Neutrophil α -Defensins Cause Lung Injury by Disrupting the Capillary–Epithelial Barrier

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Rationale: The involvement of neutrophil activation in the sentinel, potentially reversible, events in the pathogenesis of acute lung injury (ALI) is only partially understood. α -Defensins are the most abundant proteins secreted by activated human neutrophils, but their contribution to ALI in mouse models is hindered by their absence from murine neutrophils and the inability to study their effects in isolation in other species.

Objectives: To study the role of α -defensins in the pathogenesis of ALI in a clinically relevant setting using mice transgenic for polymorphonuclear leukocyte expression of α -defensins.

Methods: Transgenic mice expressing polymorphonuclear leukocyte α -defensins were generated. ALI was induced by acid aspiration. Pulmonary vascular permeability was studied *in vivo* using labeled dextran and fibrin deposition. The role of the low-density lipoprotein-related receptor (LRP) in permeability was examined.

Measurements and Main Results: Acid aspiration induced neutrophil migration and release of α -defensins into lung parenchyma and airways. ALI was more severe in α -defensin-expressing mice than in wild-type mice, as determined by inspection, influx of neutrophils into the interstitial space and airways, histological evidence of epithelial injury, interstitial edema, extravascular fibrin deposition, impaired oxygenation, and reduced survival. Within 4 hours of insult, α -defensin-expressing mice showed greater disruption of capillary–epithelial barrier function and ALI that was attenuated by systemic or intratracheal administration of specific inhibitors of the LRP.

Conclusions: α -Defensins mediate ALI through LRP-mediated loss of capillary–epithelial barrier function, suggesting a potential new approach to intervention.

Keywords: acute lung injury; capillary–epithelial barrier; α -defensins; low-density lipoprotein–related receptor; receptor-associated protein

Acute lung injury (ALI) is a complex syndrome that complicates diverse systemic disorders and intrapulmonary injuries, including aspiration and pneumonia (1–4). ALI remains a common cause of morbidity and mortality (1, 5). Early in the process, inflammation and disruption of capillary–alveolar barrier function leads to rapid ingress of plasma proteins and hematopoietic cells (1, 6–8). However, therapy is limited by an incomplete understanding of the sentinel events involved in this loss of barrier function, including both the relevant soluble

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The mechanism by which neutrophil activation contributes to the sentinel, potentially reversible events that initiate acute lung injury (ALI) are only partially understood. α -Defensins are the most abundant proteins secreted by activated human PMNs, but understanding of their contribution to ALI is hindered by their absence from murine PMNs.

What This Study Adds to the Field

The studies presented in this article demonstrate that α -defensins contribute to the pathogenesis of ALI through low-density lipoprotein–related receptor–mediated loss of capillary–epithelial barrier function, and suggest a potential new intervention to protect against lung dysfunction and mortality associated with ALI.

mediators and cognate receptors that are operational when the process is most likely to be reversible.

Migration of polymorphonuclear leukocytes (PMNs) into the lungs is central to the pathogenesis of ALI (4, 6, 7, 9). PMNs emigrate through the alveolar capillary bed in response to inflammation. Lungs from patients with ALI show marked accumulation of PMNs, diffuse alveolar damage (DAD), epithelial injury, and pulmonary edema (4, 10). Transmigration of PMNs into the bronchoalveolar lavage (BAL) is a marker of poor prognosis in acute respiratory distress syndrome caused by sepsis (9, 10). In animal models of ALI, intrapulmonary sequestration and BAL content of PMNs correlates with severity, and injury is mitigated by depletion of PMNs (6, 11–14). However, understanding the contribution of PMNs in murine models of ALI (15) is hindered by their lack of α -defensins (16, 17), the most abundant proteins secreted by activated human PMNs (18), whereas, in other species, the contribution of α -defensins to ALI cannot be studied in isolation.

Human α -defensins are a closely related family of six peptides involved in innate immunity. α -Defensins 1–4, also called human neutrophil peptides (HNPs), are sequestered within intracellular granules and released when PMNs are activated, whereas human defensins 5 and 6 are expressed by Paneth cells of the small intestine (17–20). HNP-1 through -4 are 29–33 amino acid cationic peptides containing six cysteines that form three canonical intermolecular disulfide bonds which stabilize surface β -sheet motifs (19, 21, 22). HNP-1 through -3 comprise 97% of the total PMN pool. HNP-1 and -3 differ only in the N-terminal amino acid, which, in HNP-1, is proteolyzed to generate HNP-2 (23). There is no known functional difference among HNP-1 through -4 with respect to effects on host tissue.

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α -Defensins comprise 40–50% of the content of azurophilic granules (24, 25). Plasma levels in healthy volunteers approximate 50 ng/ml, but reach 900 ng/ml–170 μ g/ml in sepsis (25, 26). Elevated levels of α -defensins are found in plasma and in BAL fluid of patients with inflammatory lung disease (27–31), and reach 1 mg/ml in sputum from patients with cystic fibrosis (32).

Previous approaches to study α -defensin in ALI based on administration of isolated proteins have yielded diverse outcomes depending on concentration, route of administration, and duration of exposure (25, 33–35). Intratracheal administration of α -defensin initiates an acute inflammatory response in mice, characterized by increased influx of PMNs into the lung and increased elastase and TNF- α in the BAL (25). *In vitro*, α -defensins, but not β -defensins, induce synthesis of several chemokines, including IL-8 (36), IL-1 β (37), and the monocyte chemoattractant protein-1 (MCP-1) (38), and these peptides initiate apoptosis of airway and other epithelial cells (38) via direct and indirect effects on mitochondrial membrane permeability (39). However, α -defensins have also been reported to promote lung epithelial proliferation (40) and elements of epithelial repair (33).

We have previously observed that α -defensins mediate vasodilation through the low-density lipoprotein-related receptor (LRP) at concentrations several orders of magnitude below those required for cytotoxicity (41). LRP mediates endocytosis and degradation of diverse ligands (42, 43), but also initiates signal transduction pathways within the vasculature that regulate vascular permeability in the central nervous system and elsewhere (41, 44). As such, we hypothesized that α -defensin released from activated PMNs may disrupt LRP-mediated endothelial–epithelial barrier function and contribute to the development of ALI.

In this study, we investigated the effects of α -defensins on the development of ALI in a clinically relevant model using transgenic mice that express human α -defensins in their PMNs. Our studies show that ALI mediated by aspiration is more severe in α -defensin-expressing mice. α -Defensin contributes to ALI by increasing PMN recruitment and increasing lung permeability, in part by signal transduction through LRP.

METHODS

Generation of *Def*^{+/+} Mice

Transgenic mice expressing HNP-1 and -2 in their neutrophils (*Def*^{+/+}) were generated using the bacterial artificial chromosome 10 (BAC-10) 10 (540N10) construct (45). The construct carries four full-length copies of the HNP-1 gene (DEFA1), and one truncated copy of the HNP-3 gene (DEFA3) verified by sequencing of the insert (GenBank accession no. AF200455). Approximately 20 constructs are integrated in the mouse genome determined by Southern blotting. The pattern of transgene inheritance suggests a single integration site. The transgene was introduced into B6SJL (F1) mice and backcrossed onto a C57BL/6N background for over 10 generations to generate a homozygous colony. Control mice on the same genetic background were purchased from Charles River Laboratories (Wilmington, MA). All experiments were conducted in accordance with protocols approved by the institutional review boards of the University of California Los Angeles and the University of Pennsylvania.

Characterization of *Def*^{+/+} Mice

Mice homozygous for the transgene for human α -defensin were genotyped by PCR. Northern blot was used to detect expression of defensin messenger RNA in myeloid cells from *Def*^{+/+} bone marrow, and the expression of the mature protein was detected in myeloid marrow precursors and peripheral blood PMNs by immunostaining using anti-human defensin antibodies compared with control Ig (46, 47), and in PMNs by migration on acid urea gels followed by Western blotting (23). Detailed methods and characterization of *Def*^{+/+} mice are presented in the online supplement.

Recombinant Proteins

Escherichia coli BL21 cells expressing the fusion protein glutathione *s*-transferase tagged receptor associated protein (GST-RAP) receptor-associated protein (RAP) in the plasmid, pGEX-PUC-3T (kind gift of Dr. T. Willnow, University of Potsdam, Potsdam, Germany), were induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside. Complementary DNA encoding the constant heavy chain (CH2) and CH3 domains of human IgG1 was the kind gift of Dr. M. Tykocinski (Thomas Jefferson University, Philadelphia, PA). The cysteines in the hinge region were previously mutated to prevent interchain dimerization (48). Fc-RAP was expressed in pGEX-PUC-3T as a GST fusion protein, purified on glutathione sepharose columns, and the GST was cleaved by thrombin. The final products were passed three times through ReductoTox columns (Sterogene Bioseparations, Carlsbad, CA) to remove endotoxin. Endotoxin levels, determined by limulus amoebocyte lysate assay using Endo-safe PTS (Charles River Laboratories), were less than 0.15 EU/ml. RAP and Fc-RAP migrated as single approximately 39 kD and approximately 65 kD bands as expected on SDS-PAGE (additional methodologic details are provided in the online supplement).

Acid-induced ALI

Mice 8 to 12 weeks of age were anesthetized with an intraperitoneal injection of ketamine/xylazine (100/10 μ g/g body weight) and placed under a heating lamp. To induce ALI, an intratracheal 0.1 N HCl (pH $\sim$ 2; 2.5 μ l/g body weight) was injected, followed by a 300- μ l bolus of air. Control mice received 0.9% phosphate-buffered saline (PBS). Mice were killed at the indicated times, a catheter was introduced via the trachea, and the chest was exposed for gross inspection to determine the ALI score (49). BAL fluid was collected by washing the lungs twice with 0.5 ml ice-cold PBS/5 mM ethylenediaminetetraacetic acid containing protease inhibitor cocktail (1:100; cat. no. P8340; Sigma, St. Louis, MD). In some experiments, blood was flushed from the pulmonary vasculature with 20 ml cold saline by perfusing the right ventricle, and lungs were kept frozen until processing. White blood cells (WBCs) in the BAL were enumerated in a Coulter Multisizer M3 counter (size range 4–7 μ m; Beckman Coulter, Fullerton, CA). Cells were pelleted at 1,500 $\times$ g for 10 minutes at 4°C, and total protein in the supernatant was measured using the bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Rockford, IL). To measure lethality, mice were given 0.5N or 1.0N HCl and survival was measured over the ensuing 24 h.

In Vivo Vascular Permeability

Pulmonary vascular permeability was measured using fluorescein isothiocyanate (FITC)-dextran (50, 51) *in vivo*. At 5 minutes after aspiration, a 200- μ l solution of 1% FITC-dextran (40 kD, FITC-dextran, catalog no. FD40S; Sigma) in PBS was injected into the jugular vein. After 4 hours, animals were killed, and heparinized blood was withdrawn. BAL was collected as described previously here. Blood was flushed from the pulmonary vasculature. The lungs were harvested, homogenized, lysed, and the FITC-dextran content was measured in the BAL and lung lysates using a fluorometer (Multi-detection Microplate Reader Synergy 2; BioTek Instruments, Winooski, VT) with filters 485/20 nm (excitation) and 528/20 nm (emission).

Intervention with Fc-RAP

Systemic administration. A 200- μ l solution of PBS containing 3 μ M Fc-RAP or bovine serum albumin (BSA; 2 μ g/g body weight) in endotoxin-free Dulbecco's PBS was injected intraperitoneally 5 minutes before acid injury. An additional 10% of the dose (100 μ l of a 0.3- μ M solution) was injected intravenously as a bolus immediately before aspiration. This dose was chosen based on the estimated initial concentration in whole blood of 300 nM Fc-RAP (injected dose/blood volume, assuming 100% absorption). After 4 hours, mice were killed, and FITC-dextran in lung lysates and BAL fluid was measured as described previously here.

Intratracheal administration. A 50- μ l solution of Fc-RAP or BSA (1 μ M final concentration) or anti-LRP (catalog no. 3,402; American Diagnostica, Stamford, CT) or an IgG1 isotype control (0.5 μ g/g body weight) was injected intratracheally 20 minutes before acid aspiration, and FITC-dextran in the BAL fluid and lung parenchyma was measured.

Systemic Oxygenation

Continuous monitoring of oxygen tension in the systemic blood was measured using a noninvasive phosphorescence quenching method (52, 53) (additional methodological details are provided in the online supplement).

Histology and Immunohistochemistry

Sections (5 μ m) cut from formalin-fixed and paraffin-embedded lung tissues were immunostained using rabbit anti-human α -defensin antisera or normal rabbit sera (1:3,000), goat antimouse fibrin(ogen) (2 μ g/ml; Nordic Immunol., Tilburg, The Netherlands) or rabbit anti-FITC antibodies (5 μ g/ml; AbD Serotec Raleigh, NC), or with the appropriate IgG as a negative control (additional methodological details are provided in the online supplement). Sections were also stained by hematoxylin and eosin for histological inspection by investigators blinded to treatment.

Statistical Analysis

Differences between groups were compared using the two-tailed Student's *t* test. Statistical analyses were performed using GraphPad Prism software version 4.0 (GraphPad Software Inc., LaJolla, CA). Significance was set at a *P* value less than 0.05.

RESULTS

Defensin Transgenic Mice

We examined the role of α -defensins in the pathogenesis of ALI *in vivo* and specifically their effect on endothelial-epithelial barrier function. To do so, transgenic mice were generated using a BAC 10 construct (45) that carries four full-length copies of the DEFA1 gene encoding HNP-1 and a truncated DEFA3 gene that encodes HNP-3. We anticipated that these mice would express HNP-1, and potentially HNP-2, through N-terminal trimming, but not HNP-3. Tissue-specific expression was affirmed by Northern blot analysis; messenger RNA encoding HNP-1 was present in bone marrow at levels comparable to human chronic myeloid leukemia (CML) cells, but was not detected in other tissues or in wild-type (WT) animals (see Figure E1A in the online supplement). α -Defensin was detected by immunohistochemistry in peripheral blood PMNs (Figure 1B) from *Def^{+/+}* mice and human PMNs (Figure 1C), but not PMNs from WT mice (Figure 1D).

Transgenic mice were crossbred onto a C57BL/6N background for over 10 generations. Immunostaining confirmed expression of α -defensin in bone marrow and peripheral blood myeloid cells (Figures E1E and E1F). Fully processed protein was detected in extracts from PMNs isolated from the peritoneal cavity of *Def^{+/+}* mice on acid urea gels (23). Western blots show two major bands consistent with expression of HNP-1 and

HNP-2; HNP-3 was not detected (Figure E1G). *Def^{+/+}* PMN expressed approximately 10% of the total mature α -defensin in human PMNs based on Western immunoblots (data not shown). *Def^{+/+}* mice were viable, showed no gross morphological defects, and histological inspection of all organs examined to date, including the lung, was normal. Females carried to term, and the number and composition of the litters were normal.

Defensin Is Released into the Lungs during ALI

To study the contribution of α -defensin to the development of ALI, we studied acid aspiration, a model that is neutrophil dependent (15, 54). We first asked whether α -defensin is released from PMNs recruited into the lungs after intratracheal injection of HCl. At 4 hours after aspiration, α -defensin was found in lungs of *Def^{+/+}* mice exposed to acid (Figures 1A–1D); no staining was seen using preimmune sera (data not shown). α -Defensin was associated with numerous PMNs adherent to pulmonary epithelial cells, but was also found diffusely throughout the bronchiolar mucosa, alveolar parenchyma (particularly on type II pneumocytes), and on the luminal and abluminal surfaces of large and small vessels. Minimal staining of type II pneumocytes was seen in lungs from *Def^{+/+}* mice exposed to saline (Figure 1E), and none was detected in lungs of WT mice exposed to acid (Figure 1F).

We next asked whether release of α -defensin contributed to the development of ALI. To do so, we compared the severity of lung injury in *Def^{+/+}* and WT mice after acid aspiration. At 4 hours after aspiration, the severity of injury was scored visually (49). Lungs from *Def^{+/+}* mice exhibited greater swelling and rubor (Figure 2B). The ALI score was 8 ($\pm$ 0.9) in *Def^{+/+}* mice versus 4 ($\pm$ 0.5) in WT mice (Figure 2A, *P* = 0.001). Total WBC count was increased in the BAL fluid of acid-injured WT and *Def^{+/+}* mice compared with saline exposed counterparts. The total WBC count in BAL from acid-injured *Def^{+/+}* mice was approximately twofold greater than similarly treated WT control animals (*P* < 0.0001) (Figure 2C). Total protein in BAL was increased approximately threefold in *Def^{+/+}* mice, and approximately 2.5-fold in WT mice compared with uninjured animals, a difference that did not reach statistical significance (Figure 2D, *P* = 0.42). However, an approximately twofold increase in total protein in the BAL fluid of *Def^{+/+}* mice was evident by 16 hours after injury (data not shown), indicating that the contribution of protein transudation into airways exceeded the release of proteins from damaged epithelial and other parenchymal cells.

Histological analysis affirmed greater lung damage in *Def^{+/+}* mice than WT control animals by 4 hours after insult (Figure 2E). The airspaces of acid-injured *Def^{+/+}* mice were filled with proteinaceous edema fluid and increased numbers of WBCs, and showed thickened alveolar septa and more severe bronchi-

Figure 1. α -Defensin is released into the lungs of *Def^{+/+}* mice during acute lung injury (ALI). ALI was induced by intratracheal administration of HCl (0.1 N) or saline. After 4 hours, the lungs were inflated with 10% buffered formalin phosphate, isolated, washed, and fixed. Paraffin-embedded sections from *Def^{+/+}* mice exposed to acid aspiration (A–D) or saline (E) were stained using rabbit anti-human defensin sera or preimmune rabbit sera as the negative control. Wild-type mice exposed to acid aspiration show no staining for α -defensin (F). Sections shown at 100 $\times$ (A, E, and F), 200 $\times$ (B and D), and 400 $\times$ (C) magnifications. Results are representative of three mice from each exposure group.

Figure 2. Acute lung injury (ALI) is more severe in *Def^{+/+}* mice after acid aspiration. Acid aspiration was induced in *Def^{+/+}* and wild-type (WT) mice, as described previously here. After 4 hours, the lungs were inspected visually and scored for severity of injury (as in Reference 49). (A) ALI score (scale, 1–10). Mean ($\pm$ SEM) of 5–10 mice in each experimental group from two different experiments are shown. $**P = 0.0012$; Student's *t* test. (B) Visual appearance of injured lungs from *Def^{+/+}* and WT mice 4 hours after acid aspiration (AA). Blood was flushed from the pulmonary vasculature with 20 ml of cold saline by perfusing the right ventricle, and the lungs were photographed under a dissecting microscope. Results shown are representative of three mice from each group. Total white blood cells (WBCs) and protein are increased in bronchoalveolar lavage (BAL) fluid from *Def^{+/+}* mice after acid aspiration. Acid aspiration was induced in *Def^{+/+}* and WT mice. After 4 hours, BAL fluid was analyzed for total WBCs (C) and total protein content (D). Means ($\pm$ SEM) from 8–12 mice in each experimental group from three different experiments are shown. $***P < 0.0001$, $^{\$}P = 0.42$. (E) *Def^{+/+}* mice exhibit more intense histological evidence of tissue injury after acid aspiration. Acid aspiration was induced in *Def^{+/+}* and WT mice. After 4 hours, the lungs were inflated with formalin, and fixed in 10% buffered formalin phosphate. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Low-power (40 $\times$) and

medium-power (200 $\times$) images of H&E-stained *Def^{+/+}* (upper panels) and WT (lower panels) mouse lungs after saline or acid aspiration. Tissues from acid-injured *Def^{+/+}* lungs show increased alveolar septal thickening and congestion compared with the acid-injured WT control animals. Lungs from saline-injected *Def^{+/+}* (left upper panel) and WT mice (left lower panel) show normal parenchymal architecture. The images are representative of three to four mice from each treatment group.

olar mucosal injury than acid-injured WT mice. Saline-treated WT and *Def^{+/+}* mice showed little to no damage.

Defensin-expressing Mice Develop Enhanced Vascular Permeability

We hypothesized that the discrepancy between the severity of lung injury in *Def^{+/+}* mice, and the modest increase in BAL protein, compared with WT control animals reflects superimposition of proteins released from damaged epithelial and other

cells onto increased microvascular permeability. To distinguish between protein influx and local release, we measured permeability directly. At 5 minutes after aspiration, a 200- μ l solution containing 1% FITC-dextran in PBS was injected intravenously. After 4 hours, the lungs were lavaged, blood was flushed from the pulmonary vasculature, and accumulation of FITC-dextran was measured in the BAL fluid and lung lysates. FITC-dextran in the BAL fluid was barely detectable in WT mice after intratracheal saline, and increased approximately 15-fold after

Figure 3. *Def^{+/+}* mice show greater vascular permeability after acid aspiration: kinetics. Acute lung injury (ALI) was induced by acid aspiration. At 5 minutes after acid or saline aspiration, a 200- μ l solution containing 1% fluorescein isothiocyanate (FITC)-dextran solution in phosphate-buffered saline was injected intravenously. At 4 hours after aspiration, the lungs were lavaged and the blood was flushed from the lung vasculature by perfusing the right ventricle with 20 ml cold saline. The lungs were homogenized and FITC-dextran content was measured in the bronchoalveolar lavage (BAL) (A) and lung lysates (B). BAL and lung lysates from *Def^{+/+}* mice contained increased FITC-dextran ($**P = 0.0023$, $***P = 0.0008$, respectively). Mean ($\pm$ SEM) from 9–12 mice in each experimental group from three independent experiments is shown. (C) *Def^{+/+}* mice show greater vascular permeability after acid aspiration/parenchymal deposition of fibrin and FITC-dextran. Paraffin-embedded sections, prepared from formalin-fixed lungs of *Def^{+/+}* and wild-type (WT) control mice 4 hours after acid aspiration and intravenous injection of FITC-dextran, were subjected to antigen retrieval and immunostained using rabbit anti-FITC antibodies (5 μ g/ml) to detect the tissue distribution of FITC-dextran (upper panels) as a measure of barrier function,

or with goat anti-fibrin(ogen) antibodies (2 μ g/ml) (lower panels). This was followed by incubation with biotinylated secondary antibodies and horseradish peroxidase-conjugated streptavidin. Color was detected using the avidin-biotin complex ABC Kit and counterstained with hematoxylin. Medium power (200 $\times$) images showing acid-injured *Def^{+/+}* (left panels) and WT mouse (middle panels) lungs and uninjured *Def^{+/+}* mouse lung controls (right panels). Sections from acid-injured *Def^{+/+}* lungs shows strong immunopositivity, with anti-FITC distributed diffusely in the bronchiolar mucosa and alveolar septa, and strong immunopositivity with anti-fibrin involving peribronchiolar and perivascular tissues. In contrast, acid-injured WT mouse lungs show weak mucosal positivity with anti-FITC and weak alveolar septal positivity with anti-fibrin. Uninjured *Def^{+/+}* mouse lung controls are negative for FITC and fibrin(ogen). Results are representative of two to three mice from each treatment group.

acid aspiration. In *Def^{+/+}* mice, FITC-dextran in the BAL fluid was comparable to that in WT mice in the absence of injury, but increased approximately 30-fold after aspiration ($P = 0.002$ compared with injured WT mice) (Figure 3A). FITC-dextran increased threefold in lung lysates from injured WT mice, and sixfold in *Def^{+/+}* mice ($P < 0.001$, injured *Def^{+/+}* versus WT mice) (Figure 3B).

Serial lung sections from FITC-dextran-injected *Def^{+/+}* and WT mice after aspiration or their uninjured counterparts were immunostained with anti-FITC (Figure 3C, upper panels) and anti-fibrin/fibrinogen antibodies (Figure 3C, lower panels) to confirm disruption of barrier function and assess tissue distribution. FITC-dextran was abundant in the edematous lung parenchyma in proximity to damaged capillaries and in the airways of *Def^{+/+}* mice after aspiration (upper left panel). Less FITC-dextran was seen in injured WT mice (upper middle panel). Little or no FITC-dextran was seen in uninjured *Def^{+/+}* (upper right panel) and WT mice (data not shown). Fibrin deposition followed the same pattern (Figure 3C). These results show that release of α -defensin after aspiration is associated with increased endothelial and epithelial permeability, permitting FITC-dextran and fibrinogen to diffuse from the circulation into and through the lung parenchyma to the BAL fluid.

Survival Is Shortened in *Def^{+/+}* Mice

We next measured survival of *Def^{+/+}* and WT mice after induction of severe ALI (1 N HCl, 2 μ l/g body weight, intratracheal). *Def^{+/+}* mice exhibited significantly shorter survival times, with 75% dying within 20 minutes after induction of injury compared with 20% mortality in WT mice ($P = 0.0035$, Logrank test; Figure E2A). At a lower dose of HCl (0.5N HCl), survival of *Def^{+/+}* mice remained significantly shortened, with a median survival of 420 minutes compared with 1,200 minutes for WT mice ($P = 0.047$, Logrank test; Figure E2B).

Role of LRP in Vascular Permeability and ALI

We then investigated the mechanism by which vascular permeability is disrupted in WT and *Def^{+/+}* animals, and, specifically, the involvement of LRP, which has been implicated in vascular contractility (41) and maintenance of blood-brain barrier function (44). To do so, we studied the effect of intravenous RAP on pulmonary vascular permeability using FITC-dextran. To help circumvent the need for extraordinary doses or multiple injections due to its rapid clearance from the circulation, we developed a fusion protein, Fc-RAP. Fc-RAP inhibited binding and internalization of 125 I-human urokinase plasminogen acti-

Figure 4. The low-density lipoprotein-related receptor antagonist, Fc-receptor-associated protein (RAP), reduces vascular permeability and acute lung injury (ALI) in wild-type (WT) mice. A 200- μ l solution containing 3 μ M Fc-RAP or bovine serum albumin (BSA; 1.9 μ g/g body weight) in phosphate-buffered saline was injected intraperitoneally 5 minutes before induction of acid injury in WT mice. An additional 10% of this dose (100 μ l of a 0.3- μ M solution) was injected intravenously as a bolus immediately before acid aspiration. Acid aspiration and intravenous injection of fluorescein isothiocyanate (FITC)-dextran were performed as described in the legend to Figure 3. At 4 after acid aspiration, mice were killed, and the content of FITC-dextran in bronchoalveolar lavage (BAL) (A) and lung lysates (B) was measured, as described in the legend to Figure 3. Fc-RAP significantly reduced FITC-dextran content in the BAL fluid (** $P = 0.0043$) and in lung lysates ($P = 0.05$). Total white blood cells (WBCs) (C) and total protein (D) in the BAL fluid were measured as described in the legend to Figure 3. The mean ($\pm$ SEM) from six to nine mice in each experimental group from two independent experiments is shown.

vator (uPA) and 125 I-RAP by murine fibroblasts that lack urokinase plasminogen receptor (uPAR) (CD87), but express LRP to the same extent as RAP itself (data not shown). We then examined its effects on vascular permeability in WT mice. A 200- μ l solution of 3 μ M Fc-RAP or BSA (1.9 μ g/g body weight) was injected intraperitoneally 5 minutes before aspiration along with 10% of this dose injected as an intravenous bolus immediately before aspiration and FITC-dextran injection. After 4 hours, FITC-dextran in BAL fluid and perfused lung lysates was measured. Fc-RAP reduced FITC-dextran in BAL fluid from WT mice by 65.6 ($\pm$ 6.2)% (mean $\pm$ SEM) compared with mice given BSA, expressed relative to uninjured control animals (Figure 4A, $P = 0.004$). Mice given BSA before injury showed a twofold increase in FITC-dextran in lung lysates relative to uninjured mice, whereas no increase was seen in mice given Fc-RAP (Figure 4B, $P = 0.05$). Mice given Fc-RAP also showed an approximately 70% reduction in total WBC in the BAL, similar to the reduction in FITC-dextran, but a somewhat more modest approximately 50% reduction in total protein (Figures 4C and 4D).

Fc-RAP Reduces Vascular Permeability and ALI in *Def*^{+/+} Mice

We then asked if increased vascular permeability in *Def*^{+/+} mice (Figures 2 and 3) is also mediated by LRP. Acid aspiration

caused an approximately 30-fold increase in FITC-dextran in BAL fluid and a fourfold increase in lung lysates of *Def*^{+/+} mice given BSA relative to uninjured mice. Fc-RAP reduced FITC-dextran in the two compartments by 65 ($\pm$ 6.6)% and 70 ($\pm$ 4.5)%, respectively (mean $\pm$ SEM), using uninjured mice as the baseline (Figures 5A and 5B). Mice given Fc-RAP also showed an approximately 55% reduction in BAL WBC and total protein (Figures 5C and 5D). The protective effect of Fc-RAP extended beyond reducing vascular permeability. The lungs of Fc-RAP-treated mice inspected 4 hours after aspiration showed less swelling and rubor than those given BSA, having an appearance closer to lungs from uninjured mice (Figure 5E). Hematoxylin and eosin-stained sections from injured, BSA-treated mice showed the expected accumulation of cells within the interstitial spaces, proteinaceous edema fluid, influx of WBCs in the airways, thickened alveolar septa, and injured airway epithelium (Figure 5F, upper left panel), whereas animals given Fc-RAP (Figure 5F, right panels) showed less capillary and epithelial cell damage, and WBC infiltration was comparable to uninjured control animals. Immunostaining revealed abundant FITC-dextran in edematous lung parenchyma in proximity to damaged capillaries and airways in mice pretreated with BSA (Figure 5F, lower panels) compared with mice pretreated with Fc-RAP; minimal or no FITC-dextran was seen

Figure 5. Fc-receptor-associated protein (RAP) reduces vascular permeability and acute lung injury (ALI) in *Def^{+/+}* mice. *Def^{+/+}* mice received intraperitoneal and intravenous injections of Fc-RAP or bovine serum albumin (BSA) control, followed by acid injury and intravenous injection of fluorescein isothiocyanate (FITC)-dextran, as described in the legend to Figure 3. At 4 hours after acid aspiration, mice were killed, and the content of FITC-dextran in bronchoalveolar lavage (BAL) (A) and lung lysates (B) was measured. Fc-RAP significantly reduced FITC-dextran in BAL fluid (* $P = 0.043$) and lung lysates (** $P = 0.004$). Total white blood cells (WBCs) (C) and total protein (D) in the BAL fluid were measured as described in the legend to Figure 2. The mean ($\pm$ SEM) from six to eight mice in each experimental group from two independent experiments is shown. Another cohort of *Def^{+/+}* and wild-type mice were treated with Fc-RAP or BSA control, as described previously here, and acid aspiration was induced, followed by intravenous injection of FITC-dextran, as described in the legend to Figure 3. At 4 hours after aspiration, the lungs were inflated with formalin, washed, and fixed with 10% formalin. (E) Gross appearance. The lungs were harvested and photographed. Lungs of *Def^{+/+}* mice given BSA I (left panel) or Fc-RAP (middle panel) 4 hours after injury are shown; no injury control (right panel). (F) Histology and immunostaining. Paraffin-embedded sections prepared from the same lungs were stained by hematoxylin and eosin (H&E) (upper panels), or immunostained with goat anti-fibrin(ogen) antibodies (middle panels) or rabbit anti-FITC antibodies (lower panels), and binding was detected as described in the legend to Figure 3. H&E staining of lung from a BSA-treated, acid-injured *Def^{+/+}* mouse at 10 $\times$ magnification showing more extensive edema and alveolar septal thickening compared with lung from a Fc-RAP-treated, acid-injured *Def^{+/+}* mouse. Strong anti-FITC and anti-fibrin immunopositivity is seen in the lung from the BSA-treated mouse compared with focal and weak immunopositivity in the Fc-RAP-treated mouse lung. Results are representative of three mice from each treatment group.

in lungs from uninjured *Def^{+/+}* mice (see also Figure 3C). Fibrin(ogen) was also more prominent in lungs from BSA-treated animals (Figure 5F, middle panels) than in uninjured mice, and was reduced by pretreatment with Fc-RAP (Figure 5F, middle right panel).

Fc-RAP and Anti-LRP Attenuate ALI-induced Lung Permeability

The protective effect of Fc-RAP endothelial–epithelial barrier integrity could be mediated by blocking either endothelial and/or epithelial LRP. To address the latter possibility directly, we examined the effects of intratracheal Fc-RAP on lung permeability. We also reasoned that intratracheal administration has

potential pharmacokinetic advantages over systemic administration, including a smaller volume of distribution and higher local concentrations permitting administration of lower doses, reduced peripheral clearance or metabolism by bypassing the liver, and less potential for off-target toxicity. To test this hypothesis, WT mice were injected intratracheally with a 50- μ l solution of PBS containing: 1 μ M Fc-RAP (0.16 μ g/g body weight) or Fc control, anti-LRP-1 antibody (0.5 μ g/g body weight = 1.33 μ M solution) or IgG1 isotype control. Acid injury was induced 20–30 minutes later, followed by intravenous injection of FITC-dextran as described in Figure 3. After 4 hours, FITC-dextran in BAL fluid (Figure 6A) and lung lysates (Figure 6B) was measured. Fc-RAP, given at 10% of the intravenous dose, and anti-LRP-1 reduced BAL FITC-dextran

Figure 6. Fc-receptor-associated protein (RAP) and anti-low-density lipoprotein-related receptor (LRP) antibody attenuate aspiration-induced vascular permeability. A 50- μ l solution of phosphate-buffered saline (PBS) containing 1 μ M Fc-RAP (0.16 μ g/g body weight) or human-Fc control, anti-LRP antibody (0.5 μ g/g body weight) or an IgG1 isotype was injected intratracheally in wild-type (WT) mice. Acid injury was induced 20–30 minutes later, followed by intravenous injection of fluorescein isothiocyanate (FITC)-dextran, as described in the legend to Figure 5. At 4 hours after aspiration, mice were killed, and the content of FITC-dextran in bronchoalveolar lavage (BAL) fluid (A) and lung lysates (B) was measured, as described in the legend to Figure 5. Fc-RAP caused a significant reduction in FITC-dextran content of the BAL fluid and lung lysates (* P = 0.026 and * P = 0.027, respectively). The mean ($\pm$ SEM) from five to eight mice in each experimental group from two independent experiments is shown. Fc-RAP attenuates hypoxemia after acute lung injury (ALI). Oxygen tension in the systemic blood (PaO₂) before and after induction of acid injury was measured using phosphorescence quenching. A-100 μ l solution containing 200 μ M Oxyphor G3 was injected intravenously into WT mice. Phosphorescent lifetimes, which correlate directly with PaO₂, were measured at 1-minute intervals using a phosphorometer in a transillumination setup placed on the mouse paw. After initial baseline measurements for 5–10 minutes, mice were given either intratracheally Fc-RAP or bovine serum albumin (BSA), as described in the legend to Figure 4. After 20 minutes, HCl (0.5 N HCl, 2 μ l/g body weight) or PBS was administered and PaO₂ was measured for an additional 3 hours. Results representative of three mice from each group are shown.

by 47 (± 12)% and 30 (± 9)%, respectively ($P = 0.026$ for anti-LRP and 0.027 for Fc-RAP) compared with mice given Fc or IgG1. The FITC-dextran content in lung lysates was unaffected by anti-LRP, and was modestly reduced by Fc-RAP. Anti-LRP administered 10 minutes after aspiration provided similar protection (data not shown), suggesting a potential therapeutic effect of intervention given soon after injury. These results demonstrate that Fc-RAP: (1) when given intratracheally, protected the epithelium directly; (2) was effective at a lower dose than required with systemic delivery; and (3) mediates its effect through a member of low-density lipoprotein superfamily of receptors, most likely LRP-1.

Fc-RAP Attenuates Hypoxemia after ALI

ALI leads to deterioration in gas exchange, reducing PaO_2 . Therefore, we followed PaO_2 in real-time using a noninvasive phosphorescence quenching technique that obviates the need for repetitive arterial blood sampling (52, 53). WT mice were given intratracheal Fc-RAP or BSA and then injected intravenously with a 100- μl solution containing 200 μM oxyphor G3. Phosphorescent lifetimes, which correlate directly with PaO_2 , were measured in the paw over the ensuing 30 minutes before induction of injury, and for an additional 2–3 hours thereafter. Representative PaO_2 profiles from the three treatments groups are shown in Figure 6C. Mice given BSA show a rapid initial fall in PaO_2 that returned to baseline within approximately 5 minutes. Acid aspiration was followed by a gradual, persistent drop ($\sim 40\%$) in systemic PaO_2 over the ensuing 2 hours. In contrast, mice given Fc-RAP showed an initial fluctuation in PaO_2 after injury, followed by a recovery to baseline by 5 minutes. Uninjured mice showed stable oxygen tensions after the initial fluctuation.

Defensin Is Released into Human Lung Parenchyma after Lung Injury

Finally, we asked whether α -defensin is released into human lung parenchyma after ALI. Sections of normal-appearing lungs from patients undergoing resection and patients with DAD were stained using rabbit anti-human defensin or preimmune sera. Lung sections exhibiting DAD showed abundant staining for α -defensin in endothelium, subendothelium, and epithelium (Figures 7A–7D), whereas, in normal-appearing sections, α -defensin was essentially confined to PMNs (Figures 7E and 7F). No staining was seen using preimmune rabbit sera (data not shown).

DISCUSSION

The involvement of PMNs in disrupting capillary–alveolar barrier function, an early event in the pathogenesis of ALI (1, 6–8), is only partially understood. Although α -defensins are the most abundant proteins secreted by activated human PMNs, their contribution to ALI cannot be assessed in murine models that lack this class of peptides. Previous approaches based on administering purified α -defensin have yielded diverse outcomes (25, 33–35). Although the advantages of using purified proteins to control administration are clear, there is a need to study α -defensins in the more complex and clinically relevant setting of ALI. This is especially true in the case of proteins released in a temporally and geographically restricted manner in response to extrapulmonary or intrapulmonary insults.

We studied transgenic mice that express human α -defensins in their neutrophils to assess their contribution to ALI. *Def^{+/+}* mice exhibit no gross or histological defects in all major organs examined to date, including the lungs. Myeloid-specific expression of HNP was confirmed by Northern blot, Western blots, and immunohistochemistry of peripheral and bone marrow PMNs. *Def^{+/+}* PMNs express mature HNP-1 and HNP-2 and two higher molecular weight bands, consistent with pre-proHNP and proHNP found in myeloid progenitors (55, 56). HNP-1 was expressed at approximately 10% of the total concentration of mature α -defensins in human PMN, a finding that might underestimate their contribution to ALI, but enabled us to study the mechanism of action, which might have been precluded by more intense release.

That *Def^{+/+}* mice express only the HNP-1 gene does not appear to compromise their utility as a model of human PMN. First, *Def^{+/+}* mice appear to process HNP-1 to HNP-2. Second, HNP1 and HNP-2 are the predominant α -defensins expressed by human PMN. Third, we are unaware of differences in the effect of HNP-1 and HNP-3 (differing only in the first amino acid) on host tissue. Fourth, humans express variable copy numbers of HNP-1 and HNP-3 genes (23), with 10–37% of the population lacking the HNP-3 gene entirely without impaired inflammatory responses (23, 57, 58).

No animal model recapitulates all causes and identifies aspects of ALI in humans (15). We studied acid aspiration (15, 54), because it occurs commonly and is associated with a relatively high mortality (52, 54, 59). The model is reproducible, reliably generates an inflammatory response involving PMNs, increases microvascular permeability, and causes alveolar edema (15, 54).

Figure 7. α -Defensin is released into human lung parenchyma after lung injury. Paraffin-embedded sections from human lung showing diffuse alveolar damage (DAD) (A–D) and normal-appearing section of control lung (E and F) were stained using rabbit anti-human α -defensin antisera or preimmune rabbit sera as the negative control, as described in the legend to Figure 1. High-power (400 $\times$) images of DAD and normal-appearing lung showing bronchiolar epithelial, endothelial, and subendothelial immunopositivity with α -defensin. In contrast, control sections showed no staining of bronchiolar epithelium, endothelium, or subendothelial tissue. Results shown are representative of three DAD cases and two normal control sections.

By 4 hours after aspiration, α -defensin was distributed throughout the damaged lungs. α -Defensin was associated with PMNs that had immigrated into interstitial compartments and those adherent to lung epithelium. α -Defensin was also found diffusely in the surrounding alveolar parenchyma and on the luminal and abluminal surfaces of large and small vessels in human lungs damaged by ALI. Thus, *Def^{+/+}* PMNs migrate into the lung in response to chemotactic stimuli resulting from acid injury, where they are activated and discharge α -defensin from their granules into the surrounding parenchyma. α -Defensins are highly cationic proteins that bind to diverse components of the extracellular matrix, integrins, and undoubtedly other proteins critical to lung function (47, 60, 61), and no degradative pathway for their removal from tissue has been identified.

ALI was more severe in *Def^{+/+}* mice than in WT animals as evidenced by: (1) increased vascular permeability; (2) increased edema and rubor; (3) greater accumulation of PMNs in interstitial and alveolar compartments, accompanied by increased interstitial edema; (4) greater accumulation of fibrin(ogen), consistent with exudation of plasma proteins into the interstitium, coupled with inhibition of fibrinolysis characteristic of ALI (35, 62) and also mediated by defensin as we described (63, 64); (5) histological evidence of more intense epithelial damage; (6) greater impairment of gas exchange; and (7) reduced survival. These data demonstrate that α -defensins contribute to the pathogenesis of ALI in this model.

A 30-fold increase in pulmonary vascular permeability in *Def^{+/+}* mice, measured using intravenous FITC-dextran, was evident within 4 hours of injury, indicating that endothelial injury occurs soon after epithelial damage. Immunostaining for FITC-dextran and analysis of BAL fluid content confirmed influx of dextran into all lung compartments. The absence of a significant increase in total protein in the BAL fluid in *Def^{+/+}* mice at this early time point might reflect intense parenchymal injury by acid, leading to protein release into damaged airways. This interpretation is supported by the approximately twofold increase in BAL protein in *Def^{+/+}* mice by 16 hours after injury. The delayed increase in BAL protein in *Def^{+/+}* mice, and evidence of protein-rich edema on histology, suggests that transudation of FITC-dextran provides a more sensitive index of capillary-alveolar permeability.

Therefore, we measured permeability to intravenous dextran to investigate the mechanism by which microvascular/epithelial barrier function is disrupted in ALI. Specifically, we examined the involvement of LRP, which has been implicated in vascular contractility (41) and regulation of blood-brain barrier function (44). To do so, we developed a fusion protein, Fc-RAP, designed to enhance the survival of the LRP antagonist in the circulation. As a proof of principle, we administered Fc-RAP intraperitoneally and intravenously 5 minutes before injury. Intravenously administered Fc-RAP inhibited the enhanced influx of FITC-dextran into lung lysates and airways by 60–70% 4 hours after injury. Similar protection was afforded to *Def^{+/+}* mice, which exhibit higher baseline influx of FITC-dextran after injury. As the lung content of FITC-dextran in *Def^{+/+}* mice was twofold greater after aspiration than in injured WT mice, the absolute protection afforded by Fc-RAP was higher, assuming similar clearance and distribution of Fc-RAP. The reduction in vascular permeability by Fc-RAP sufficed to attenuate ALI, evidenced by gross appearance of the lungs, reduction in BAL WBCs and total protein, reduced edema, PMN infiltration, and fibrin(ogen) deposition. This indicates that LRP helps maintain the integrity of the capillary-alveolar barrier, and its disruption contributes to the injury caused by α -defensin.

The protective effect of intravenous Fc-RAP on pulmonary vascular integrity could be mediated by blocking endothelial or epithelial LRP. LRP is expressed widely, and intravenous Fc-RAP might affect diverse processes mediated by LRP in other organs that affect lung function indirectly (42). Thus, we reasoned that intratracheal instillation of Fc-RAP acting locally and more rapidly might permit lower doses to be employed and limit extrapulmonary activity. In support of this concept, Fc-RAP, given intratracheally at 10% of the dose used systemically, and anti-LRP-1, given before injury, reduced BAL FITC-dextran compared with Fc or IgG1 control animals. Intratracheal Fc-RAP had less effect on reducing lung dextran than on BAL fluid content, unlike after intravenous administration, suggesting a more pronounced effect on epithelial than endothelial function at the doses employed. Nevertheless, intratracheal Fc-RAP preserved oxygenation, affirming the importance of maintaining epithelial integrity on the outcome of ALI (65). Initial experiments also showed that intratracheal anti-LRP given 10 minutes after injury similarly reduced transudation of FITC-dextran, suggesting that early intervention might attenuate edema and lung dysfunction after aspiration or other forms of ALI.

In summary, our data demonstrate that release of α -defensin by activated PMNs contributes to the initiation of ALI by increasing both the inflammatory response (PMN recruitment) and lung (microvascular and epithelial) permeability. The effects of α -defensin are not attenuated by plasma or tissue proteins that inhibit cytotoxicity (66–68), likely because of the high local concentrations attained when the protein is rapidly released from PMNs adherent to endothelial and epithelial cells. The effect of α -defensin is mediated, at least in part, by signal transduction through LRP, an effect that is reversible. The data also show that loss of alveolar barrier function is an early, critical, and potentially remediable feature of ALI.

Conflict of Interest Statement: K.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.A.-A.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. I.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.C.-S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.A.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.C.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.I. has received lecture fees paid by Brahms (\$5,001–\$10,000), and has received an industry-sponsored grant from Attenuon, LLC (\$10,001–\$50,000). R.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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