for 1 h at room temperature in a humidity chamber. Slides were rinsed and incubated with a biotinylated mouse anti-rat IgG2a antibody for 30 min at room temperature and then rinsed. Slides were then incubated with a streptavidin Alexa Fluor 488 conjugate for 30 min, rinsed, and compared with negative control slides (without primary). Slides were then coverslipped with a mounting medium for fluorescence microscopy with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), for visualization of intact cells. Confocal images were acquired with a Nikon PCM 2000 (Nikon, Melville, NY) using the argon (488) and the green helium-neon (543) lasers. Serial optical sections were performed with Simple 32, C-imaging computer software (Compix, Cranberry Township, PA). Z-series sections were collected at 0.6 μm with a ×60 PlanApo lens and a scan zoom of ×2 and a ×40 PlanApo lens and a scan zoom of ×1. Images were processed, overlaid, and reconstructed/merged in NIH Image J (National Institutes of Health, Springfield, VA). Adobe Photoshop was used in the assembly of figures.

Statistical Analysis

Data was expressed as means ± SE of five mice examined in each group. Statistical error was determined by one-way ANOVA; the post hoc test was Tukey’s. Calculations were performed with SigmaStat for Windows version 2.03. P values <0.05 were considered significant.

RESULTS

Absence of Both Peripheral Blood Neutrophils and Mature Macrophage Suppress Proinflammatory Response

Levels of IL-6 were assessed in lung tissue from antibody-depleted neutropenic mice (Gr1−) and macrophage-deficient mice (op−/−) (Fig. 1). Mice subjected to hemorrhage (Hem)/CLP, but not antibody depleted of neutrophils (Ctrl Hem/CLP) or op background animals (op−/+ Hem/CLP), showed a significant increase in lung tissue IL-6 compared with their respective SHem/CLP groups. Both the deficiency of circulating neutrophils (induced by anti-Gr1 pretreatment) as well as a lack of chemokine-synthesizing macrophages (op−/− mice) significantly reduced the increase in Hem/CLP lung tissue levels of IL-6 compared with background/controls.

Suppression of Chemokine Production Was Observed in Both Neutrophil-Depleted and Macrophage-Deficient Mice

The increased lung tissue levels of neutrophil chemotactic chemokines, KC and MIP-2, observed in IgG-treated Ctrl Hem/CLP or op−/+ Hem/CLP background mice were significantly suppressed in lung tissue from neutropenic mice (Gr1−) and macrophage-deficient mice (op−/−) following Hem/CLP (Fig. 2, A and B).

Neutrophil Depletion but not Macrophage Deficiency Restores IL-10

In those Ctrl and op−/+ mice subjected to Hem/CLP, there was a small but consistent decline in lung tissue IL-10 levels vs. Ctrl and op−/+ animals subjected to sham/CLP. Mice depleted of peripheral blood neutrophils before Hem/CLP (Gr1−) showed a significant increase in lung tissue levels of anti-inflammatory cytokine IL-10 above that of their SHem/CLP control mice with or without prior anti-Gr1 treatment (Fig. 3). However, in the absence of mature tissue macrophages, such an increase above background op−/+ SHem/CLP mouse levels was not observed in the op−/− Hem/CLP mice.

Macrophage Deficiency Significantly Attenuates TNF-α Production in Lung Tissue

Lung tissue levels of TNF-α were significantly elevated above SHem/CLP levels for all Hem/CLP groups (Fig. 4). Furthermore, this effect of Hem/CLP was not attenuated by the depletion of PMN. However, macrophage deficiency (op−/−) attenuated overall TNF-α levels in both the Hem/CLP and SHem/CLP mice.
**Neutrophil Influx Significantly Blocked**

Subjecting Ctrl and op−/− mice to Hem/CLP markedly increased lung tissue MPO levels compared with respective SHem/CLP groups (Fig. 5A). This increase in tissue MPO was significantly attenuated by both anti-Gr1 treatment (Gr1−) and the genetic deficiency in mature tissue macrophages (op−/−) in mice subjected to Hem/CLP.

**Decrease in Neutrophil-Specific Esterase+ Cells in Lung Tissue Sections Is Similar in Both Neutrophil Depletion and Macrophage-Deficient Mice**

Supporting MPO data, lung tissue histology from (Gr1−) Hem/CLP (neutropenic mice) and op−/− Hem/CLP (Hem/macrophage-deficient) mice showed a significant reduction in the percentage of neutrophil-specific esterase+ cells compared with their respective Hem/CLP control/background groups (Fig. 5B).

**Decrease in BAL Fluid Protein Similar in Both Neutrophil Depletion and Macrophage-Deficient Mice**

Protein concentration in BAL fluid was measured to assess lung leakage, an indicator of lung tissue injury. Irrespective of whether the mice were Ctrl or were op−/− background animals, subjecting them to Hem/CLP as opposed to SHem/CLP markedly increased the level of BAL fluid protein (Fig. 6). Further, neutrophil depletion (Gr1−) or macrophage deficiency (op−/−) significantly reduced levels of protein in lung lavage fluid compared with their respective Hem/CLP control/background groups.

**ICAM-1 Expression Suppressed on Endothelial Cells in Lungs of Macrophage-Deficient Mice**

A low level of ICAM-1 is constitutively expressed on endothelial cells in lung tissue (38). In the merged images

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**Fig. 2.** A-B. Lung tissue levels of chemokines KC and MIP-2 were significantly elevated in the Hem/CLP control/background groups relative to their respective SHem/CLP groups in both the neutrophil depletion and macrophage deficiency studies. A suppression of KC and MIP-2 to SHem/CLP levels was observed in both the neutrophil depleted (Gr1−) and macrophage deficient (op−/−) Hem/CLP mice. *P < 0.05 vs. respective SHem/CLP group. @P < 0.05 vs. op−/− Hem/CLP; n = 6 mice/group. #P < 0.05 vs. (Gr1−) Hem/CLP; 8 mice/group.

**Fig. 3.** Lung tissue IL-10 content in the macrophage deficient SHem/CLP, Hem/CLP and control Hem/CLP groups remained at control SHem/CLP levels. Alternatively, lung tissue IL-10 levels were significantly elevated in neutrophil depleted (Gr1−) Hem/CLP mice compared with control Hem/CLP mice. *P < 0.05 vs. respective SHem/CLP group. n = 6 mice/group. #P < 0.05 vs. (Gr1−) Hem/CLP; 8 mice/group.

**Fig. 4.** Lung tissue levels of TNF-α increased in all Hem/CLP groups relative to their respective SHem/Hem group. However, a significant reduction of TNF-α was observed in the macrophage deficient (op−/−) SHem/CLP and Hem/CLP groups compared with (op−/−) SHem/CLP and Hem/CLP controls. *P < 0.05 vs. respective SHem/CLP group. @P < 0.05 vs. op−/− Hem/CLP; n = 6 mice/group. #P < 0.05 vs. (Gr1−) Hem/CLP; 8 mice/group.
deficient mice subjected to Hem/CLP (Fig. 7) not visualized in lung tissue sections from the macrophage-endothelial cell-associated ICAM-1 (yellow) expression was rable to the Ctrl Hem/CLP mice (Fig. 7)

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Lung Tissue MPO pUnits/mg protein

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Fig. 5. A: myeloperoxidase activity increased in lung tissue from the (op−/−) and Ctrl Hem/CLP mice compared with their respective SHem/CLP groups. Less of an increase was observed in the macrophage deficient (op−/−) Hem/CLP mice, however, there was no significant increase in the (Gr1−) Hem/CLP mice. B: % neutrophil-specific esterase+ cells in lung tissue sections increased above SHem/CLP levels in both the op−/+ background and Ctrl control groups. Macrophage deficiency (op−/−) and neutrophil depletion (Gr1−) suppressed this increase in these mice subjected to Hem/CLP. *P < 0.05 vs. respective SHem/CLP group. @P < 0.05 vs. op−/+ Hem/CLP; n = 6 mice/group. #P < 0.05 vs. (Gr1−) Hem/CLP; 8 mice/group.

shown in Fig. 7, endothelial cells stain fluorescent green, and ICAM-1 stains fluorescent red; the colocalization of these colors is visualized by the pseudocolor yellow. In the presence of significantly elevated IL-10 and neutropenia, lung tissue sections from (Gr1−) Hem/CLP mice (Fig. 7B) expressed levels of endothelial cell associated ICAM-1 (yellow) comparable to the Ctrl Hem/CLP mice (Fig. 7A). An upregulation of endothelial cell-associated ICAM-1 (yellow) expression was not visualized in lung tissue sections from the macrophage-deficient mice subjected to Hem/CLP (Fig. 7D) compared with their Hem/CLP background (Fig. 7C).

DISCUSSION

We have previously demonstrated that hemorrhagic shock (90 min at 35 ± 5 mmHg) followed 24 h by septic challenge (CLP) induces, in the lungs of mice, changes in cytokine/chemokine profiles, neutrophil infiltration, and sequestration, and lung tissue injury similar to ALI encountered in a clinical setting of the shock/trauma patient (3, 28). In this study we have used this unique model of murine ALI to assess the neutrophil and macrophage contribution to associated inflammatory lung injury seen following hypotensive shock. Depletion of peripheral blood neutrophils was achieved through treatment with anti-Gr1 antibody 48 h before an initial priming stimulus, hemorrhage, thus preventing hemorrhage activation of neutrophils in circulation. Macrophage-associated studies were performed on mice genetically deficient in M-CSF-1. The op−/− mice (6–7 wk of age) used in our study lack, due to a point mutation, a major macrophage growth factor gene, colony stimulating factor-1 (M-CSF-1) (6, 8, 12, 42, 46). M-CSF-1 is the major growth factor associated with the maturation of tissue macrophages. Although M-CSF-1-independent macrophages have been identified, their phagocytic response is poor and population density low (13, 30, 46). This strain has been characterized by a number of investigators over the last 15 years (8, 12, 34, 35, 41, 46, 47). Labeled “osteopetrotic” due to their low number of osteoclasts, this murine strain has been shown to have defective differentiation of monocytes into osteoclasts and mature macrophage that exhibit reduced cytokine/chemokine expression (18).

These models, neutrophil depleted/mature macrophage deficient, and their backgrounds produce strain-specific perturbations in the local pulmonary immune response following Hem priming and subsequent septic challenge. Through comparison within each strain of their altered response following hemorrhagic shock and sepsis, a clearer picture can be resolved as to the contribution these cells make to the pathogenesis of ALI. Antibody depletion of peripheral blood neutrophils greatly reduced indices of inflammation in these mice. This supports the hypothesis that neutrophils via hemorrhage induced priming have an enhanced capacity to traffic to the lung, where if subsequently stimulated (as with CLP) can contribute to local tissue injury. Because the depletion of neutrophils with anti-Gr1 and their absence from the lungs of (Gr1−) Hem/CLP mice markedly attenuated proinflammatory cytokine, IL-6, and levels of the chemokines KC and MIP-2, a potential require-

Fig. 6. Protein concentration in bronchoalveolar lavage fluid from op−/+ and Ctrl Hem/CLP mice increased significantly compared with their respective SHem/CLP groups. The absence of mature macrophage (op−/−) and neutrophil depletion (Gr1−) in mice subjected to Hem/CLP diminished lavage fluid protein concentration to SHem/CLP levels. *P < 0.05 vs. respective SHem/CLP group. @P < 0.05 vs. op−/+ Hem/CLP; n = 6 mice/group. #P < 0.05 vs. (Gr1−) Hem/CLP; 8 mice/group.
ment for the presence of neutrophils to potentiate the local lung inflammatory response is suggested. Theoretically, the sequence of events in the lung during an inflammatory response is recognized as progressing from MIP-2 production by stimulated macrophage to the targeting of PMN across the lung endothelium, which results in IL-10 production. This cascade of mediator and cellular interactions produces an efficient and regulated inflammatory response resolving in the elimination of invading pathogen and clearance of activated neutrophils. However, interference in the cellular interactions necessary for an effective immune response disrupts not only downstream events, but also those occurring concurrently. In the series of experiments reported in this study we found that by depleting the animal of neutrophils before shock, a broader disruption of inflammatory signaling was observed. By severely reducing the number of neutrophils in the peripheral blood, the signaling pathways stimulated through cellular interactions with activated neutrophils were disrupted. A decrease in circulating neutrophils with an increase of IL-10 is consistent with our previous findings where mice treated with anti-MIP-2 immediately posthemorrhage showed elevated IL-10, decreased MIP-2, and decreased MPO (PMN influx)(28). This data highlights the necessity of cell-cell interactions in the regulation of mediator expression.

The absence or diminution of neutrophil/endothelial cell (ICAM-1) interaction presents an additional disruption in the immune signaling pathway. Systemic activation of ICAM-1, via complement and/or TNF-α and IL-1β increases intrapulmonary recruitment of neutrophils via lymphocyte function-associated Ag (LFA-1) receptor binding to ICAM-1(9, 23, 45). Peripheral blood lymphocytes also express the LFA-1 receptor and have been reported to migrate across the lung endothelium in response to antibody against LFA-1 as well as interactions with B and T cells (1, 5, 17, 21, 39). Additionally, as reported by van der Meeren et al. and others (1, 5, 21, 43), these emigrating lymphocytes were found to have the capacity to release IL-10. Our data showed an upregulation of ICAM-1 on lung endothelial cells in the presence of elevated IL-10 in neutrophil-depleted mice following hemorrhage and subsequent septic challenge (Fig. 7); this is in agreement with recent studies by Ledeboer et al. and Lisinski et al. (22, 25). Their studies show that although IL-10 suppressed extravasation of neutrophils and cytokine production, adhesion molecule expression was not similarly affected in response to chronic inflammation (22) or TNF-α and IL-1β stimulation (22). In this scenario, despite neutropenia, the transendothelial migration of lymphocytes presents a level of complexity that although existing in the background control mice, prevents more than a speculative explanation for this outcome.

With respect to the macrophage-deficient mouse lungs, ICAM-1 expression was not upregulated compared with its background. However, in these mice neither IL-10 nor TNF-α
was upregulated following hemorrhage and septic challenge. These findings suggest that disruption of macrophage-associated signaling represents a broader impact on the process and intensity of the inflammatory response.

Lung tissue levels of TNF-α remained elevated in the anti-Gr1 treatment group compared with Ctrl Hem/CLP group despite the significant reduction in neutrophils detected in the lungs of (Gr1−) Hem/CLP mice. The finding of increased expression of TNF-α in the presence of IL-10 is also not without precedence, as the neutrophil-depleted septic mice maintain a full complement of mature IL-10 producing macrophages in their lung compartment. In a recent study of trauma/hemorrhage in mice, Knoferl et al. (20) found both IL-10 and TNF-α to be produced by Kupffer cells in the liver.

Mice genetically deficient in M-CSF-1, a protein associated with macrophage survival, proliferation, and differentiation (6), also showed marked reduction in levels of inflammatory and chemotactic proteins. Alveolar macrophage are a major source of neutrophil chemokine MIP-2 (7, 48). Thus elimination of this reservoir should have also served to reduce the number of neutrophils chemotaxing to the lung, as we have observed here.

Additionally, macrophage are thought to be a potentially significant source for or respondent to IL-10 (40). The inability of macrophage-deficient (op−/−) mice to produce increased IL-10 levels as opposed to the neutrophil-deficient (Gr1−) Hem/CLP mice [as op−/−Hem/CLP mice had similarly reduced MPO levels/numbers esterase/Hem/CLP mice as opposed to Ctrl Hem/CLP group] maintain a full complement of mature IL-10 producing macrophages in their lung compartment. In a recent study of trauma/hemorrhage in mice, Knoferl et al. (20) found both IL-10 and TNF-α to be produced by Kupffer cells in the liver.

These findings suggest that disruption of macrophage-associated signaling represents a broader impact on the process and intensity of the inflammatory response.

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In summary, the results described in this study support our hypothesis that the absence or suppression of either neutrophil or macrophage response reduce indexes of inflammation and lung injury following hemorrhage and subsequent septic challenge. Thus both cell types are essential to the pathological development of lung injury resultant from shock and sepsis. However, their contributions while interrelated are not wholly redundant.


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