Neutrophil-mediated lung permeability and host defense proteins

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Kantrow SP, Shen Z, Jagneaux T, Zhang P, Nelson S. Neutrophil-mediated lung permeability and host defense proteins. Am J Physiol Lung Cell Mol Physiol 297: L738–L745, 2009. First published July 31, 2009; doi:10.1152/ajplung.00045.2009.—Neutrophil recruitment to the alveolar space. Neutrophils are recruited to the lung and contribute to increased epithelial permeability. The present study investigated in mice whether neutrophil recruitment to the lung leads to accumulation of plasma-derived host defense proteins in the alveolar space and whether respiratory burst contributes to this increase in permeability. Albumin, complement C1q, and IgM were increased in bronchoalveolar lavage (BAL) fluid 6 h after intratracheal LPS challenge. Neutrophil depletion before LPS treatment completely prevented this increase in BAL fluid protein concentration. Respiratory burst was not detected in neutrophils isolated from BAL fluid, and BAL proteins were increased in mice deficient in a key subunit of the respiratory burst apparatus, gp91phox, similar to wild-type mice. Neutrophil recruitment elicited by intratracheal instillation of the chemokines macrophage inflammatory protein-2 and keratinocyte-derived chemokine was also accompanied by accumulation of albumin, C1q, and IgM. During neutrophil recruitment to the alveolar space, epithelial permeability facilitates delivery of host defense proteins. The observed increase in epithelial permeability requires recruitment of neutrophils, but not activation of the respiratory burst, and occurs with chemokine-induced neutrophil migration independent of LPS exposure. complement; NADPH oxidase

MIGRATION OF CIRCULATING NEUTROPHILS to the alveolar space requires sequestration in the pulmonary vasculature in response to inflammatory challenge. Reorganization of cytoskeletal components markedly decreases deformability of neutrophils and arrests their transit through pulmonary capillaries (14). Participation of specific adhesion molecules (selectins and integrins) is dependent on the inflammatory stimulus (30), but receptor-ligand interactions prolong retention of neutrophils in the vasculature (21). Movement out of the capillary (13) into the interstitial space follows CXC chemokine gradients (36) and occurs preferentially via tricellular endothelial junctions (10). Migration across the interstitium to the epithelial surface may follow myofibroblasts that extend from the endothelium and epithelium (2), and emergence into the alveolar space can be observed at tricellular junctions between type I and type II epithelial cells (9). Movement across the epithelium through the paracellular route likely requires interaction with and disruption of tight junction proteins, including occludin and junctional adhesion molecules, which are linked by zona occludens to the epithelial actin cytoskeleton (9).

Numerous experimental models of pulmonary inflammation demonstrate that neutrophil recruitment to the lung is associated with increased permeability of the alveolocapillary barrier. Increased epithelial permeability to albumin is observed in animals and humans by 6 h after intrapulmonary LPS challenge (11, 31, 39). Early studies of inflammation during pneumonia attempted to determine a mechanism for formation of serous exudate in the alveolar space and proposed mechanical disruption of the alveolar epithelial barrier (28). Recent investigations have suggested that permeability defects during acute lung inflammation are due to neutrophil release of oxidants and proteases (1, 15, 16, 37, 41). NADPH oxidase generates high levels of superoxide anion during the respiratory burst in inflammatory cells, and reactive oxygen species may contribute to microbial killing and tissue injury (41). Although a dense antiprotease shield appears to protect normal tissues, oxidative stress can disable this redox-sensitive enzymatic defense (33), and neutrophil-derived proteases may cause local tissue injury (20). However, the contribution of neutrophil activation and migration to permeability events in the lung is not fully understood.

The epithelial permeability during lower respiratory tract infection leads to exudation of protein-rich fluid into the alveolar space (29) and may be a tightly regulated component of pulmonary host defense. The delivery of antimicrobial proteins, including fibrinogen, complement, and immunoglobulin, from intravascular and interstitial sites to the alveolar space may be critical to host defense at epithelial surfaces (3). In this study, we tested the hypothesis that neutrophil recruitment to the alveolar space increases local permeability to antimicrobial proteins. We measured alveolar content of two large plasma-derived host defense proteins, complement C1q and IgM, after intrapulmonary challenge with LPS. To determine the importance of alveolar neutrophil recruitment in the observed permeability events, we depleted neutrophils systematically before the intrapulmonary challenge. To determine whether respiratory burst contributes to lung epithelial permeability in this model, we measured superoxide anion production in neutrophils recruited to the alveolar space and the effect of deficiency of a functional respiratory burst apparatus on the observed permeability events. Finally, we determined whether chemokine-stimulated neutrophil recruitment can increase alveolar permeability independent of LPS exposure.

METHODS

Mice. gp91phox (Nox2)-deficient and wild-type male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME), maintained on a standard laboratory diet, and housed in a controlled environment with a 12:12-h light-dark cycle. Experiments were performed when mice were 8–12 wk old. Approval for these experiments was obtained from the Institutional Animal Care and Use Committee.

Neutrophil recruitment to the alveolar space. Mice were anesthetized by injection of pentobarbital sodium (80 mg/kg ip), and after direct exposure of the trachea, 25 μg of LPS from Escherichia coli (serotype 0111.B4, Sigma Aldrich, St. Louis, MO) in 50 μl of PBS or PBS alone were injected into the airway (39). Mice were held upright for 2 min after the injection. For intrapulmonary exposure to chemo-
kines, animals were injected intratracheally with 0.25 μg of macrophage inflammatory protein-2 (MIP-2) and 0.25 μg of keratinocyte-derived chemokine (KC; Invitrogen Biosource, Carlsbad, CA) in 50 μl of PBS.

**Distribution of intrapulmonary challenge.** Mice were injected intratracheally with 50 μl of trypan blue (0.4%; GIBCO BRL, Grand Island, NY) using the technique described for LPS and euthanized at 6 h for assessment of distribution of the intrapulmonary challenge.

**Neutrophil depletion.** Neutrophils were depleted by intraperitoneal injection of 20 μg of anti-Gr-1 (clone RB6-8C5, rat isotype IgG2b, BD Pharmingen, San Diego, CA) 24 h before intratracheal LPS challenge.

**Bronchoalveolar lavage.** Mice were anesthetized by injection of pentobarbital sodium (80 mg/kg ip), the trachea was exposed and cannulated, and the animals were euthanized by exsanguination through the abdominal aorta. The bronchoalveolar lavage (BAL) method was adapted from Ye et al. (42) and performed with 0.5 ml volume of PBS for a total of 3 ml. The first aliquot recovered was centrifuged at 300 g for 10 min, and the supernatant was stored at -80°C for measurement of albumin, immunoglobulin, and complement. Subsequent lavage aliquots were pooled and centrifuged at 300 g for 10 min, and pellets were resuspended for total white blood cell counts, cytocentrifugation, and flow cytometric analyses.

**Isolation of peripheral blood leukocytes.** Blood was collected by cardiac puncture, anticoagulated with EDTA, and mixed with Puregene RBC lysis solution (Gentra Systems, Minneapolis, MN). Cell samples were then counted or washed with PBS for assessment of the respiratory burst (see below).

**Total cell count and differential.** Total cell counts in BAL and blood were determined using a hemocytometer, and a cell monolayer was prepared by cytocentrifugation. Wright's Giemsa stain was used to differentiate macrophages, neutrophils, and lymphocytes on the cell monolayer.

**Respiratory burst.** To determine fluorescence attributable to NADPH oxidase activation during respiratory burst, circulating leucocytes and recruited alveolar neutrophils from gp91phox−/− deficient and wild-type mice were incubated with a peroxide-sensitive fluorophore, hydroethidine (HE; Sigma Aldrich) (40). Leukocytes from BAL (~80% neutrophils) or lysis of peripheral blood were suspended in 10 μmol/l of HE in RPMI (GIBCO BRL) for 15 min at 37°C with continuous shaking. PMA (10 μM final concentration; Sigma Aldrich) was added to individual cell suspensions for 30 min to stimulate the respiratory burst. Cells were fixed in 1% paraformaldehyde, washed, and stored in the dark at 4°C for flow cytometric analysis. HE fluorescence was analyzed on a FACSCaliber (Becton Dickinson) flow cytometer for the population of cells with forward and side scatter characteristic of neutrophils (488-nm excitation and 585-nm emission wavelengths).

**Wet-to-dry ratio.** The animal was anesthetized with pentobarbital sodium, and the left lung was removed, rinsed in PBS, blotted briefly, and placed on foil. Lung weight was measured before and after incubation at 60°C for 48 h.

**Albumin.** Albumin concentration in BAL fluid was determined using a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX).

**IgM.** IgM concentration in BAL fluid was determined using a mouse IgM ELISA kit (Immunology Consultants Lab, Newberg OR).

**C1q ELISA.** We established an indirect ELISA for measurement of C1q with the anti-human C1q antibody utilized for Western blot, inasmuch as we observed excellent cross-reactivity with murine C1q with little nonspecific staining. Briefly, we coated wells of a polystyrene microtiter plate with 50 μl of purified C1q standard (US Biological, Swampscott, MA; range 0–100 ng/ml) or 50 μl of sample for 2 h at room temperature. Wells were washed three times with PBS, and the remaining protein-binding sites on the polystyrene microtiter surface were blocked by addition of 200 μl of BSA for 2 h at room temperature. Wells were washed twice with PBS and incubated with 100 μl of primary antibody (goat anti-human C1q; US Biological) diluted 1:1,000 in conjugate diluent (Bethyl Laboratories) for 2 h at room temperature. The wells were washed four times with PBS and then incubated with 100 μl of horseradish peroxidase-conjugated secondary antibody (rabbit anti-goat IgG; US Biological) diluted 1:4,000 in conjugate diluent for 1 h at room temperature. Wells were washed four times, incubated with 3’,5’,5’-tetramethylbenzidine solution (Bethyl Laboratories) for 15–30 min, and then treated with 2 M H2SO4, and the optical density was determined at 450 nm (Multiskan Ascent, Thermo Scientific, Waltham, MA).

**Statistical analysis.** All numerical results are expressed as means ± SE. Statistical analyses for comparison of multiple groups were conducted using the one-way ANOVA or the Kruskal-Wallis test when the assumptions of ANOVA were not met. Differences were considered statistically significant at P < 0.05. Spearman rank-correlation coefficients were computed for BAL fluid neutrophil counts and protein concentrations. We compared the correlation coefficients using Fisher’s z-transformation method for dependent correlations (23).

**RESULTS**

**Neutrophil recruitment into the alveolar space.** Using an intratracheal injection of trypan blue, we characterized the distribution of the intrapulmonary challenge and observed a diffuse accumulation of dye in most lobes of the lung at 6 h (Fig. 1). Neutrophil recruitment to the lung was determined in BAL fluid recovered after intratracheal challenge with LPS or PBS (Fig. 2). Marked neutrophil recruitment was observed at 6 h after LPS (760 ± 151 × 103) compared with controls (2 ± 1 × 103), and total neutrophil counts in lavage fluid increased an additional fourfold by 18 h (2,900 ± 400 × 103).

For determination of the effect of neutrophil depletion on permeability events in the lung, mice were treated with anti-Gr-1, an antibody directed at the Ly-6G epitope highly expressed on neutrophils. Circulating neutrophils in anti-Gr-1-treated mice decreased by >90% at 6 h after LPS challenge compared with controls (data not shown). The increase in alveolar neutrophils observed 6 h after LPS challenge was abolished by anti-Gr-1 pretreatment. Macrophage recovery from animals treated with anti-Gr-1 antibody (111 ± 81 × 103) was similar to that from isotype antibody-treated controls (122 ± 34 × 103), consistent with high-level expression of Gr-1 on recruited neutrophils but not resident alveolar macrophages (27).

To determine the contribution of NADPH oxidase to lung epithelial permeability in our model, we used intratracheal...
challenge with LPS to recruit neutrophils to the alveolar space in gp91phox-deficient mice. Total neutrophil counts in BAL from gp91phox-deficient mice 6 h after treatment with LPS (1,294 ± 460 × 10³) did not differ statistically from those in LPS-treated wild-type mice.

To determine whether the permeability events measured in this model accompany neutrophil migration into the lung independent of LPS, we used the potent CXC chemokines MIP-2 (0.25 μg) and KC (0.25 μg) in combination to recruit neutrophils (43). Neutrophil counts in BAL fluid at 6 h were significantly higher in animals challenged intratracheally with MIP-2 + KC (1,802 ± 114 × 10³) than in wild-type animals challenged with LPS.

Respiratory burst in circulating and alveolar neutrophils.

To estimate the activation of respiratory burst in neutrophils recruited to the alveolar space, we measured HE fluorescence in circulating and alveolar neutrophils in wild-type animals after intratracheal LPS challenge (Fig. 3). Mean channel fluorescence (arbitrary units) did not differ between circulating and alveolar neutrophils (275 ± 35 and 323 ± 53, respectively). To confirm the signal for respiratory burst in this system, we treated neutrophils in vitro with PMA, a potent activator of protein kinase C and the NADPH oxidase respiratory burst apparatus. Marked increases in mean fluorescence were observed for circulating and alveolar neutrophils (613 ± 128 and 738 ± 112, respectively).

HE may fluoresce after reaction with reactive species other than superoxide derived from NADPH oxidase. To evaluate the contribution of sources other than NADPH oxidase to the fluorescence signal and to confirm the deficiency of gp91phox in the knockout mice, we measured unstimulated and PMA-stimulated HE fluorescence in alveolar and circulating neutrophils isolated from gp91phox-deficient mice (Fig. 3). Mean fluorescence of unstimulated alveolar neutrophils did not differ between wild-type and gp91phox-deficient mice. Similarly, HE fluorescence in unstimulated circulating neutrophils did not differ between wild-type and gp91phox-deficient animals. Stimulation of circulating or alveolar neutrophils with PMA was not accompanied by increased HE fluorescence in gp91phox-deficient animals. Our findings are consistent with the absence of a high level of respiratory burst in neutrophils recruited to the alveolar space in wild-type animals. In addition, when HE is used as a superoxide detection system, nearly all fluorescent signal measured in unstimulated circulating and alveolar neutrophils from wild-type animals appears to be derived from sources other than gp91phox-dependent NADPH oxidase.

Lung permeability in wild-type, neutrophil-depleted, and respiratory burst-deficient mice.

We characterized lung permeability after neutrophil recruitment in two ways. First, we determined the wet-to-dry ratio for whole lung, an indirect measurement of lung permeability that reflects changes in blood volume, interstitial edema, and alveolar edema (Table 1). In wild-type animals, LPS treatment was followed by an increased wet-to-dry ratio at 6 h (5.0 ± 0.1 vs. 4.3 ± 0.1, P < 0.05) that returned to control levels by 18 h (data not shown). Neutrophil depletion by pretreatment with anti-Gr-1 significantly, but incompletely, opposed the LPS-induced increase in wet-to-dry ratio. A modest increase in wet-to-dry ratio after LPS treatment was observed in respiratory burst-deficient animals compared with PBS-treated controls. An increase in wet-to-dry ratio was also observed in wild-type animals challenged intratracheally with MIP-2 + KC compared with PBS-challenged controls.

Our second method more directly assessed epithelial integrity by measuring concentrations of albumin (68 kDa), IgM (900 kDa), and C1q (440 kDa) in BAL fluid. The albumin concentration in BAL fluid was increased 6 h after LPS challenge compared with controls (Fig. 4). Neutrophil depletion with anti-Gr-1 prevented the increase in albumin concentration, whereas treatment with isotype antibody had no effect. An increase in albumin concentration in BAL after LPS challenge similar to that in wild-type animals was observed in respiratory burst-deficient animals. The mean BAL fluid albumin concentration was modestly higher in PBS-treated gp91phox-deficient than in PBS-treated wild-type mice (243 ± 52 vs. 135 ± 41 μg/ml). Albumin concentrations in BAL fluid
were higher in mice treated with MIP-2 + KC than in LPS-treated wild-type mice.

The IgM concentration in BAL fluid was increased at 6 h after LPS challenge compared with PBS controls (Fig. 5). Pretreatment with anti-Gr-1 decreased the BAL fluid IgM concentration to control levels, as observed for albumin, and treatment with isotype antibody had no effect. The increased IgM concentration in BAL fluid from wild-type-animals treated with LPS was also observed in respiratory burst-deficient animals. Mean IgM concentration in BAL was significantly higher in mice treated with MIP-2 + KC than in LPS-treated animals.

We assessed murine C1q concentration semiquantitatively in BAL fluid by Western blot using an anti-human C1q antibody and observed a single 70- to 80-kDa band, consistent with migration of the protein under nonreducing conditions (Fig. 6). C1q protein was increased in BAL samples from LPS-challenged mice compared with PBS controls by densitometry (net intensity = 3.9 ± 0.1 vs. 2.8 ± 0.1 arbitrary units). We developed an indirect ELISA to quantify C1q in BAL samples and found that intratracheal LPS challenge was followed by an increase in BAL fluid C1q concentration by 6 h. Pretreatment with neutrophil-depleting anti-Gr-1 antibody, but not isotype control antibody, completely prevented this increase in BAL fluid C1q concentration. An increase in BAL fluid C1q concentration in respiratory burst-deficient mice was similar to that in wild-type animals after LPS treatment. Concentrations of C1q in BAL fluid were similar in mice challenged with MIP-2 + KC and wild-type animals treated with LPS.

Total protein, albumin, IgM, and C1q accumulation in the alveolar space were measured at 6 and 18 h after challenge (Fig. 7). The IgM concentration in BAL fluid was increased 7.0-fold at 6 h and 15.2-fold at 18 h compared with PBS-treated controls. In contrast, the total protein, albumin, and C1q concentration in BAL fluid increased 4.1-, 3.6-, and 2.5-fold, respectively, at 6 h and remained unchanged at 18 h. We analyzed the relationship between protein concentration and neutrophil count in the alveolar space and observed that IgM exhibited the strongest correlation with total neutrophil count (r = 0.83 vs. 0.68 for albumin, P = 0.01; Fig. 8). To determine when IgM enters the alveolar space in relation to neutrophil migration, we measured total neutrophil counts and IgM concentration.

**Table 1. Lung wet-to-dry ratio after intrapulmonary challenge**

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>LPS</th>
<th>LPS + anti-Gr-1</th>
<th>MIP-2 + KC</th>
<th>gp91phox(-/-)</th>
<th>PBS</th>
<th>LPS</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>4.3 ± 0.1</td>
<td>5.0 ± 0.1*</td>
<td>4.6 ± 0.1†</td>
<td>4.7 ± 0.1†</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>5.0 ± 0.1*</td>
</tr>
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Values are means ± SE. Lung wet-to-dry ratios were determined 6 h after intrapulmonary challenge in wild-type, anti-Gr-1-pretreated, and gp91phox-deficient mice (n = 6–7). MIP-2, macrophage inflammatory protein-2; KC, keratinocyte-derived chemokine. *P < 0.05 vs. wild-type or gp91phox-deficient control. †P < 0.05 vs. PBS-treated wild-type mice.

Fig. 3. Hydroethidine (HE) fluorescence in alveolar and circulating neutrophils isolated from wild-type and gp91phox-deficient mice. A: representative flow cytometric study of stimulated and unstimulated alveolar and circulating neutrophils from wild-type and gp91phox-deficient mice. B: fluorescence of neutrophils from 4 wild-type and 4 gp91phox-deficient mice. *P < 0.05 vs. unstimulated neutrophils.

Fig. 4. Albumin concentration in BAL fluid 6 h after LPS in wild-type, anti-Gr-1-treated wild-type, and gp91phox-deficient mice and 6 h after MIP-2 + KC in wild-type mice (n = 5–10). *P < 0.05 vs. PBS in wild-type mice. #P < 0.05 vs. LPS in wild-type mice.
concentration in BAL fluid at time 0 and 2 h after intratracheal administration of MIP-2 + KC. Total neutrophil counts increased from 1 ± 1 to 160 ± 60 (× 10⁶) at 2 h, and IgM concentration increased from 155 to 726 ng/ml (5-fold increase, n = 5).

**DISCUSSION**

Neutrophil depletion largely prevented the increase in lung water and alveolar protein content observed early after intrapulmonary LPS in our experiments. In humans exposed to LPS via bronchial instillation, neutrophil counts and albumin concentration are increased in the BAL by 6 h. In a study of intrapulmonary LPS challenge in mice, increased BAL protein concentration measured at 3 h was opposed by neutrophil depletion, whereas BAL protein concentration at 24 h was relatively unaffected (11). Our data in a mouse model are consistent with the concept that epithelial permeability to protein is increased within hours of intrapulmonary LPS challenge and that this early increase in permeability requires neutrophil participation. However, influx of other leukocytes may also contribute to lung permeability during inflammation. Monocyte recruitment to the lung increases permeability indirectly by amplifying neutrophil recruitment and can mediate a modest increase in lung permeability in the absence of neutrophils (27). An independent effect of monocyte migration on permeability has not been excluded in our model.

We used a murine model of NADPH oxidase deficiency to investigate the contribution of the respiratory burst to permeability in the lung. The systemic deletion of gp91phox yields phagocytic cells incapable of increased superoxide anion production via the respiratory burst apparatus (34). gp91phox is also a component of vascular NADPH oxidase, and effects of deletion in this animal model may be in part attributable to the absence of reactive oxygen species signaling in the vasculature (7, 22). On arrival to the air spaces, activation of neutrophils depends on the nature of the recruitment stimuli. In humans, neutrophils in lung parenchyma during bacterial pneumonia are more metabolically active than neutrophils in bronchiectatic airways (19). In human neutrophils recruited to the lung within 4 h of a chemotactic stimulus (leukotriene B₄), no superoxide anion production was detectable (26); in neutrophils isolated 16 h after intrapulmonary LPS, superoxide anion release was increased (12). Free radical formation was detected in lungs from wild-type but not p47phox-deficient mice after intrapulmonary LPS challenge, providing evidence that NADPH oxidase can be a source of reactive oxygen species in this model (37). However, the contribution of the respiratory burst and superoxide release to lung permeability during neutrophil recruitment to the alveolar space is not clear.

Our study characterized the requirement for NADPH oxidase in lung permeability events after intrapulmonary LPS challenge and found that permeability accompanies early neutrophil recruitment independent of respiratory burst-derived oxidants. This observation differs from findings during systemic bacterial challenge, in which NADPH oxidase appears to contribute to increased endothelial permeability (15). Intravascular leukocyte accumulation after systemic inflammatory challenge is accompanied by comparatively modest neutrophil migration into the alveolar space (15, 32), and the permeability events observed after systemic challenge are likely different from those observed in our model.
from those that accompany the rapid migration of neutrophils into the interstitial space and across the epithelial surface after intrapulmonary challenge (32, 35). Although neutrophils may contribute to increased lung permeability directly by disrupting epithelial cell junctions, we have not excluded a role for more proximal events, such as neutrophil-endothelial interactions, in this process. In addition, Chignard and Balloy (11) observed increased alveolocapillary permeability in the absence of neutrophil recruitment to the lung late after intrapulmonary LPS challenge and concluded that neutrophil-dependent and -independent mechanisms participate.

NADPH oxidase may play a role in neutrophil recruitment. In a model of acid aspiration-induced lung inflammation, neutrophil and albumin in the alveolar space were increased by 6 h in NADPH oxidase-deficient mice compared with wild-type animals, suggesting a role for NADPH oxidase in attenuating the inflammatory response (38). In NADPH oxidase-deficient mice challenged with *Pneumococcus*, host defense was not compromised and increased neutrophil counts were present in BAL samples at 24 and 48 h after infection (25). The reported effect of NADPH oxidase deficiency on neutrophil accumulation suggests that phagocytic and/or vascular superoxide production may inhibit downstream recruitment signals. We found a modest trend toward increased BAL neutrophil recruitment after intrapulmonary LPS challenge in gp91phox−/−, deficient mice compared with wild-type animals. This difference from previous observations may reflect more limited participation of NADPH-derived oxidants in inflammation and leukocyte recruitment in this model.

MIP-2 and KC are murine chemokines produced under inflammatory conditions by myeloid cells in the lung in response to activation of pattern recognition receptors. These chemokines can participate in directional gradients for chemotaxis (4) and can activate neutrophils directly (23) but are not known to regulate lung epithelial cell function. Previous studies have yielded conflicting results about neutrophil recruitment and lung epithelial permeability stimulated by endogenous inflammatory mediators. A study of lung permeability in normal humans 4 h after instillation of leukotriene B4, a chemotactic and activating factor for neutrophils, found that neutrophil recruitment to the alveolar space was not accompanied by a marked increase in protein permeability (26). In contrast, lung epithelial permeability to albumin was increased in rats after intratracheal instillation of interleukin-1, and the permeability changes were neutrophil dependent (24). The increase in albumin, IgM, and C1q concentrations in BAL fluid after MIP-2 + KC challenge in our model is consistent with an increase in lung epithelial permeability with neutrophil recruitment, independent of LPS signaling.

We observed that 68- to 900-kDa proteins accumulated in the alveolar space during neutrophil recruitment. IgM concentration increased approximately sevenfold early after intrapulmonary challenge, whereas albumin and C1q increased by two- to fourfold. The alveolar protein accumulation we report differs qualitatively from that described in hydrostatic pulmonary edema, in which higher-molecular-weight proteins do not increase in the alveolar space. The permeability in our model also differs quantitatively from the leak observed in acute respiratory distress syndrome patients, who have massive, sustained injury to epithelial surfaces and 10- to 90-fold increases in measured proteins (17). However, our finding of a greater increase in high-molecular-weight proteins than in lower-molecular-weight proteins is similar to that reported for acute respiratory distress patients. This may be due to a greater partitioning of high-molecular-weight proteins in serum than in alveolar lining fluid in normal lung, leading to a more marked increase in these proteins when permeability is nonselective. In normal mouse serum the albumin concentration is ~100-fold higher than IgM concentration, whereas in our BAL fluid measurements the albumin concentration was ~1,000-fold higher than the IgM concentration in control animals. In our model, a relatively nonselective increase in permeability would be predicted to have a greater effect on IgM than on albumin content in the alveolar space.

It is interesting that, among the proteins measured, IgM concentration correlated most directly with total neutrophil count in BAL fluid. Albumin and C1q concentration in BAL fluid did not increase between 6 and 18 h, even as neutrophil counts continued to rise. These observations raise the possibility that IgM is preferentially transported during neutrophil
migration. In addition to facilitating a relatively nonselective protein movement through epithelial pores, neutrophils could increase immunoglobulin transport directly via a transient physical association (e.g., Fc receptors). Additional explanations for the differences in protein accumulation over time may include more efficient clearance of albumin from the lung on the basis of its small molecular weight and binding or consumption of C1q via complement activation during epithelial passage or in the alveolar space. The plateau in total protein accumulation as neutrophil migration continues may also be explained by competition of neutrophils and proteins for flux through limited transport sites (e.g., paracellular junctions).

Lung permeability with accumulation of antimicrobial proteins in air spaces may serve a fundamental host defense function. Complement proteins, in particular, play a critical role in amplifying host responses, appear in the lung within hours of LPS challenge (6), and play an important early role defending the host against pneumococcal pneumonia (8). Native IgM plays an important role in controlling pulmonary infection with influenza virus (18) and *Pneumococcus* (5, 8) and is an efficient initiator of the complement cascade via C1q activation (44). Our research adds to existing knowledge by demonstrating that plasma-derived host defense proteins accompany neutrophil migration into the lung and that this protein accumulation does not require activation of the respiratory burst. These findings suggest that increased lung permeability during neutrophil migration contributes to host defense and that the increased permeability is not mediated by neutrophil-derived oxidants.

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