Neutrophil-Mediated Lung Permeability and Host Defense Proteins

Stephen P. Kantrow, Zhiwei Shen, Tonya Jagneaux, Ping Zhang and Steve Nelson

Section of Pulmonary and Critical Care Medicine, Department of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA

Address correspondence to:
Stephen P. Kantrow, M.D.
1901 Perdido Street
Suite 3205
New Orleans, LA 70112
Email: skantr@lsuhsc.edu

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ABSTRACT

Neutrophil recruitment to the alveolar space is associated with 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 immunoglobulin M (IgM) were all increased in bronchoalveolar lavage fluid six hours after intratracheal LPS challenge. Neutrophil depletion prior to LPS treatment completely prevented this increase in BAL fluid protein concentration. Respiratory burst was not detected in neutrophils isolated from the BAL fluid, and mice deficient in a key subunit of the respiratory burst apparatus, gp91phox, had increases in BAL proteins similar to wild type mice. Neutrophil recruitment elicited by intratracheal instillation of chemokines MIP-2 and KC 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.
INTRODUCTION

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, integrins) is dependent upon 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 epithelium through the paracellular route likely requires interaction with and disruption of tight junction proteins, including occludin and junctional adhesion molecules that are linked by zonula 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 alveolo-capillary barrier. Increased epithelial permeability to albumin is observed in animals and humans by 6 hours after intrapulmonary lipopolysaccharide (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 both to microbial killing and tissue injury (41). While a dense anti-protease 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.

Epithelial permeability that occurs 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 from intravascular and interstitial sites to the alveolar space, including fibrinogen, complement and immunoglobulin, 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 immunoglobulin M (IgM), after intrapulmonary challenge with LPS. To determine the importance of alveolar neutrophil recruitment on the observed permeability events, we depleted neutrophils systemically prior to 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 measured 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-hour light/dark cycle. Experiments were performed when mice were 8-12 weeks 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 intraperitoneal injection of pentobarbital (80 mg/kg), and after direct exposure of the trachea, 25 ug LPS from E. coli (serotype 0111.B4; Sigma Aldrich, St. Louis, MO) in 50 uL phosphate buffered saline (PBS) or PBS alone were injected into the airway(39). Mice were held upright for 2 minutes after the injection. For intrapulmonary exposure to chemokines, animals were injected intratracheally with 0.25 ug MIP-2 and 0.25 ug KC (Invitrogen Biosource, Carlsbad, CA) in 50 uL PBS.

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

Neutrophil depletion. Neutrophils were depleted by intraperitoneal injection of 20 ug anti-Gr-1 (clone RB6-8C5, rat isotype IgG2b) (BD Pharmingen, San Diego, CA) 24 hours before intratracheal LPS challenge.
**Bronchoalveolar lavage.** Mice were anesthetized by intraperitoneal injection of pentobarbital (80 mg/kg), the trachea was exposed and cannulated, and the animals were euthanized by exsanguination through the abdominal aorta. Bronchoalveolar lavage method was adapted from Ye et al (42) and performed with 0.5 ml volumes of PBS for a total of 3 mL. The first aliquot recovered was centrifuged at 300g for 10 min and the supernatant stored at – 80 °C for measures of albumin, immunoglobulin and complement. Subsequent lavage aliquots were pooled, centrifuged at 300g 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 as described 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. The Wright-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 leukocytes and recruited alveolar neutrophils from gp91phox−/− and wild type mice were incubated with a superoxide sensitive fluorophore, hydroethidine (HE, Sigma Aldrich)(40). Leukocytes from BAL (~80% neutrophils) or lysis of peripheral blood were suspended in 10 umol/l of HE in RPMI (Gibco BRL, Grand Island, NY) for 15 minutes at 37 °C
with continuous shaking. Phorbol 12-myristate 13-acetate (PMA) (10 uM 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 FACSCalibur (Becton Dickenson) flow cytometer for the population of cells with forward and side scatter characteristic of neutrophils using 488 nm excitation and 585 nm emission wavelengths.

**Wet-to-dry ratio.** After administration of anesthesia with pentobarbital, the left lung was removed, rinsed in PBS, blotted briefly and placed on foil. Weights were measured before and after incubation at 60 °C for 48 hours.

**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).

**Complement C1q Western blot.** Equal volumes (12.5 ul) of mouse BAL were separated by electrophoresis on a 10% SDS-PAGE gel under non-reducing conditions. Samples were then transferred for 1 hour from the gel to nitrocellulose membrane (Biorad, Hercules, CA) and blots were blocked with TBS-0.1% Tween 20 (Sigma Aldrich) (TBS-Tween) containing 5% dry milk for 1 hour at room temperature. Blots were then washed three times with TBS-Tween and then incubated with the primary antibody, goat anti-human C1q (concentration 1:2000, Complement
Technology, Tyler, TX), in TBS-Tween containing 5% dry milk at 4°C overnight. Blots were washed three times with TBS-Tween, and then incubated with peroxidase conjugated secondary antibody, rabbit anti-goat IgG (concentration 1:10,000, Calbiochem, San Diego, CA), in TBS-Tween containing 5% dry milk at room temperature for 1 hour. Blots were washed as above, and then incubated in Opti-4CN substrate (Biorad) for up to 30 minutes. Semiquantitative analysis of bands was performed using a Kodak Gel Logic 2200 Imaging System (Carestream Molecular Imaging, New Haven, CT). Data are presented as net intensity.

**C1q ELISA.** We established an indirect ELISA for measurement of C1q ELISA with the anti-human C1q antibody utilized for Western blot, as we observed excellent cross reactivity with murine C1q with little nonspecific staining. Briefly, we coated wells of a PVC microtiter plate with 50 μl of purified C1q standard (US Biological, Swampscott, MA) (range 0 to 100 ng/ml) or 50 μl of sample for 2 hours at room temperature. Wells were washed three times with PBS, and the remaining protein binding sites on the PVC surface were blocked by adding 200 μl of BSA for two hours at room temperature. Wells were washed twice with PBS and incubated with 100 μl of primary antibody (goat anti-human C1q, US Biological) diluted 1:1000 in conjugate diluent (Bethyl Laboratories) for 2 hours at room temperature. After washing four times with PBS, wells were incubated with 100 μl of horseradish peroxidase conjugated secondary antibody (rabbit anti-goat IgG, US Biological) diluted 1:4000 in conjugate diluent for 1 hour at room temperature. Wells were washed four times, incubated with 3,3′,5,5′-tetramethylbenzidine solution (Bethyl Laboratories) for 15-30 min, 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 mean ± SEM. The sample size is indicated in the legend of each figure. 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 a Fisher z-transformation method for dependent correlations (23).
RESULTS

*Neutrophil recruitment into the alveolar space.*

We characterized the distribution of the intrapulmonary challenge using an intratracheal injection of trypan blue and observed a diffuse accumulation of dye in most lobes of the lung at 6 hours (Figure 1). Neutrophil recruitment to the lung was determined in bronchoalveolar lavage fluid recovered after intratracheal challenge with LPS or PBS (Figure 2). We observed marked neutrophil recruitment at 6 hours after LPS (760 ± 151 x 10³) compared with controls (2 ± 1 x 10³) and total neutrophil counts in lavage fluid increased an additional 4 fold by 18 hours (2,900 ± 400 x 10³).

To determined 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 hours after LPS challenge compared with controls (data not shown). The increase in alveolar neutrophils observed 6 hours after LPS challenge was abolished by anti-Gr-1 pretreatment. Macrophage recovery from animals treated with anti-Gr-1 antibody (111 ± 81 x 10³) was similar to isotype antibody treated controls (122 ± 34 x 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 recruited neutrophils to the alveolar space in gp91phox⁻/⁻ mice using intratracheal challenge with LPS. Total neutrophil counts in BAL from gp91phox⁻/⁻ mice 6 hours after treatment with LPS (1,294 ± 460 x 10³) did not differ statistically from those observed 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 recruited neutrophils using the potent CXC chemokines MIP-2 (0.25 ug) and KC (0.25 ug) in combination (43). Neutrophil counts in BAL fluid at 6 hours were significantly higher in animals challenged intratracheally with the combination of MIP-2 and KC \((1,802\pm114 \times 10^3)\) 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 treated after intratracheal LPS challenge (Figure 3). Mean channel fluorescence (arbitrary units) did not differ between circulating and alveolar neutrophils \((275\pm35 vs 323\pm53, \text{ respectively})\). 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 both circulating and alveolar neutrophils \((613\pm128 \text{ and } 738\pm112, \text{ respectively})\).

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 observed fluorescence signal, and to confirm the deficiency of gp91<sup>phox</sup> in the knockout mice, we measured unstimulated and PMA stimulated HE fluorescence in alveolar and circulating neutrophils isolated from gp91<sup>phox</sup><sup>-/-</sup> mice (Figure 3). No difference in mean fluorescence was
seen between unstimulated alveolar neutrophils from wild type compared to gp91\textsuperscript{phox-/-} mice. Similarly, HE fluorescence in unstimulated circulating neutrophils did not differ between wild type and gp91\textsuperscript{phox} deficient animals. Stimulation of circulating or alveolar neutrophils with PMA was not accompanied by increased HE fluorescence in gp91\textsuperscript{phox} deficient animals. Our findings are consistent with absence of a high level of respiratory burst in neutrophils recruited to the alveolar space in wild type animals. In addition, nearly all fluorescent signal measured in unstimulated circulating and alveolar neutrophils from wild type animals using HE as a superoxide detection system appears to be derived from sources other than gp91\textsuperscript{phox} dependent NADPH oxidase.

\textit{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 hours (5.0 ± 0.1 vs 4.3 ± 0.1, p< 0.05 ) that returned to control levels by 18 hours (data not shown). Neutrophil depletion by pretreatment with anti-Gr-1 significantly but incompletely opposed the increase in wet-to-dry ratio observed with LPS. Respiratory burst deficient animals had a modest increase in wet-to-dry ratio after LPS treatment compared with PBS treated controls. Wild type animals challenged intratracheally with MIP-2 and KC also had an increase in wet-to-dry ratio compared with PBS challenged controls.
Our second method more directly assessed epithelial integrity by measuring concentrations of albumin (68 kD), IgM (900 kD) and C1q (440 kD) in BAL fluid. The albumin concentration in BAL fluid was increased 6 hours after LPS challenge compared with controls (Figure 4). Neutrophil depletion with anti-Gr-1 prevented the observed increase in albumin concentration, while treatment with isotype antibody had no effect. Respiratory burst deficient animals had an increase in albumin concentration in BAL after LPS challenge similar to that observed in wild type animals. The mean BAL fluid albumin concentration was modestly higher in PBS treated gp91phox−/− mice compared with PBS treated wild type mice (243±52 vs 135±41 ug/ml). MIP-2 + KC treated mice had higher concentrations of albumin in BAL fluid than LPS treated wild type mice.

The IgM concentration in BAL fluid was increased at 6 hours after LPS challenge compared with PBS controls (Figure 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 observed in BAL fluid from wild type animals treated with LPS was also observed in respiratory burst deficient animals. Mice treated with MIP-2 + KC had a significantly higher mean IgM concentration in BAL than LPS treated animals.

We assessed murine C1q concentration semi-quantitatively in BAL fluid by Western blot using an anti-human C1q antibody, and observed a single band with a molecular weight 70-80 kD, consistent with migration of the protein under non-reducing conditions (Figure 6). C1q protein was increased in BAL samples from LPS challenged mice compared with PBS controls by
densitometry (net intensity 3.9±0.1 after LPS vs 2.8±0.1 in controls, 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 C1q concentration by 6 hours. Pretreatment with neutrophil depleting anti-Gr-1 antibody, but not isotype control antibody, completely prevented this increase in BAL fluid C1q concentration. Respiratory burst deficient mice had an increase in BAL fluid C1q concentration similar to that observed in wild type animals after LPS treatment. Mice challenged with MIP-2 + KC had similar concentrations of C1q in BAL fluid compared to wild type animals treated with LPS.

Total protein, albumin, IgM and C1q accumulation in the alveolar space were measured at 6 and 18 hours after challenge (Figure 7). The IgM concentration in BAL fluid was increased 7.0 fold at 6 hours and 15.2 fold at 18 hours 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 hours and remained unchanged at 18 hours. 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) (Figure 8). To determine when IgM enters the alveolar space in relation to neutrophil migration, we measured total neutrophil counts and IgM concentration in BAL fluid at zero and two hours after intratracheal administration of MIP-2 KC. Total neutrophil counts increased from 1±1 to 160±60 (x 10³) at two hours 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 bronchoalveolar lavage by six hours. A study of intrapulmonary LPS challenge in mice found that increased BAL protein concentration measured at 3 hours was opposed by neutrophil depletion, while BAL protein concentration at 24 hours 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 vasculature (7, 22). Upon arrival to the airspaces, activation of neutrophils depends upon 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).
Human neutrophils recruited to the lung within 4 hours of a chemotactic stimulus (LTB4) have no detectable superoxide anion production (26), while neutrophils isolated 16 hours after intrapulmonary LPS have increased superoxide anion release (12). Free radical formation is 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 that accompany the rapid migration of neutrophils into the interstitial space and across the epithelial surface after intrapulmonary challenge (32, 35). While 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 both 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 six hours 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 hours 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 that gp91phox-/- mice had a modest trend toward increased BAL neutrophil recruitment after intrapulmonary LPS challenge compared with wild type animals. This difference from previous observations may reflect more limited participation of NADPH derived oxidants on 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 four hours 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 and KC challenge in our model is consistent with an increase in lung epithelial permeability with neutrophil recruitment, independent of LPS signaling.

We observed that proteins ranging in size from 68 kD to 900 kD accumulated in the alveolar space during neutrophil recruitment. IgM concentration increased approximately 7 fold early after intrapulmonary challenge, while albumin and C1q increased by 2 to 4 fold. 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 ARDS patients, who have massive, sustained injury to epithelial surfaces and 10 to 90 fold increases in measured proteins (17). However, our finding of a relatively greater increase in high molecular weight proteins compared with smaller proteins is similar to that reported for ARDS patients. This may be due to a greater partitioning of high molecular weight proteins in serum compared with alveolar lining fluid in normal lung, leading to a more marked increase in these proteins when permeability is non-selective. In normal mouse serum the albumin concentration is ~100 fold higher than IgM, while in our BAL fluid measurements the albumin concentration was ~1000 fold higher than the IgM concentration in control animals. In our model, a relatively non-selective increase in permeability would be predicted to have a greater effect on IgM than on albumin content in the alveolar space.

Interestingly, IgM concentration correlated most directly with total neutrophil count in BAL fluid among the proteins measured. Albumin and C1q concentration in BAL fluid did not
increase between 6 and 18 hours, 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 non-selective 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 based upon 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 observed 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 airspaces 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.
Figure 1. **Distribution of intratracheal challenge at six hours.** Representative image of lungs from three mice 6 hours after intratracheal injection of 50 uL of trypan blue (0.04%).

Figure 2. **Neutrophil recruitment to the alveolar space after intrapulmonary challenge.**

Total BAL neutrophil counts 6 hours after LPS in wild type, anti-Gr-1 treated wild type and gp91phox⁻/⁻ mice, and 6 hours after MIP-2 / KC in wild type mice (n=5-10). (*p < 0.05 compared with PBS in wild type mice, #p < 0.05 compared with LPS in wild type mice)

Figure 3. **Hydroethidine (HE) fluorescence in alveolar and circulating neutrophils isolated from wild type mice and gp91phox⁻/⁻ mice.** Representative flow cytometric study of stimulated and unstimulated alveolar and circulating neutrophils from wild type and gp91phox⁻/⁻ mice (A). Mean value for fluorescence of neutrophils from four wild type and four gp91phox⁻/⁻ mice (B). (*p < 0.05 compared with unstimulated neutrophils)

Figure 4. **Albumin concentration in BAL fluid after intrapulmonary challenge.** Albumin concentration in BAL fluid 6 hours after LPS in wild type, anti-Gr-1 treated wild type and gp91phox⁻/⁻ mice, and 6 hours after MIP-2 / KC in wild type mice (n=5-10). (*p < 0.05 compared with PBS in wild type mice, #p < 0.05 compared with LPS in wild type mice)

Figure 5. **IgM concentration in BAL fluid after intrapulmonary challenge.** IgM concentration in BAL fluid 6 hours after LPS in wild type, anti-Gr-1 treated wild type and
gp91phox/- mice, and 6 hours after MIP-2 / KC in wild type mice (n=5-10). (*p < 0.05 compared with PBS in wild type mice, #p < 0.05 compared with LPS in wild type mice)

Figure 6. **Complement C1q in BAL fluid after intrapulmonary challenge.** Western blot of C1q protein was performed on equivalent volumes (25 ul) from BAL fluid obtained from mice 6 hours after challenge with PBS or LPS (A). C1q concentration in BAL fluid 6 hours after LPS in wild type, anti-Gr-1 treated wild type and gp91phox/- mice, and 6 hours after MIP-2 / KC in wild type mice (n=5-7) (B). (*p < 0.05 compared with PBS in wild type mice, #p < 0.05 compared with LPS in wild type mice)

Figure 7. **Protein concentration in BAL fluid at 6 and 18 hours after intrapulmonary challenge.** Total protein, albumin, IgM and C1q concentration in BAL fluid expressed as fold increase compared with PBS treated controls at 6 and 18 hours (n=5-10 for each group per time point).

Figure 8. **Correlation between protein concentration and total neutrophil count in BAL fluid after intrapulmonary challenge.** Albumin, IgM and C1q concentration are plotted against total neutrophil count for BAL fluid samples obtained 6 and 18 hours after intrapulmonary LPS challenge.
Table 1. **Lung wet-to-dry ratio after intrapulmonary challenge.** Lung wet-to-dry ratios were determined 6 hours after intrapulmonary challenge in wild type, anti-Gr-1 pre-treated and gp91phox⁻/⁻ mice (n=6-7). (*p < 0.05 compared with wild type or gp91phox⁻/⁻ control, #p < 0.05 compared with LPS treated wild type mice)


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Figure 2

![Bar graph showing neutrophil counts](image-url)
Figure 3

A

unstimulated

wild type

gp91phox(-/-)

alveolar      circulating

PMA

B

mean channel fluorescence

wild type

gp91phox(-/-)

Alveolar      Alveolar + PMA      Circulating      Circulating + PMA

*
Figure 4

The figure shows a bar graph representing Albumin (µg/mL) levels for different groups:
- PBS
- LPS
- antiGr1 + LPS
- gp91phox(-/-) + LPS
- MIP-2 / KC

Significance levels are indicated:
- * signifies a significant difference compared to the control (PBS)
- # signifies a significant difference compared to the LPS group

The x-axis represents the different experimental groups, and the y-axis indicates the Albumin levels in µg/mL.
Figure 5
Figure 6

A

B

Figure 6

A

PBS
LPS

131 kD
85 kD
41 kD

B

C1q (ng/mL)

PBS  LPS  antiGr1 + LPS  gp91phox(-/-) + LPS  MIP-2 / KC

*  #  *

*
Figure 7
Figure 8

![Graphs showing protein concentration vs. total neutrophil count at 6 hours and 18 hours.](image)
Table 1

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