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Inhibition of Neutrophil Exocytosis Ameliorates Acute Lung Injury in Rats

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Abstract

Exocytosis of neutrophil granules contributes to acute lung injury (ALI) induced by infection or inflammation, suggesting that inhibition of neutrophil exocytosis *in vivo* could be a viable therapeutic strategy. This study was conducted to determine the effect of a cell-permeable fusion protein that inhibits neutrophil exocytosis (TAT-SNAP-23) on ALI using an immune complex deposition model in rats. The effect of inhibition of neutrophil exocytosis by intravenous administration of TAT-SNAP-23 on ALI was assessed by albumin leakage, neutrophil infiltration, lung histology, and proteomic analysis of bronchoalveolar lavage fluid (BALf). Administration of TAT-SNAP-23, but not TAT-Control, significantly reduced albumin leakage, total protein levels in the BALf, and intra-alveolar edema and hemorrhage. Evidence that TAT-SNAP-23 inhibits neutrophil exocytosis included a reduction in plasma membrane CD18 expression by BALf neutrophils and a decrease in neutrophil granule proteins in BALf. Similar degree of neutrophil accumulation in the lungs and/or BALf suggests that TAT-SNAP-23 did not alter vascular endothelial cell function. Proteomic analysis of BALf revealed that components of the complement and coagulation pathways were significantly reduced in BALf from TAT-SNAP-23-treated animals. Our results indicate that administration of a TAT-fusion protein that inhibits neutrophil exocytosis reduces *in vivo* ALI. Targeting neutrophil exocytosis is a potential therapeutic strategy to ameliorate ALI.

Keywords

Complement; proteomics; immune complex

INTRODUCTION

Acute lung injury (ALI) is a life threatening complication associated with sepsis, pneumonia, aspiration, multiple blood transfusions, trauma, and acute pancreatitis. Despite improvements in management of critically ill patients, mortality remains about 40%, and

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survivors often do not return to a normal life (1). Neutrophils are the first immune cells to accumulate in the lung during ALI, recruited by a complex network of cytokines and chemokines produced by tissue macrophages (2). Evidence supporting a causative role for neutrophils in lung injury includes a correlation between the degree of neutrophil accumulation in bronchoalveolar lavage fluid (BALf) and the severity of ALI in humans, and a reduction in the severity of ALI in animal models following neutrophil depletion or inhibition of neutrophil accumulation in the lungs (3–7). The major systems by which neutrophils induce lung injury include the release of granule components, formation of neutrophil extracellular traps (NETs), and generation of reactive oxygen species (ROS) (2, 8–10). A number of neutrophil granule components, including serine proteases, matrix metalloproteinases, lactoferrin, LL-37, azurocidin, and defensins, have been shown to contribute to ALI (2) and direct evidence for a role of neutrophil exocytosis was provided by studies in which injection of granule products produced ALI (8).

Although a role for neutrophils in ALI is well established, strategies to control neutrophil activation to prevent tissue and organ injury have not followed. We recently developed a TAT-fusion protein containing a SNARE domain from SNAP-23 (TAT-SNAP-23) that inhibited secretory vesicle, gelatinase granule, and specific granule exocytosis in human neutrophils (11). Using that TAT-fusion protein we showed that inhibition of neutrophil exocytosis reduced phagocytosis-stimulated hydrogen peroxide production by 60% and inhibited tumor necrosis factor (TNF) α and platelet activating factor-induced priming of the respiratory burst by up to 80% (11). To determine if administration of TAT-SNAP-23 was able to inhibit inflammatory injury *in vivo*, the present study used a model of ALI induced by immune complex (IC) deposition in rats. That model is dependent on complement activation, cytokine production, and chemokine-induced neutrophil infiltration (12, 13). Our results show that intravenous administration of TAT-SNAP-23 inhibited the development of increased vascular permeability without affecting neutrophil accumulation in the lungs, and that effect was associated with reduced accumulation of complement components.

MATERIALS AND METHODS

Rodent Model of ALI

ALI was induced in pathogen-free male Long-Evans rats (275 to 300 g) as previously described (3–7). Briefly, rabbit polyclonal IgG anti-bovine serum albumin (BSA; 2.5 mg, MP-Biomedics, Solon, OH) was injected intratracheally in 0.3 ml of PBS, pH 7.4. Immediately thereafter, 10 mg of BSA (Sigma-Aldrich, St. Louis, MO) with 250 μ g of FITC-BSA (Invitrogen, Carlsbad, CA) in 0.5 ml of PBS was injected intravenously. Two h later, 0.05 mg/kg of TAT-SNAP-23 or a control TAT-fusion protein of similar molecular size (TAT-Control) (11) was injected intravenously. Animals were euthanized four h after initiation of injury. In separate studies intravenous administration of TAT-SNAP-23 or TAT-Control showed no side effects after 24 h. Animal care was performed following the National Institute of Health guidelines. Animal studies were approved by the University of Louisville Institutional Animal Care and Use Committee.

In Vitro Assays

BALf was collected by instilling and withdrawing 5 ml of sterile PBS three times via an intratracheal cannula. Lung injury was quantified by measuring leakage of FITC-BSA into lung parenchyma by fluorometry and by measuring the BALf albumin concentration. Cells were recovered from BALf by centrifugation and Wright-stained following a cytospin. A cell differential count was performed and the total number of neutrophils calculated. BALf was stored at -80°C for mass spectrometry analysis and measurement of cytokine/chemokine levels (Multi-Analyte ELISA array, SABiosciences, Frederick, MD). For

immunoblot analysis, BALf cells were lysed in 40 μ l of ice-cold lysis buffer as previously described (11, 14). Lysate or BALf proteins were separated by 4–12% gradient SDS-PAGE, followed by immunoblot analysis for His-tag (1:1000, Abcam, Cambridge, MA), C-reactive protein (CRP, Santa Cruz, Santa Cruz, CA, 1:1000), or complement C3 (1:1000, Pierce, Rockford, IL). Lung homogenates were separated by 4–12% gradient SDS-PAGE, followed by immunoblot analysis for caspase-3 (1:500, Cell Signaling Technology, Danvers, MA). Protein signal was visualized by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Plasma membrane expression of CD18 was measured in BAL cells by FITC-conjugated monoclonal anti-CD18 (Abcam, Cambridge, MA); secretory vesicle and specific granule exocytosis by isolated human neutrophils was determined by measuring the increase in plasma membrane expression of FITC-conjugated monoclonal anti-CD35 (secretory vesicles, clone E11; Pharmingen, San Diego, CA), and FITC-conjugated monoclonal anti-CD66b (specific granule, clone CLB-B 13.9; Accurate Chemical, Westbury, NY) in human neutrophils. All the plasma membrane markers were analyzed by flow cytometry as previously described (11, 14).

Human neutrophils were isolated from healthy donors using plasma-Percoll gradients as previously described (11, 14, 16). Wright staining performed on cytopspins of isolated cells confirmed that >95% of the cells were neutrophils. Trypan blue exclusion staining confirmed >97% of cells were viable. The Institutional Review Boards of the University of Louisville approved the use of human donors for the study.

Lung histology

In a separate group of rats, lungs were fixed by intratracheal instillation of 10% buffered formaldehyde. Sections of embedded lung were stained by hematoxylin and eosin. The extent and location of neutrophils in the lungs was determined by immunohistochemistry for myeloperoxidase (MPO) using a polyclonal anti-rat MPO antibody (1:500; Abcam, Cambridge, MA).

Mass spectrometry analysis of BALf

To achieve greater sensitivity for identification of low-abundance proteins, BALf supernatants were immunodepleted of seven high-abundance serum proteins (albumin, IgG, transferrin, fibrinogen, IgA, alpha-2 macroglobulin, IgM) using a ProteomeLab™ IgY R7 LC-2 column according to manufacturer guidelines. Proteins in the flow-through fractions were reduced, alkylated, and digested with trypsin, as previously described (14, 15). Tryptic peptides were separated by 2DLC, and MS/MS data generated by nanospray ionization into a LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific Waltham, MA). The acquired mass spectrometry data were searched against a rat refseq protein database modified to include bovine serum albumin using the SEQUEST (version 27 revision 11) algorithm, as described previously (14, 15). The database analysis was performed with SequestSorcerer (Sage-N Research, San Jose, CA) and high-probability peptide and protein identifications were assigned from the SEQUEST results using the ProteinProphet (tools.proteomecenter.org/software.php) and SageN Sorcerer statistical platforms. Scaffold 3 proteomic analysis software (ProteomeSoftware, Inc, Portland, OR) was used for quantitative comparison using a label-free spectral counting method (14, 15). Qualitative comparison of protein expression patterns was performed by Ingenuity Pathways Analysis software (<http://ingenuity.com>) (16).

Statistical analysis

All data are expressed as mean $\pm$ SEM. Statistical analysis was performed using ANOVA with the Tukey-Kramer multiple-comparison test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Administration of TAT-SNAP-23 ameliorates ALI

To determine an effective dose of TAT-SNAP-23, a preliminary study was performed in which 0.01 mg/kg, 0.05 mg/kg, or 0.1 mg/kg was injected intravenously 2 h after IC deposition in the lung. BALf was collected at 4 h and vascular permeability was measured by the amount of FITC-BSA fluorescence. Induction of ALI resulted in a 5-fold increase in fluorescence in BALf that was reduced by 54% following administration of 0.01 mg/kg of TAT-SNAP-23, by 57% with 0.05 mg/kg, and 35% with 0.1 mg/kg (data not shown). Based on those results, 0.05 mg/kg TAT-SNAP-23 was used in all subsequent experiments.

To determine the effect of inhibiting exocytosis with TAT-SNAP-23 on ALI, four groups of rats were used. A control group received intratracheal and intravenous saline, while the other 3 groups received anti-BSA intratracheally and BSA and FITC-labeled BSA intravenously. One group also received an intravenous injection of 0.05 mg/kg TAT-SNAP-23 and a second received 0.05 mg/kg TAT-Control 2 h after IC deposition. At 4 h rats were euthanized, and bronchoalveolar lavage was performed or lung tissue obtained for histology. Figure 1A shows the BALf fluorescence resulting from leakage of FITC-labeled BSA from blood into the lungs. As seen in the preliminary study, ALI was associated with a significant increase in BALf fluorescence that was significantly reduced following administration of TAT-SNAP-23, but not TAT-control. Figure 1B shows total protein in BALf from rats with ALI with and without treatment with TAT-Control or TAT-SNAP-23. Those results confirm that treatment with TAT-SNAP-23, but not TAT-Control, significantly reduced the increased vascular permeability associated with ALI. A differential cell count was performed in the BALf cells recovered from the four groups. The average number of neutrophils was very low in the control animals ($0.07 \times 10^6 \pm 0.02 \times 10^6$, n=8 animals). About 95% of the cells recovered from rats with ALI were neutrophils, as previously described (12, 13). ALI was associated with a significant increase in total neutrophil count ($5.2 \times 10^6 \pm 2 \times 10^6$, n=8 animals) that was not affected by treatment with TAT-SNAP-23 ($8 \times 10^6 \pm 2.8 \times 10^6$, n=8 animals) or TAT-Control ($9 \times 10^6 \pm 2.2 \times 10^6$, n=8 animals). No statistical difference was found between the number of cells recovered from BALf of ALI, or TAT-SNAP-23, or TAT-Control treated animals. Thus, TAT-SNAP-23 treatment significantly reduced the increased vascular permeability and total protein levels following IC deposition, while neutrophil infiltration was not affected.

Figure 1C shows representative lung histology from four groups. Rats administered saline showed a normal lung structure, while rats with untreated ALI demonstrated intraalveolar edema and hemorrhage and neutrophil infiltration. Treatment with TAT-SNAP-23 resulted in a reduction in lung edema and hemorrhage, although infiltrating neutrophils were still observed. Lung histology in rats treated with TAT-Control was similar to that seen in rats with untreated ALI. To confirm the observation that neutrophil infiltration was not impaired in TAT-SNAP-23 treated animals, immunohistochemistry for MPO was performed. Figure 1D shows that the number and location of neutrophils was similar with or without TAT-SNAP-23 treatment. Thus, histology supported the BALf findings that TAT-SNAP-23, but not TAT-control, inhibited the increased vascular permeability produced by IC deposition, while neutrophil migration into lungs was not affected.

To determine if neutrophils infiltrating the lungs contained TAT-fusion proteins, immunoblot analysis for the His-tag present in the TAT-fusion proteins was performed in cells contained in BALf. Figure 2 shows that cell lysates from rats treated with TAT-SNAP-23 and TAT-Control contained proteins at the appropriate molecular size that stained for the His-tag (11). As the majority of cells contained in BALf were neutrophils, we

interpret those results to indicate that TAT-fusion proteins entered circulating neutrophils that subsequently migrated into the lungs.

Effect of TAT-SNAP-23 on neutrophil exocytosis and generation of inflammatory stimuli

To determine if intravenous administration of TAT-SNAP-23 inhibited neutrophil exocytosis, plasma membrane expression of a granule marker CD18 ($\beta 2$ integrin) by BALf neutrophils was analyzed by flow cytometry. Table 1 shows that neutrophils from rats with ALI showed a significant increase in the plasma membrane expression of CD18, indicating that exocytosis had occurred in infiltrating neutrophils. BALf neutrophils obtained from animals with ALI that were also treated with TAT-SNAP-23, but not TAT-Control, showed a small but significant reduction in the plasma membrane expression of CD18 (Table 1).

Although the inhibitory effect of TAT-SNAP-23 on CD18 expression suggests that intravenous administration of TAT-SNAP-23 partially inhibited neutrophil exocytosis, other possibilities are that TAT-SNAP-23 treatment reduced expression of neutrophil stimuli in BALf. To determine if BALf from TAT-SNAP-23 treated animals contained stimuli of neutrophil exocytosis, *in vitro* degranulation assays were performed with isolated human neutrophils incubated with BALf from the four different animal groups. Secretory vesicle and specific granule exocytosis was determined by an increased plasma membrane expression of CD35 and CD66b, respectively. Figures 3A and 3B show that BALf obtained from rats with ALI with or without treatment with TAT-SNAP-23 or TAT-Control stimulated exocytosis equivalent to that stimulated by fMLF, while human neutrophils incubated with BALf from control animals showed no increase in CD35 and CD66b expression compared to basal levels. These data indicate that there are sufficient stimuli in the BALf of TAT-SNAP-23 treated animals to activate neutrophils; however those animals showed less severe tissue injury and reduced upregulation of CD18. *In vitro* degranulation assays using human neutrophils pre-treated with TAT-SNAP-23 followed by incubation with the different BALf showed inhibition of secretory vesicle and specific granule exocytosis (data not shown). No difference in TNF α , and IL-6 levels were observed in BALf from rats with ALI with or without treatment with TAT-SNAP-23 or TAT-Control (data not shown). No change in caspase-3 levels was observed in lung homogenates from rats with ALI with or without treatment with TAT-SNAP-23 or TAT-Control (data not shown). Taken together, the results suggest that administration of TAT-SNAP-23 resulted in amelioration of ALI by reducing neutrophil exocytosis, while generation of pro-inflammatory stimuli, apoptosis, and infiltration of neutrophils into the lungs was not affected.

Proteomic analysis of BALf

To examine potential mechanisms by which inhibition of neutrophil exocytosis reduced ALI, identification and quantification of proteins contained in BALf from rats with untreated ALI or ALI treated with TAT-SNAP-23 or TAT-Control was performed by mass spectrometry. A total of 117 proteins were identified from the three groups that had spectral counts greater than 1 (see Supplemental Table 1 which contains the accession number and spectral counts for all the proteins identified in the BALf). Those proteins were subjected to Ingenuity Pathways Knowledge Base (IPKB) analysis to assign functional and canonical pathways. Table 2 shows the seven functional and canonical pathways contained the largest number of identified proteins. This analysis indicates that a number of proteins present in BALf from rats with ALI are associated with pathways related to an immune or inflammatory response. Functional pathways included antigen presentation (23 proteins), humoral immune response (9 proteins), inflammatory response (30 proteins), and immune cell trafficking (23 proteins). Canonical pathways included the acute phase response (24 proteins), the complement system (12 proteins), and the coagulation system (6 proteins).

To determine if TAT-SNAP-23 treatment altered protein expression in BALf, the spectral counts of all 117 proteins were normalized to the total protein concentration. Of the 117 proteins, 78 (67%) proteins were reduced by at least 50% in the TAT-SNAP-23 treated rats, 32 proteins (28%) had similar spectral counts among the three groups, and 6 proteins (5%) showed at least a 50% increase in the spectral counts in TAT-SNAP-23 treated animals (see Supplemental Table 1). Of the 78 proteins reduced in BALf from TAT-SNAP-23 treated rats, 12 proteins were components of the complement cascade, 3 proteins components of the coagulation pathway, 4 were neutrophil granule proteins, and one was an acute phase protein (Table 3). Validation of the proteomic data was performed by immunoblot analysis for two identified proteins, the acute phase protein C-reactive protein and the complement protein C3. As shown in Figure 4, a reduction of C-reactive protein (Fig 4A) and C3 (Fig 4B) was observed in BALf from TAT-SNAP-23 treated animals compared to TAT-Control or untreated ALI.

DISCUSSION

The major finding of the present study is that *in vivo* administration of a novel fusion protein containing a SNARE domain from SNAP-23 resulted in amelioration of injury in a neutrophil-dependent ALI rat model. Using a well characterized rat model of neutrophil-dependent ALI induced by alveolar deposition of IC (3–7, 12, 13, 17–19), intravenous administration of that fusion protein inhibited the development of lung injury, as measured by a reduction in albumin leakage and by reduced alveolar hemorrhage and edema on histologic examination. Additionally, proteomic analysis of BALf suggested that one mechanism by which inhibition of neutrophil exocytosis reduced ALI is via reduced activation of the complement and coagulation cascades. The data suggest that targeting SNARE protein-mediated exocytosis may be an effective therapeutic strategy for neutrophil-mediated diseases.

SNARE proteins mediate exocytosis in a variety of cell types, including neuronal cells, leukocytes, endothelial and epithelial cells (20, 21). We recently showed that incubation of isolated human neutrophils with a novel TAT-fusion protein containing the SNARE domain of SNAP-23 significantly inhibited stimulated exocytosis of secretory vesicles, gelatinase granules, and specific granules, but not azurophilic granules (11). That inhibition of exocytosis was associated with a reduction in phagocytosis-stimulated respiratory burst activity and with inhibition of TNF α - and platelet activating factor-induced priming of fMLF-stimulated superoxide release. Based on the ability of TAT-SNAP-23 to bind SNARE protein complexes *in vitro*, the mechanism of inhibition of exocytosis was proposed to be interference with SNARE protein coiled-coil formation (11). The current study provides evidence that TAT-SNAP-23 inhibited neutrophil exocytosis *in vivo*. Neutrophils obtained from BALf of TAT-SNAP-23 treated rats contained TAT-SNAP-23 and showed reduced plasma membrane expression of CD18. The increase in plasma membrane expression of CD18 in stimulated neutrophils results from exocytosis of gelatinase and specific granules (22, 23). Additionally, quantitative proteomic analysis of BALf found reduced levels of several neutrophil granule proteins, including myeloperoxidase, collagenase, and lysozyme.

As SNARE proteins are involved in protein trafficking in all cells, the possibility that TAT-SNAP-23 inhibited ALI through an action on cells other than neutrophils was considered. N-ethylmaleimide-sensitive factor (NSF), like SNAP-23, is another molecule involved in vesicular trafficking (21). Morrel et al. showed that intravenous administration of a TAT-fusion protein containing an inhibitory peptide derived from NSF inhibited vascular endothelial cell exocytosis and blocked neutrophil infiltration into the peritoneum in a mouse model of inflammatory peritonitis (24). If TAT-SNAP-23 acted similarly, reduced neutrophil recruitment into the lungs would be predicted. The presence of similar numbers

of neutrophils in the lungs and BALf of TAT-SNAP-23 treated and untreated animals in our study argues against an effect of TAT-SNAP-23 on vascular endothelial cell exocytosis. Additionally, the presence of similar levels of cytokines, including TNF α and IL-6, in BALf of all three groups of rats with alveolar IC deposition indicates that administration of TAT-SNAP-23 had no effect on cytokine production and release by resident macrophages, lung parenchymal cells, and infiltrating neutrophils. *In vitro* studies performed with isolated human neutrophils exposed to BALf from all three groups of rats showed that the reduced plasma membrane expression of CD18 in rat neutrophils derived from BALf was not due to the absence of adequate pro-inflammatory stimuli. Taken together, our data suggest that TAT-SNAP-23 inhibits development of ALI by blocking exocytosis of neutrophils that have infiltrated into the lung in response to the inflammatory response initiated by immune complex deposition.

The proteomic analysis of BALf identified complement and coagulation proteins, as well as C-reactive protein, and the neutrophil-derived proteins myeloperoxidase, collagenase, gelatinase, and vitamin-D binding protein in rats with ALI. Quantitative analysis found that a number of proteins in the complement and coagulation systems were reduced in BALf from TAT-SNAP-23-treated animals. Complement activation is necessary for neutrophil recruitment and IC-induced ALI in the rodent model used in this study (3–7, 17–19, 25). Complement activation and generation of C5a results in activation of alveolar macrophages stimulating the release of pro-inflammatory cytokines such as TNF- α , IL-1 and chemokines such as MIP-2 and KC (12, 13, 19). Additionally, C5a and the membrane attack complex (C5b-9) are involved in up-regulation of endothelial cell adhesion molecules which participate in neutrophil migration into the lung parenchyma (17). This pro-inflammatory environment results in enhanced neutrophil recruitment and activation leading to tissue damage. It has been shown that intratracheal administration of a neutralizing antibody to C5a or inducing the IC-ALI in a C5 knockout mice resulted in attenuation of neutrophil infiltration and amelioration of lung injury (26–28). Our data show that blocking neutrophil exocytosis by administration of TAT-SNAP-23 results in reduction of complement components and amelioration of lung injury. Previous reports showed that intra-tracheal injection of neutrophil granule components produced ALI (8) and that specific granule contents, including serine proteases, matrix metalloproteinases, LL-37, lactoferrin, defensins, and azurocidin, may contribute to ALI (2). An interaction of those granule components with the complement cascade has not been examined. Our findings suggest the novel hypothesis that neutrophil exocytosis participates in ALI through release of granule components that, among other actions, regulate complement activation. Neutrophils and alveolar macrophages cleave C5 to generate active C5a, and serine proteases participate in C5a generation (29, 30). Our findings suggest that inhibition of neutrophil exocytosis not only limits the release of granule components capable of directly inducing tissue injury, but also prevents activation of an amplification loop which results from activation of the complement cascade.

The ability of BALf from both treated and untreated groups to stimulate equal neutrophil exocytosis *in vitro* indicates IC deposition results in the generation of equivalent amounts of neutrophil stimuli. We have reported previously that TNF α is a potent stimulus of neutrophil exocytosis (11). The presence of substantial amounts of TNF α in TAT-SNAP-23-treated animals supports the conclusion that TAT-SNAP-23 inhibited ALI by blocking neutrophil exocytosis. Although the mechanism by which exocytosis contributed to complement activation was not examined in our study, Camous et al. recently described a novel mechanism by which the alternative complement pathway is activated on the surface of neutrophils (31). Neutrophils primed by TNF α and stimulated by fMLF demonstrated increased plasma membrane expression of properdin released from granules, which activated the alternative complement pathway resulting in plasma membrane deposition of

C3. We reported previously that properdin is a component of neutrophil specific granules (22). Ritis et al. reported that activation of neutrophil C5a receptors triggers activation of the extrinsic coagulation cascade via release of tissue factor (32). It has been shown that high levels of C5a result in upregulation of factor H, a key complement inhibitor, suggesting that C5a controls its own generation to maintain homeostasis (33). Flierl et al. reported that neutralization of C5a *via* systemic infusion of anti-C5a resulted in reduction of factor H, and amelioration of trauma-induced ALI (34). Similarly, in the present study inhibition of neutrophil exocytosis resulted in a reduction in both C5 and factor H and amelioration of IC-induced ALI. Thus, neutrophil degranulation might contribute to complement activation leading to tissue damage through a mechanism that remains to be determined.

A proteomic study performed on BALf of patients with acute respiratory distress syndrome (ARDS) found higher levels of complement components C3, C4 and C9 compared to normal subjects (35). In the same study, BALf from ARDS patients showed increasing expression of C3 as the disease progressed (35). Additionally, this study showed increased S100 proteins (S100 A8 and S100 A9) at Day 1 in BALf of ARDS patients compared to control subjects. We reported previously that S100 A8 and S100 A9 proteins are components of neutrophil specific and gelatinase granules (22). Those reports and our data support a pathophysiologically relevant link between neutrophil exocytosis and activation of the complement and coagulation cascade following IC deposition. Understanding the mechanisms involved in regulation of neutrophil exocytosis may lead to new therapeutic strategies to improve the outcome of ALI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Lung injured animals treated with TAT-SNAP-23 showed amelioration of lung injury without altering neutrophil infiltration into the lungs

(A) Fluorescence of FITC-BSA was measured in BALf samples from 4 animal groups: control, lung injury (LI), lung injury followed by TAT-SNAP-23 (LI + TAT-SNAP-23), or lung injury followed by TAT-Control (LI + TAT-Control). Data were expressed as mean relative fluorescent units $\pm$ SEM, $n = 6$ animals per group. (B) Total protein was measured in BALf samples from 3 animal groups: lung injury (LI), lung injury followed by TAT-SNAP-23 (LI + TAT-SNAP-23), or lung injury followed by TAT-Control (LI + TAT-Control). Data were expressed as mean $\pm$ SEM, $n = 4$ animals per group. (C) Hematoxylin & Eosin (H&E) staining was performed on lung sections from 4 animal groups: control, lung injury, lung injury followed by TAT-SNAP-23, or lung injury followed by TAT-Control;

original magnification 10 $\times$ and 100 $\times$. (D) Immunohistochemistry for myeloperoxidase (MPO) was performed on lung sections from lung injured animals, and lung injured treated with TAT-SNAP-23; original magnification 40 $\times$ and 100 $\times$.

Figure 2. BAL cells have TAT-SNAP-23 or TAT-Control

BAL cells collected from 3 animal groups: lung injury (LI), lung injury treated with TAT-SNAP-23 (LI + TAT-SNAP-23), or lung injury followed by TAT-Control (LI + TAT-Control), were lysed and proteins were separated by SDS-PAGE followed by immunoblot analysis for His-tag. A representative immunoblot from three different and independent experiments is shown.

Figure 3. Effect of BALF from TAT-SNAP-23 treated animals' on human neutrophil exocytosis Human neutrophils (4×10^6 cell/ml) were incubated with buffer (Basal), stimulated with fMLF (300 nM, 5 min), or with BALF (1:100 dilution, 30 min) from 4 animal groups: control, lung injury (LI), lung injury followed by TAT-SNAP-23 (LI + TAT-SNAP-23), or lung injury followed by TAT-Control (LI + TAT-Control). (A), exocytosis of secretory vesicles was measured as the increase in plasma membrane expression of CD35. (B), exocytosis of specific granule was measured as the increase in plasma membrane expression of CD66b. Data are expressed as mean $\pm$ SEM of the mean channel fluorescence (MCF) for 7 independent experiments.

Figure 4. Less expression of CRP and C3 protein in the BALf of TAT-SNAP-23 treated animals
Immunoblot for C-reactive protein (CRP), and complement component 3 (C3) was performed on the BALf from 4 animal groups: control, lung injury (LI), lung injury followed by TAT-SNAP-23 (LI + TAT-SNAP-23), or lung injury followed by TAT-Control (LI + TAT-Control). Proteins present in the BALf were separated by SDS-PAGE and the immunoblot analysis performed with anti-CRP (A), or anti-C3 (B).

Table 1

Administration of TAT-SNAP-23 reduced CD18 plasma membrane expression in BAL cells.

	CD18MCF (mean $\pm$ SEM)	N=number of rats
Control	305 $\pm$ 37 **	5
IC-ALI	611 $\pm$ 20	7
TAT-SNAP-23-IC-ALI	562 $\pm$ 16 *	9
TAT-Control-IC-ALI	619 $\pm$ 12	9

*
p<0.05 compared to TAT-Control;

**
p<0.001 compared to IC-ALI

Table 2Distribution of proteins by functional and canonical pathways^a

Function	# of proteins	Canonical	# of proteins
Antigen presentation	23	Acute phase response signaling	24
Humoral immune response	9	Complement system	9
Inflammatory response	30	Coagulation system	4
Cellular movement	24	Neuroprotective role of THOP1 in Alzheimer's disease	4
Hematological system development and function	26	Hepatic fibrosis/hepatic stellate cell activation	4
Immune cell trafficking	23	LXR/RXR activation	3
Cell-to-cell signaling and interaction	23	Intrinsic prothrombin activation pathway	2

^aProteins were categorized by functional and canonical pathways based on Ingenuity analysis.

Table 3

Administration of TAT-SNAP-23 resulted in a reduction in BALf proteins identified by proteomic analysis.

Identified Proteins	Accession Number	Spectral counts normalized to total protein		
		LI	LI + TAT-SNAP-23	LI + TAT-Control
<i>Classical Complement Pathway Proteins</i>				
Complement C3	gi 158138561	434.63	160.9	387.7
Complement factor H	gi 77861917	56.23	22.03	43.76
Complement factor B	gi 218156285	51.5	19.3	44.83
Complement C9	gi 16924006	29.6	12.03	27.76
Complement C4 precursor	gi 158186621	33.63	12.5	25.2
Predicted: Complement C5	gi 109468076	15.23	6.2	11.26
Complement factor I precursor	gi 13162353	13.03	4.6	13.4
Complement C8 alpha chain	gi 157821013	8	3.63	8.13
Complement C8 gamma chain	gi 157817131	5.06	2.7	5.13
Complement C6 precursor	gi 28570180	4	2.56	7.86
Complement factor D precursor	gi 117647198	3.36	1.93	4.06
Complement C4, gene 2	gi 50657362	22.16	7.1	15.03
<i>Coagulation Factors</i>				
Plasminogen	gi 16758216	129	56.06	132.1
Prothrombin	gi 161333847	15.56	8.33	14.83
Coagulation factor XII	gi 62078741	4.4	2.73	5
<i>Acute Phase Proteins</i>				
C-reactive protein precursor	gi 8393197	60.43	32.7	65.76
<i>Neutrophil Granule Proteins</i>				
Myeloperoxidase	gi 157820285	43.6	22.36	31.33
Lysozyme C-1 precursor	gi 40254796	52.26	30.46	53.63
Neutrophil collagenase precursor	gi 11560008	4.16	2	7.2
Vitamin D-binding protein	gi 6978879	109.2	47.7	111.5

Note: LI= Lung Injury