ORIGINAL RESEARCH

Rhesus θ-Defensin-1 Attenuates Endotoxin-induced Acute Lung Injury by Inhibiting Proinflammatory Cytokines and Neutrophil Recruitment

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Abstract

Acute lung injury (ALI) is a clinical syndrome characterized by acute respiratory failure and is associated with substantial morbidity and mortality. Rhesus θ-defensin (RTD)-1 is an antimicrobial peptide with immunomodulatory activity. As airway inflammation and neutrophil recruitment and activation are hallmarks of ALI, we evaluated the therapeutic efficacy of RTD-1 in preclinical models of the disease. We investigated the effect of RTD-1 on neutrophil chemotaxis and macrophage-driven pulmonary inflammation with human peripheral neutrophils and LPS-stimulated murine alveolar macrophage (denoted MH-S) cells. Treatment and prophylactic single escalating doses were administered subcutaneously in a well-established murine model of direct endotoxin-induced ALI. We assessed lung injury by histopathology, pulmonary edema, inflammatory cell recruitment, and inflammatory cytokines/chemokines in the BAL fluid. *In vitro* studies demonstrated that RTD-1 suppressed CXCL8-induced neutrophil chemotaxis, TNF-mediated neutrophil–endothelial cell adhesion, and proinflammatory cytokine release in activated murine alveolar immortalized macrophages (MH-S) cells. Treatment with RTD-1 significantly inhibited *in vivo* LPS-induced ALI by reducing pulmonary edema and histopathological changes. Treatment was associated with dose- and time-dependent inhibition of proinflammatory cytokines (TNF, IL-1β, and IL-6), peroxidase activity, and neutrophil recruitment into the airways. Antiinflammatory effects were demonstrated in animals receiving RTD-1 up to 12 hours after LPS challenge. Notably, subcutaneously administered RTD-1 demonstrates good peptide stability as demonstrated by the long *in vivo* half-life. Taken together, these studies demonstrate that RTD-1 is efficacious in an experimental model of ALI through inhibition of neutrophil chemotaxis and adhesion, and the attenuation of proinflammatory cytokines and gene expression from alveolar macrophages.

Keywords: LPS; acute lung injury; airway inflammation; θ defensin; host defense peptides

Clinical Relevance

This research highlights the potential of rhesus θ-defensin (RTD)-1 as a therapy for acute lung injury. *In vitro* studies described in this article shine light on some previously unknown RTD-1 antiinflammatory mechanisms of action. Collectively, these findings support the clinical development of RTD-1 for acute lung injury and its underlying mechanism.

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Acute lung injury (ALI) is a clinical syndrome characterized by lung edema, impaired gas exchange, and respiratory failure (1). The principal cause of ALI is the excessive and prolonged activation of neutrophils in response to infection or aspiration, which cause damage to the pulmonary parenchyma and microvasculature (2). Proinflammatory cytokines are associated with the severity of ALI, underscoring the importance of inflammation as a therapeutic target (3). Despite improvements in the understanding of the pathophysiology of ALI, outcomes remain poor, with significant morbidity and mortality at 40% (2). Some have proposed a paradigm shift toward prevention or early treatment of ALI with the goal of improving outcomes (4).

Defensins are cationic antimicrobial peptides that promote key innate and adaptive immune responses within the lungs. In humans, α-defensins are primarily expressed in neutrophils, whereas the β-defensins are expressed in many tissues, including the epithelial cells lining the respiratory tract. Although first studied for their broad-spectrum activity against bacteria, fungi, and viruses, defensins also contribute to recruitment of inflammatory cells and activation of adaptive immune responses (5). Although α- and β-defensins are critical to mounting an effective immune response to a pathogen, α-defensins have been shown to contribute to the pathogenesis of ALI by disrupting the capillary–epithelial barrier (6).

θ-defensins are macrocyclic antimicrobial peptides found in leukocytes of Old World monkeys, but lost to man through the evolutionary acquisition of a premature stop codon that aborts translation (7, 8). They are 18 amino acids long and are formed by the dimeric head-to-tail ligation of two precursors and further stabilized with three disulfide bonds. Similar to the human α-defensins, rhesus θ-defensin (RTD)-1 exhibits broad-spectrum microbicidal activity against viruses, bacteria, and fungi (9–12). In contrast to the α-defensins, which are proinflammatory, θ-defensins have been shown to exhibit antiinflammatory activity *in vitro* (13, 14). Data show that RTD-1 diminishes cytokine production in leukocytes stimulated with a number of TLR agonists, including heat-killed *Listeria monocytogenes* (TLR2), flagellin (TLR5), LPS (TLR4), and live *Escherichia coli* (13). In murine models of polymicrobial sepsis and severe acute respiratory syndrome, RTD-1 treatment significantly reduced inflammatory cytokines (13, 15). Importantly, early preclinical investigations in mice, rats, and chimpanzees suggest RTD-1 to be safe and nonimmunogenic (13).

The antimicrobial and antiinflammatory properties of RTD-1 may provide a unique approach to treating both the infection and inflammation associated with ALI. In this study, we sought to investigate whether the immunomodulatory potential of RTD-1 mitigates the ALI properties of cellular airway influx and leukocyte-induced lung injury (16). To determine the effect of RTD-1 on ALI, mice received RTD-1 30 minutes before initiation of pulmonary inflammation, at the time of insult and 12 hours after initiation of inflammation using the well established murine model of intranasal LPS-induced acute lung neutrophilia (16, 17). We evaluated RTD-1 intervention through quantifying airway neutrophil burden and activation, cytokine/chemokine release, gene expression, pulmonary vascular leakage, and extent of lung injury. In addition, *in vitro* assays investigating neutrophil chemotaxis and adhesion, as well as LPS-induced alveolar macrophage and human lung epithelial inflammation provide supportive details on RTD-1–responsive cell populations. We provide evidence that RTD-1 reduces LPS-induced lung injury by inhibiting neutrophil recruitment and alveolar macrophage proinflammatory cytokine production.

This work was presented in part at the North American Cystic Fibrosis Conference, October 8–11, 2012 (18).

Methods

Detailed Methods can be found in the data supplement.

In Vitro Studies

Neutrophil migration and adhesion was assessed using peripheral blood neutrophils from healthy volunteers. Migration was conducted using a CXCL8 chemotactic gradient, as previously described (19). Adhesion of neutrophils to human microvascular endothelial lung cells was determined after stimulation with TNF and the addition of RTD-1–pretreated, calcein-AM–labeled neutrophils. Adhesion was measured by fluorescence. Alveolar macrophage (murine MH-S) and lung epithelial (human A549) cell inflammation was assessed after LPS stimulation. MH-S cells are a continuous cell line of murine alveolar macrophages, which were established after transformation of cells obtained by BAL from Balb/c mice with simian virus 40. Supernatant was collected after 24 hours and cytokines were analyzed by ELISA.

In Vivo Studies

Several experiments were performed in groups of mice to assess the pharmacokinetics, efficacy, and safety of RTD-1 in a murine model of LPS-induced ALI. ALI was induced by intranasal instillation of LPS in BALB/c mice as previously described (16, 17, 19–22). Efficacy was determined in a dose-ranging study, where mice received RTD-1 at 0, 0.2, 1, 5, or 25 mg/kg 0.5 hour before LPS challenge ($n = 6$/group). Mice were killed 24 hours after LPS administration and BAL was performed and total and differential cell counts were determined via hemocytometer (Hausser Scientific). Lung edema was assessed by total protein concentrations in BAL fluid (BALF) using the Bradford assay (17). Multiple analyte levels in BALF were determined using multiplex ELISA kits. Total matrix metalloproteinase activity was determined using a fluorogenic substrate. Peroxidase (POD) was measured by tetramethylbenzidine, as previously described (23).

In an exploratory time course study, mice ($n = 3$/group) received 5 or 25 mg/kg RTD-1 0.5 hour before LPS challenge and were killed after 0.5, 1, 3, 7, 24, 48, and 72 hours. BAL was performed for total and differential cells counts and cytokine concentrations, as described previously here. To determine the efficacy of RTD-1 after the onset of ALI, we performed an intervention study in which 25 mg/kg RTD-1 was administered at time zero (T0) or 12 hours (T12) after LPS-induced inflammation. Comparatively, 2 mg/kg dexamethasone was administered at T0. Mice were killed ($n = 9$/group) 48 hours after LPS administration and BAL was performed for total and differential cell counts and cytokine concentrations as described previously here.

Lung histology was performed in mice after PBS sham, or LPS challenge and treatment with 25 mg/kg RTD-1, or no treatment ($n = 3$/group). Lung injury was scored as recommended previously (17, 24).

To determine the effect of RTD-1 on inflammatory gene expression, mice ($n = 3$)

were challenged with LPS and killed after 3 hours. RNA was extracted from lung homogenate and differential gene expression was determined by RT-PCR.

Pharmacokinetics was performed in LPS-challenged mice receiving a single dose of 5 or 25 mg/kg RTD-1 subcutaneously. Plasma samples were obtained at serial time points ($n = 3$–7/group) and RTD-1 plasma concentrations were measured by liquid chromatography/mass spectrometry. Noncompartmental analysis was performed in Kinetica version 5.1 (Thermo Scientific).

Statistical Analysis

Statistical and graphical analyses were performed using STATA version 13 (STATA Corp.) and GraphPad Prism version 6.0 (GraphPad Software, Inc.). A significance level of P less than 0.05 was determined *a priori*.

Results

RTD-1 Inhibits Neutrophil Migration and Adhesion

Given the importance of airway neutrophilia in the pathogenesis of ALI, we began by testing the activity of RTD-1 using *in vitro* methods. First, we investigated the effect of RTD-1 on cell migration using human blood neutrophils in a transwell migration assay. RTD-1 treatment significantly reduced CXCL8-stimulated chemotaxis when compared with control with over 50% inhibition at 5 μg/ml (Figure 1A). As one possible mechanism is modification of neutrophil adhesion, we then investigated the effect of RTD-1 on neutrophils in an endothelial adhesion assay. Importantly, RTD-1 reduced neutrophil adhesion to human microvascular endothelial lung cells by over 50% at 5 μg/ml compared with untreated cells ($P < 0.01$) (Figure 1B). Representative images can be found in Figure 1C. The potent, specific Cell Adhesion Inhibitor Cyclic Peptide (SCP0111; Sigma) inhibited adhesion by greater than 85% at 100 μM ($P < 0.001$).

RTD-1 Dampens LPS-induced Alveolar Macrophage Inflammation, but Has Little Effect on Epithelial Cells *In Vitro*

To determine the effect of RTD-1 on LPS-induced alveolar macrophage inflammation, we used an *in vitro* model using MH-S cells and profiled the macrophage-relevant cytokines, macrophage inflammatory protein (MIP)-2, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein (MCP)-1, IL-6, and TNF. Treatment with RTD-1 resulted in roughly 5-, 3-, 154-, and 4-fold reductions in MIP-2 ($P < 0.0001$), MCP-1 ($P < 0.01$), TNF ($P < 0.001$), and IL-6 ($P < 0.001$), respectively, when compared with LPS-stimulated MH-S cells alone (Figures 2A–2E). There was no difference in KC levels between groups ($P > 0.05$) (Figure 2B). Given the attenuation of TNF at 100 μg/ml RTD-1, we performed a dose–response experiment. The data were best described by a sigmoidal maximum effect model with a half-maximal inhibitory concentration of 4.5 μg/ml (95% confidence interval = 3.99–5.13; $R^2 = 0.99$; Figure 2F).

To evaluate the effect of RTD-1 on epithelial inflammation, we analyzed cytokine concentrations from LPS-stimulated A549 cells. Overall, basal and LPS-stimulated cytokine expression was significantly reduced in the A549 (pg/ml) when compared with the MH-S cells (ng/ml). RTD-1 did not significantly affect IL-8, MIP-2, IFN-γ, TNF, IL-12, or IL-1β expression (Table E1). Unexpectedly, RTD-1 induced a doubling (1.07-fold increase) of IL-6 in LPS-stimulated A549 cells. No cytotoxicity was observed by the alamar blue method (data not shown), which is in agreement with published *in vitro* data demonstrating limited observed erythrocyte hemolysis or fibroblast cytotoxicity under similar conditions (i.e., 10% serum and 100 μg/ml RTD-1 [10]).

RTD-1 Pharmacokinetics in Plasma

The mean plasma concentration versus time plot after single subcutaneous RTD-1 doses at 5 or 25 mg/kg in diseased mice are depicted in Figure E2a in the data supplement. The observed mean maximum RTD-1 concentrations of 342.5 and 1,169.9 ng/ml were achieved at 1 hour indicating rapid absorption. The disposition of RTD-1

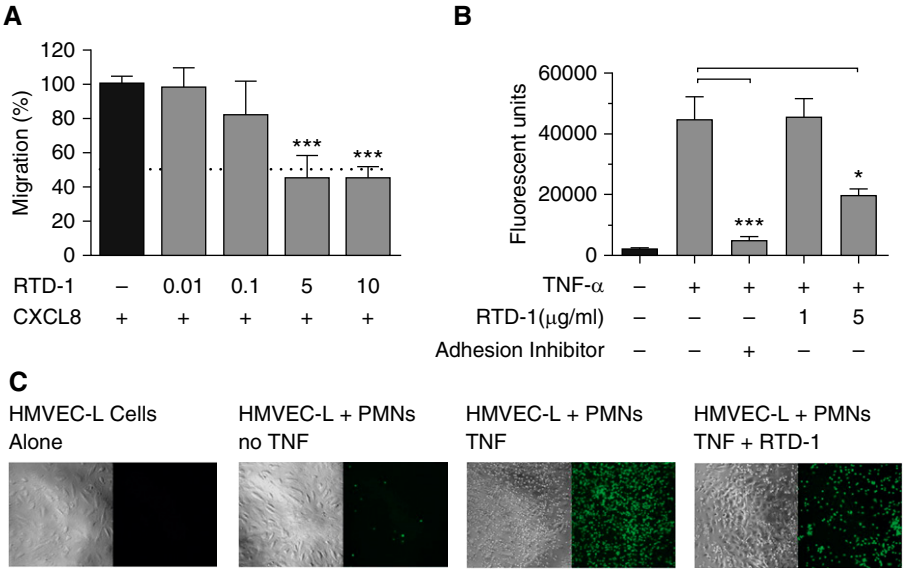

Figure 1. Dose-dependent effects of rhesus θ-defensin (RTD)-1 on neutrophil migration *in vitro*. To assess RTD-1 effects, peripheral blood neutrophils were isolated and (*A*) 4×10^5 cells in Rosewell Park Memorial Institute medium were pretreated with RTD-1 or PBS for 15 minutes and placed in the apical chamber of their respective transwells. Inserts were then placed in the presence of 15 ng/ml CXCL8 to induce chemotaxis. Cells in the basolateral chamber are expressed as percent normalized migrated cells; $n = 3$ per group; mean (±SD). Horizontal dashed line indicates 50% migration. Treatment compared with control by one-way ANOVA. Neutrophil–endothelial cell adhesion was assessed by exposing endothelial cells to 100 ng/ml TNF before incubation with drug-treated neutrophil. Adhesion was assessed by measuring fluorescence from the calcein-AM–labeled neutrophils (*B* and *C*); $n = 3$ per group (technical replicates) taken from two experiments; mean (±SD). Representative images from a third experiment (*C*). Magnification ×10. Treatment compared with control by unpaired two samples *t* test. $^*P < 0.05$, $^{***}P < 0.001$; HMVEC-L = human microvascular endothelial lung cells; PMN = polymorphonuclear leukocyte.

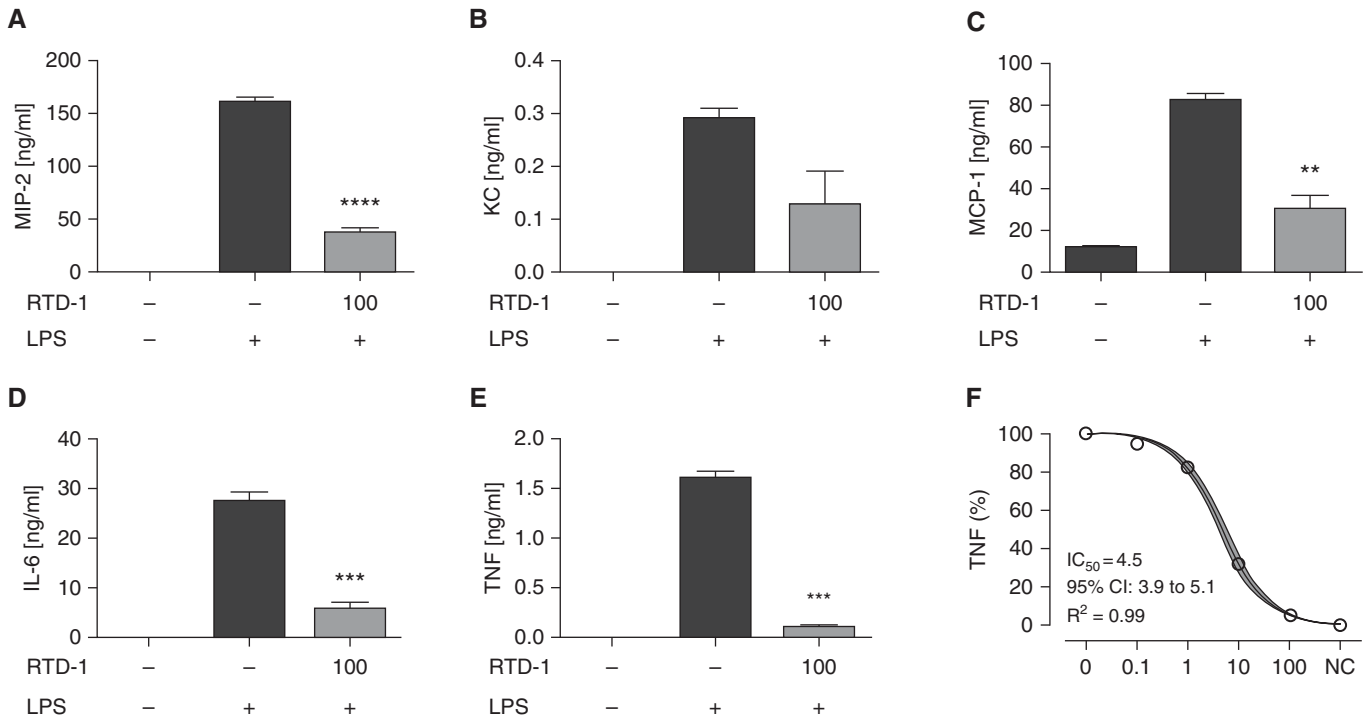

Figure 2. Dose-dependent effects of RTD-1 in LPS-induced alveolar macrophage inflammation *in vitro*. To assess the effects of RTD-1, cell culture supernatant from 24-hour 100 ng/ml LPS-induced MH-S alveolar macrophage cells (1.25×10^5) in the presence or absence of 100 μg/ml RTD-1 was analyzed for soluble cytokines, macrophage inflammatory protein (MIP)-2 (*A*), keratinocyte-derived chemokine (KC) (*B*), monocyte chemoattractant protein (MCP)-1 (*C*), IL-6 (*D*), and TNF (*E*); $n = 3$ per group; mean (±SD). Treatment compared with control by unpaired two-sample *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. In a separate investigation, supernatant from 24-hour–stimulated cells in the presence of 0, 0.1, 1, 10, and 100 μg/ml RTD-1 were analyzed for TNF dose response (*F*); $n = 5$ per group; mean (±SD). CI = confidence interval; IC_{50} = half-maximal inhibitory concentration; NC = negative control.

appears to be biphasic with a short distribution phase followed by a prolonged elimination phase with an apparent terminal half-life of around 30 hours. A less than proportional increase in the maximum concentration (3.4-fold) and area under the curve (2.3-fold) was observed when comparing the disposition between the 5- and 25-mg/kg doses (Figure E2B). Airway concentrations were detectable but not quantifiable by our assay methods.

RTD-1 Reduces Airway Neutrophil Burden and Pulmonary Edema

After LPS exposure at 24 hours, total inflammatory cell counts in BALF were increased approximately 32-fold ($P < 0.0001$) in LPS-untreated control mice compared with sham mice. Cellular differentials confirmed that neutrophils were the main infiltrative leukocyte (>90% of total cells; Figures 3A and 3B). No mortality was observed in the disease model. RTD-1 treatment resulted in a significant decrease in neutrophils in RTD-1–treated mice at doses of 5 mg/kg ($P < 0.01$) and 25 mg/kg ($P < 0.001$). Treatment with RTD-1 was not associated with any increases in airway macrophages when compared with LPS-untreated mice ($P > 0.05$) (Figure 3B).

Airway neutrophil activation status was measured by cell-free BALF POD activity and global MMP activity. RTD-1 diminished airway POD (Figure 3C) and MMP-9 activity (Figure 3D) in a dose-dependent manner in the 5-mg/kg and 25-mg/kg treatment groups when compared with LPS control mice ($P < 0.01$). A strong positive association was observed between the total neutrophil count and POD activity ($r = 0.87$; $P < 0.05$; Figure E1A), or total neutrophil count and global MMP activity ($r = 0.78$; $P < 0.05$; Figure E1B). Total protein concentrations in BALF were quantified to assess disease severity and, more specifically, alveolar–capillary barrier leakage induced by LPS. BALF protein levels were increased 3.8-fold ($P < 0.001$) after LPS challenge in untreated mice compared with sham mice. Compared with LPS-untreated mice, RTD-1 treatment resulted in significant reductions in protein levels in the 1-mg/kg ($P < 0.05$), 5-mg/kg ($P < 0.05$), and 25-mg/kg ($P < 0.001$) RTD-1 groups (Figure 3E).

RTD-1 Inhibits LPS-induced Inflammatory Cytokines/Chemokines

Given the observed effects of RTD-1 on lung neutrophilia, 24-hour airway levels of proinflammatory cytokines were measured from BALF to further evaluate immunomodulatory activity (Figures 4A–4C). TNF, IL-1α, IL-1β, IL-6, MCP-1, KC, MIP-1α, and MIP-1β were elevated in response to LPS challenge in mice compared with sham controls ($P < 0.0001$; Table E2). RTD-1 treatment at doses of 5 and 25 mg/kg achieved significant reductions in IL-1β ($P < 0.05$) and TNF ($P < 0.01$) when compared with LPS untreated mice, respectively (Figures 4A and 4B). In addition, RTD-1 at the highest treatment dose resulted in significant reductions in IL-6 ($P < 0.05$; Figure 4C). No treatment differences were observed in the levels of IL-1α, KC, MCP-1, MIP-1α, or

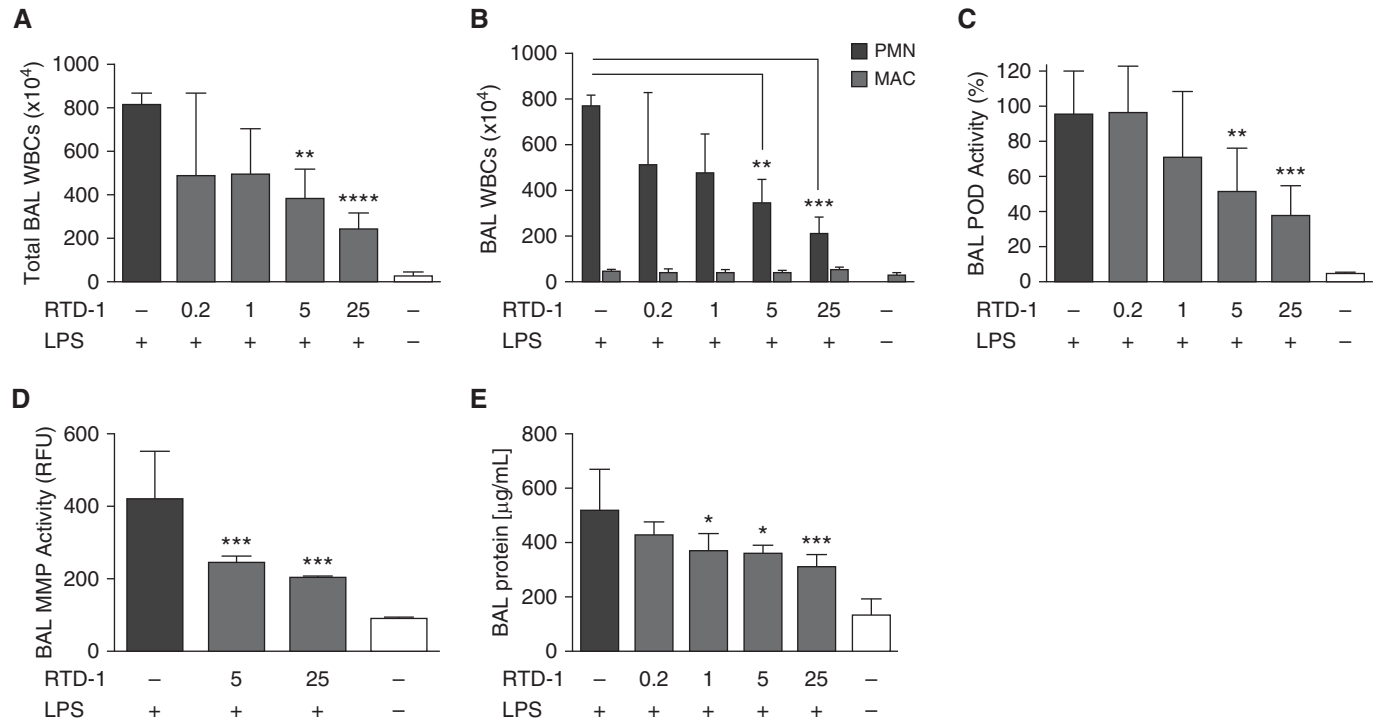

Figure 3. Dose effects of RTD-1 on airway neutrophilia, neutrophil activation, and microvascular disruption. To assess the dose response of RTD-1, mice received subcutaneous pretreatment with 0, 0.2, 1, 5, or 25 μg/ml RTD-1 0.5 hour before 1 μg intranasal LPS instillation. BAL fluid (BALF) was evaluated for (*A*) total, (*B*) differential, (*C*) peroxidase (POD) activity, (*D*) global matrix metalloproteinase (MMP) activity, and (*E*) total protein; $n = 6$ mice per group; mean (±95% confidence interval); treatment compared with control by one-way ANOVA with the Bonferroni post test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. MAC = macrophages; RFU = relative fluorescent units; WBCs = white blood cells.

MIP-1β when compared with LPS-untreated controls (Table E2).

RTD-1 Dampens Early Inflammatory Responses in the Lung

Given the promising *in vivo* treatment effect described previously here, we performed an exploratory study in LPS-challenged mice to evaluate the time course of the antiinflammatory activity of RTD-1. Led by *in vitro* findings, we evaluated TNF, IL-1β, IL-6, KC, and endogenous antiinflammatory soluble receptors. In addition, MIP-2 was evaluated due to its primary production in the resident macrophage and role in neutrophil migration to the lung space (25, 26). Comparison of cytokine concentration–time profiles demonstrated RTD-1 dose-dependently reduced cumulative airway cytokine exposure of MIP-2 ($P < 0.01$), KC ($P < 0.001$), TNF ($P < 0.001$), and IL-1β ($P < 0.01$) compared with LPS-untreated controls (Figures 5A–5D and Table E3). This reduction in cumulative exposure was largely attributed to an early (3 h) attenuation of these markers. No treatment effects were observed for IL-6, or the soluble receptor antagonists profiled (Table E3).

Next, we evaluated inflammatory cell infiltration into the airspace. Treatment with

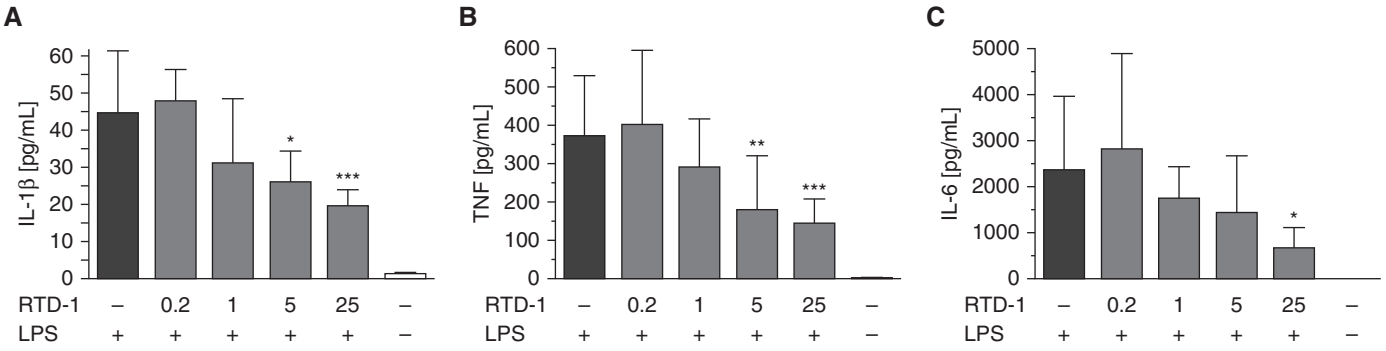

Figure 4. Dose effects of RTD-1 on airway cytokines and chemokines. To assess the dose response of RTD-1, mice received pretreatment with 0, 0.2, 1, 5, or 25 mg/kg RTD-1 0.5 hour before 1-μg intranasal LPS instillation. BAL fluid was obtained at 24 hours. Effect of RTD-1 on cytokines (*A*) IL-1β, (*B*) TNF, and (*C*) IL-6 are shown; $n = 6$ mice per group; geometric mean (±95% CI). Treatment compared with control by one-way ANOVA with the Bonferroni post test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

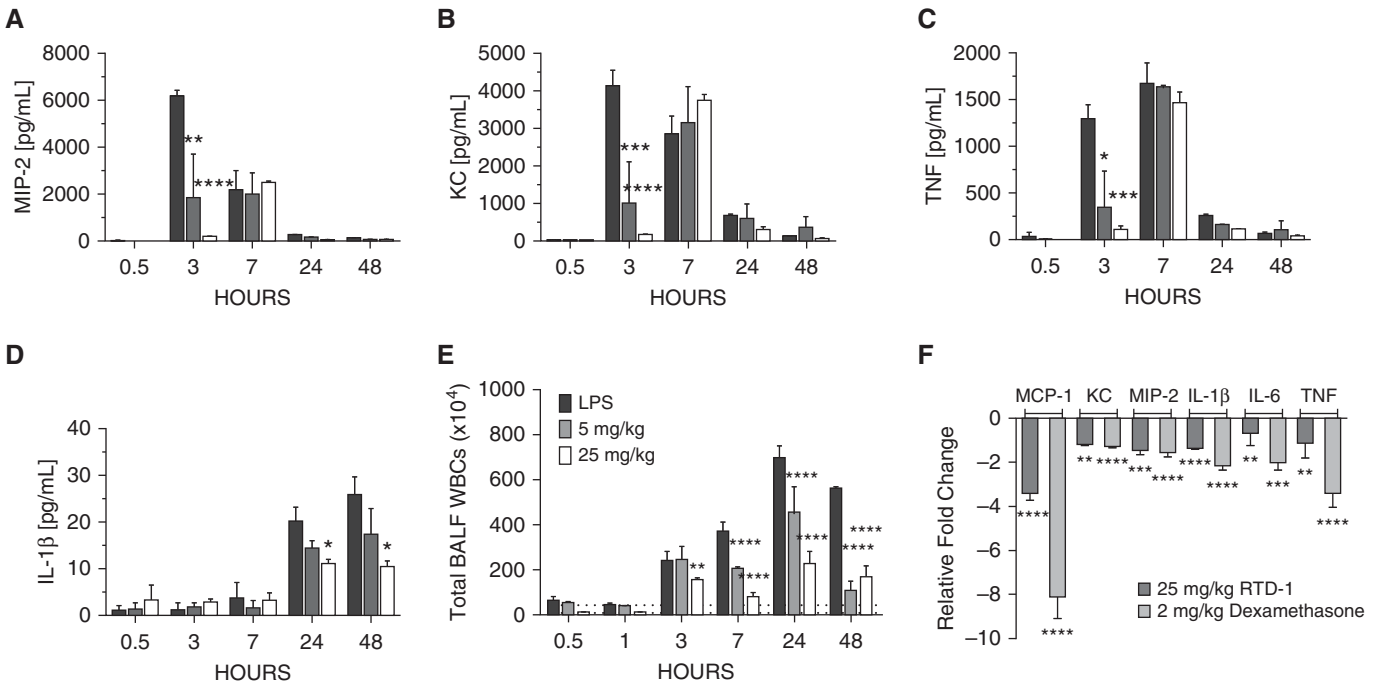

Figure 5. Dose- and time-dependent effects of RTD-1 on airway neutrophilia, cytokine proteins, and gene expression. A time course study was conducted to assess the direct effects of RTD-1s on airway neutrophils and immune mediators. RTD-1 at 0, 5, or 25 mg/kg was administered 0.5 hour before 1 μg intranasal LPS. BALF was obtained at the times noted for MIP-2 (*A*), KC (*B*), TNF (*C*), IL-1β (*D*), and total cell counts (*E*) ($n = 3$ mice per group; mean [±SD]). In a separate set of mice, changes in 3-hour lung homogenate cytokine mRNA levels were evaluated (*F*) ($n = 3$ mice per group; mean [±SD]). Treatment compared with control by two-way ANOVA with the Bonferroni post test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RTD-1 resulted in a significant dose-dependent decrease in airway leukocytes in response to LPS starting as early as 3 hours. The cumulative exposure of neutrophils to the airways was significantly reduced after RTD-1 treatment at doses of 5 and 25 mg/kg compared with LPS-untreated mice ($P < 0.0001$; Figure 5E and Table E3).

To investigate whether these actions could be explained by RTD-1–regulated gene expression, we performed a study ($n = 6$/group) to assess mRNA levels between treated (RTD-1 25 mg/kg) and untreated groups 3 hours after endotoxin challenge. RTD-1 significantly reduced mRNA levels of TNF (−1.560 fold change [FC]; $P < 0.01$), IL-1β (−1.386 FC; $P < 0.0001$), IL-6 (−1.393 FC; $P < 0.01$), MCP-1 (−3.396 FC; $P < 0.0001$), MIP-2 (−1.496 FC; $P < 0.001$), and KC (−1.277 FC; $P < 0.01$) in mouse whole-lung homogenate compared with untreated controls (Figure 5F).

RTD-1 Exhibits Antiinflammatory Effects up to 12 Hours after LPS Insufflation

After the encouraging prophylactic *in vivo* data, we tested the interventional effects of 25 mg/kg RTD-1 administered subcutaneously at 0 and 12 hours after LPS insufflation. For comparison, we tested 2 mg/kg dexamethasone. One death occurred in the RTD-1 T0 group 12 hours after LPS insufflation, which was likely a surgical complication. RTD-1 treatment showed a trend toward reducing neutrophil lung infiltration at T0 and significantly reduced lung neutrophilia at T12 ($P < 0.01$; Figure 6A). Dexamethasone also significantly reduced lung neutrophilia ($P < 0.05$). There was no statistically significant difference between the T0 and T12 cohorts (unpaired two-sample *t* test $P = 0.1360$). In addition, RTD-1 significantly reduced TNF (−0.32 FC; $P < 0.05$), IL-6 (−0.66 FC; $P < 0.001$), and inducible protein 10 kD (IP-10; −0.34 FC; $P < 0.001$) at T0 and TNF (−0.40 FC; $P < 0.01$), IL-1β (−0.43 FC; 0.01), IL-6 (−0.66 FC; 0.001), and IP-10 (−0.67 FC; $P < 0.001$) at T12 (Figures 6B–6F). MIP-2 and KC were not significantly changed by RTD-1 treatment (data not shown). Dexamethasone reduced IL-1β (−0.40 FC; $P < 0.05$), KC (−0.27 FC; $P < 0.05$; data not shown), and IP-10 (−0.50 FC; $P < 0.001$). These data show that RTD-1 treatment retains a protective effect when administered 12 hours after LPS insufflation.

RTD-1 Protects against LPS-induced ALI

Considering the maximal immunomodulatory effects observed at the higher dose, we selected the 25-mg/kg RTD-1 dose to evaluate disease severity at 24 hours as measured by histology and lung pathology scoring. We compared the presence of congestion, interstitial and alveolar leukocytes, and alveolar hyaline membranes between RTD-1–treated, LPS-untreated, and sham mice (Figure 7). RTD-1 exhibited a protective effect against LPS-induced lung injury, as evidenced by the lack of alveolar leukocyte infiltrates, absence of hyaline membranes, and normal alveolar wall thickness (Figures 7A–7I). A comparison of standardized lung injury scores demonstrated a significant reduction in lung injury in RTD-1–treated animals when compared with controls ($P < 0.01$; Figure 7J).

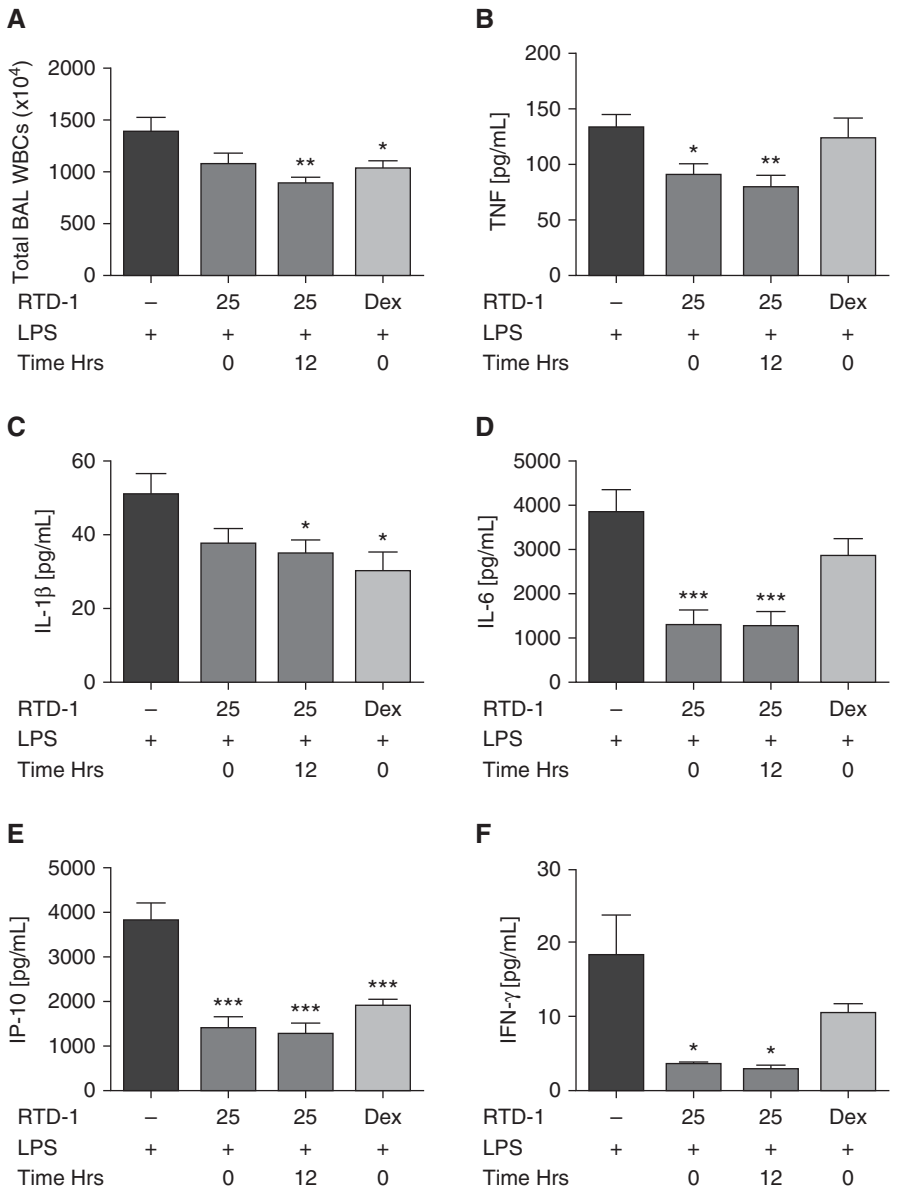

Figure 6. Time-dependent effect of RTD-1 on airway neutrophilia and cytokines. To assess timing of drug administration, 25 mg/kg RTD-1 was administered at 0 or 12 hours after 1-μg LPS instillation. As a comparator agent, 2 mg/kg dexamethasone (Dex) was given at 0 hour. LPS mice administered saline served as control. (*A*) BALF WBCs were quantified by manual counting. BALF concentrations of TNF (*B*), IL-1β (*C*), IL-6 (*D*), inducible protein 10 kD (IP-10) (*E*), and IFN-γ (*F*) were quantified by multiplex ELISA; $n = 9$ mice per group (except $n = 8$ for RTD-1 at 0 h); mean (±SD). Treatment compared with control by unpaired two-sided *t* test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Discussion

ALI is a disorder of acute inflammation characterized by loss of alveolar–capillary membrane integrity, excessive neutrophil infiltration, release of proinflammatory mediators, and pulmonary edema (2). Lung infections and/or sepsis are frequently the underlying cause of ALI. Although antibiotics remain the primary treatment, limited therapies are currently available for directly targeting the inflammatory response in ALI. Cationic antimicrobial peptides participate in bacterial killing, as well as initiation and resolution of acute inflammatory responses (27, 28). Several cationic antimicrobial peptides have demonstrated efficacy in murine models of sepsis-induced ALI (29–32). The dual antimicrobial and immunomodulatory actions of cationic antimicrobial peptides make them attractive drug candidates for treatment of ALI. However, host toxicity profiles and pharmacokinetic challenges have hampered the clinical development of earlier cationic antimicrobial peptides (28).

θ-defensins are macrocyclic cationic peptides with antimicrobial and immunomodulatory activities. Although initially studied for their antimicrobial action, recent investigations indicate that attenuation of the inflammatory response plays a significant role in the therapeutic benefit in murine models of severe acute respiratory syndrome and sepsis (13, 15). However, the effects of RTD-1 in a model of "sterile inflammation" and direct lung injury have not previously been investigated. In the current investigation, we present the first evidence that the θ-defensin, RTD-1, yields protective effects in mitigating ALI by reducing proinflammatory cytokines, neutrophil infiltration, alveolar–capillary membrane leakage, and lung injury.

After a direct insult (i.e., infection or trauma), cell-mediated amplification of proinflammatory cytokines occur in response to pathogens and/or ongoing cellular injury. Here, we used the well established bacterial endotoxin model to simulate the host lung immune response and ALI pathology. Upon intranasal LPS challenge, we found that RTD-1 treatment reduced the total airway exposure of several monocyte/macrophage–related cytokines (i.e., MIP-2, TNF, IL-1β) in a dose-dependent manner. In particular, we observed a distinct and early reduction (3 h) in soluble TNF and MIP-2. To see if these early observations could be explained by resident macrophage responses and/or lung epithelium, we conducted *in vitro* investigations with LPS-stimulated cells. These experiments revealed that RTD-1 treatment inhibited the release of soluble TNF, MIP-2, MCP-1, and IL-6 in alveolar but not epithelial cells. These data are consistent with published reports highlighting the importance of resident alveolar macrophages in mediating early inflammatory responses to LPS-induced ALI, and suggest the monocyte/macrophage as a primary cellular target of RTD-1 (33–35).

A fundamental feature of ALI is the vascular emigration and interstitial

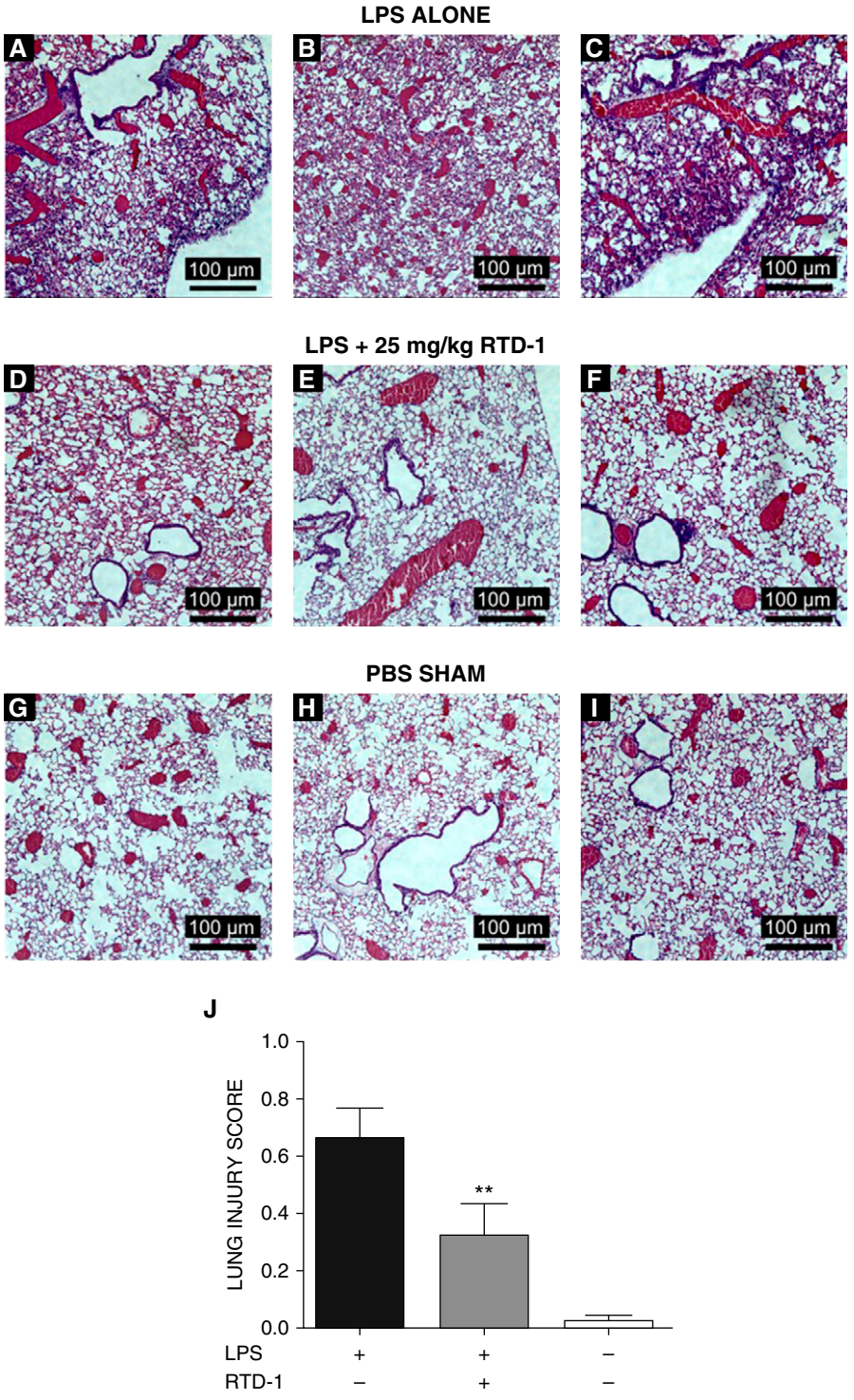

Figure 7. RTD-1 effects on severity of acute lung injury. To assess treatment effects on lung injury, mice received pretreatment with 0 or 25 mg/kg RTD-1 0.5 hour before 1-μg LPS insufflation. Lungs were formalin inflated and hematoxylin and eosin stained. Representative photomicrographs of each mouse and their respective treatment groups (*A–C*) LPS alone, (*D–F*) LPS + 25 mg/kg RTD-1, or (*G–I*) PBS sham are shown. (*J*) Pathological scoring of slides for lung injury and inflammation. Parameters included the presence of congestion, interstitial and alveolar leukocytes, and alveolar hyaline membranes; $n = 3$ mice per group; mean (±SD). Treatment compared with control by unpaired two-sample *t* test. Scale bars: 100 μm. Magnification ×10. $^{**}P < 0.01$.

migration of neutrophils into the lung compartment. After intranasal LPS challenge in mice, we found that RTD-1 dose-dependently reduced 24-hour airway neutrophil recruitment *in vivo*. The early treatment differences of airway neutrophils (3–7 h) compared with LPS control mice suggest that RTD-1 may directly inhibit neutrophil emigration from the vasculature. Supporting this direct neutrophil blockade hypothesis are *in vitro* data demonstrating that RTD-1 inhibited human CXCL8-dependent neutrophil chemotaxis. Additional *in vitro* data indicate the effect of RTD-1 on neutrophil chemotaxis may be due to inhibition of neutrophil cell adhesion. RTD-1 reduced neutrophil–endothelial cell adhesion by greater than 50% compared with the specific cell adhesion inhibitor cyclic peptide, which reduced adhesion by more than 85%. This new finding of antagonism of chemotaxis runs counter the general observation of host defense peptides known to induce migration (36). This is important, as KC and MIP-2 (murine homologs of human CXCL8) are pivotal in regulating migration and activation of neutrophils in murine LPS–induced pulmonary inflammation (37, 38). Consistent with these findings is the improved lung inflammation scores *in vivo* that were driven by reduced airway neutrophilia. Therefore, data suggest that the peripheral blood neutrophils may be another target of RTD-1, as systemic action on this cell population would reduce airway neutrophil burden.

Excessive and prolonged neutrophilia and the associated inflammatory products contribute to basement membrane destruction and increased permeability of the alveolar–capillary barrier. One measure of membrane permeability and pulmonary edema is the total protein concentration in BALF. RTD-1 treatment was associated with reduced pulmonary edema *in vivo*. Although active migration of neutrophils contribute to a "leaky" barrier, the release of proteolytic enzymes (neutrophil elastase, MMP-9), and reactive oxygen species further increase the permeability of the alveolar–capillary membrane and, thus, pulmonary edema (39). RTD-1 treatment reduced POD and MMP-9 activity in a dose-dependent manner *in vivo*. Given the observed strong positive association between neutrophil counts and their released products (POD or MMP activity), these dose-dependent effects are likely the result of

reduced neutrophil recruitment. However, we have previously demonstrated that RTD-1 is an inhibitor of MMP-9 activity *in vitro* (40). The antiinflammatory and ALI-protective effects were observed when RTD-1 was administered 12 hours after LPS insufflation supporting its potential as a treatment for ALI.

From investigations disclosed here, we postulate that multifunctional actions of RTD-1 on (*1*) early alveolar macrophage–driven inflammatory reactions and (*2*) blood neutrophil emigration and adhesion underlie the *in vivo* observation of reduced neutrophil lung recruitment from LPS challenge. The immunoregulatory action of RTD-1 is relatively unique in comparison with other cationic host defense peptides in which the antiinflammatory action is an indirect effect mediated by their endotoxin-neutralizing effects (13, 14). Recent work has elucidated several modes by which RTD-1 may exert these effects. Work in macrophage/monocyte cells demonstrates inhibition of NF-κB translocation and proinflammatory effects through PI3K/AKT pathway activation (14). RTD-1 was recently shown to inhibit NF-κB translocation through induction of phosphorylated Akt, which acts as a negative regulator of NF-κB and mitogen-activated protein kinase (14). In addition, RTD-1 inhibits IκBα degradation and p38 mitogen-activated protein kinase phosphorylation in macrophage/monocyte cells (14). This is consistent with our *in vivo* gene expression data suggesting that RTD-1 modulates TLR signaling activity and subsequently mRNA levels and soluble cytokines. Published data demonstrating fast temporal inhibition (4 h) of soluble TNF in macrophages suggest that RTD-1 is likely multiregulatory (13, 14). Ongoing mechanistic studies in our laboratories are underway to understand the multiregulatory actions of this peptide at the molecular level.

Pharmacokinetic studies designed to characterize the time course of drug concentrations in blood are key to identifying the optimal dose to maximize efficacy and safety of the compound. Examination of the plasma concentration time curves revealed several observations. First, the peak *in vivo* concentrations after single-dose subcutaneous administration were below the *in vitro* half-maximal inhibitory concentrations (e.g., neutrophil chemotaxis and inflammatory cytokines from MHS cells) indicating the potential for greater antiinflammatory activity with improved drug delivery. Second, the area under the plasma concentration curve did not increase in proportion to the dose when comparing between the 5- and 25-mg/kg doses, suggesting saturable absorption from the injection site. This observation explains the less than proportional increase in antiinflammatory activity at the higher dose, and is consistent with unpublished data from our group demonstrating low absolute bioavailability of RTD-1 in rodents. Lastly, taking into account the relatively long plasma half-life observed here, as well as previously disclosed *in vitro* plasma stability, a prolonged treatment effect is possible (13).

There are several areas worthy of future exploration. First, although LPS-induced ALI is a recommended model for testing potential therapeutics, we did not explore the utility of RTD-1 in treatment of ALI induced by other causes (e.g., acid aspiration). Second, we have investigated part of the potential mechanism of action through which RTD-1 is affecting neutrophil chemotaxis. Future studies evaluating the RTD-1 effect on specific adhesion molecules and cytoskeleton components would be informative. Finally, pharmacokinetic observations suggest that additional work on drug formulation is warranted to optimize treatment effects.

In conclusion, data described here demonstrate that the θ-defensin RTD-1 exhibited antiinflammatory effects comparable to 2 mg/kg dexamethasone in a murine model of LPS-induced ALI, and retains its protective effects 12 hours after LPS administration. *In vitro* findings suggest that RTD-1 reduced *in vivo* lung injury and airway neutrophilia by directly inhibiting neutrophil migration and attenuating keystone responses of the resident alveolar macrophages. Observations disclosed here suggest that this dual antiinflammatory and antimicrobial peptide may provide a potentially new therapeutic approach to the treatment of ALI, especially given its promising early preclinical safety profile (13). ■

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