RAGE and the innate immune response in Infection and Inflammation

van Zoelen, M.A.D.

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Neutrophil-derived S100A12 in acute lung injury and respiratory distress syndrome

Helmut Wittkowski\textsuperscript{1,2,5}, Anne Sturrock\textsuperscript{3}, Marieke A. D. van Zoelen\textsuperscript{4}, Dorothee Viemann\textsuperscript{1,2,5}, Tom van der Poll\textsuperscript{4,6}, John R. Hoidal\textsuperscript{3}, Johannes Roth\textsuperscript{1,2,5} and Dirk Foell\textsuperscript{1,2,5}

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\textsuperscript{1}Department of Pediatrics and \textsuperscript{2}Interdisciplinary Center of Clinical Research, University of Muenster, Germany
\textsuperscript{3}Division of Respiratory, Critical Care and Occupational Pulmonary Medicine, University of Utah, Salt Lake City, UT
\textsuperscript{4}Center for Infection and Immunity Amsterdam (CINIMA) and Center for Experimental and Molecular Medicine (CEMM), Academic Medical Center, University of Amsterdam, The Netherlands
\textsuperscript{5}Institute of Experimental Dermatology, University of Muenster, Germany
\textsuperscript{6}Department of Tropical Medicine and AIDS, Academic Medical Center, University of Amsterdam, The Netherlands
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Abstract

Objective
Both persistent accumulation and activation of neutrophils may contribute to the most severe form of acute lung injury (ALI), acute respiratory distress syndrome (ARDS). We analyzed the expression of neutrophil-derived S100A12 and the pro-inflammatory receptor for advanced glycation end products (RAGE) in patients suffering from ARDS. Additional in vivo and in vitro experiments were performed to further analyze the contribution of S100A12 to pulmonary inflammation.

Subjects
We included 14 patients with ARDS and 8 controls. In addition, 16 healthy subjects were included into an experimental LPS challenge model.

Interventions
Concentrations of S100A12 and soluble RAGE (sRAGE) were analyzed in bronchial alveolar lavage fluids (BALF). The expression of S100A12 and RAGE in lung biopsies from patients was analyzed by immunohistochemistry. S100A12 was also analyzed in BALF from 8 healthy subjects following challenge with lipopolysaccharide (LPS) and compared to 8 controls inhaling placebo. Effects of S100A12 on endothelial cells were analyzed in vitro.

Main Results
Patients with ARDS had significantly enhanced pulmonary S100A12 expression and higher S100A12 protein concentrations in BALF than controls. Levels of sRAGE were not significantly elevated in ARDS. S100A12 concentrations decreased with time from disease onset. In healthy volunteers, S100A12 was elevated in BALF after LPS inhalation. In vitro experiments confirmed strong pro-inflammatory effects of human S100A12.

Conclusions
S100A12 and its receptor RAGE are found at high concentrations in pulmonary tissue and BALF in ALI. S100A12 expression may reflect neutrophil activation during lung inflammation and contribute to pulmonary inflammation and endothelial activation via binding to RAGE.
**INTRODUCTION**

Acute lung injury (ALI) is a complex and devastating illness, often occurring in the setting of sepsis and trauma. ALI and its most severe consequence, acute respiratory distress syndrome (ARDS), are characterized by life-threatening congestion, atelectasis, and pulmonary edema [1]. Inflammatory mechanisms involving activation of endothelial cells and infiltration of leukocytes play a major role in the pathogenesis of ARDS. Neutrophils accumulate at sites of lung injury and mediate inflammation via production of proteases, oxidants, and cytokines [2]. In addition to these classical inflammatory mediators novel families of pro-inflammatory molecules have been identified which contribute to immune dysregulation and tissue damage. Some of these molecules, also called damage associated molecular pattern molecules (DAMPs), have been proven to be critically involved in acute and chronic inflammation, including sepsis and ALI [3-5].

DAMPs share characteristics of cytokines; they mediate systemic inflammatory responses after release by activated or necrotic cells, and they function as danger signals to recruit inflammatory cells to sites of tissue damage via receptors like the receptor for advanced glycation end products (RAGE)[3,6,7]. RAGE is expressed at high levels in the lung, and its expression is upregulated under inflammatory conditions [8-10]. The interaction of specific ligands with RAGE leads to activation of pro-inflammatory signaling cascades. RAGE knockout mice were recently reported to be resistant to septic shock, suggesting that RAGE potentially plays a role in systemic acute inflammation [11].

S100A12, also known as EN-RAGE (extracellular newly identified ligand of RAGE), has been described as a prototypic RAGE-ligand which provokes pro-inflammatory responses in endothelial cells and leukocytes [7]. It is a calcium-binding protein expressed in the cytoplasm of neutrophils [12]. Neutrophils migrate rapidly to the lungs in response to endotoxemia or hemorrhage and secrete S100A12 [13,14] as well as tumor necrosis factor alpha (TNFα) and interleukin-8 (IL-8) [15,16]. Thus, multiple positive feedback loops with other cytokines may amplify S100A12-mediated inflammatory processes. Blocking the interaction of S100A12 and RAGE resulted in improvement of inflammation in multiple experimental models [6,7,11]. In addition, S100A12 has been described as a biomarker of neutrophil activation in inflammatory diseases including sepsis and pulmonary infections [17,18]. Since neutrophil activation is a hallmark of ALI, we hypothesized that S100A12 may trigger innate immune processes during ARDS. We therefore analyzed S100A12 and RAGE in patients suffering from ARDS and in subjects challenged with endotoxin. In addition, we performed in vitro experiments to analyze the contribution of S100A12 to pulmonary inflammation. For this purpose, we studied the effects of S100A12 on endothelium as a key player in ALI.
Materials and methods

Subjects in human studies
Bronchoalveolar lavage fluid (BALF) was obtained from 14 patients with ARDS and 8 healthy controls at the University of Utah. Patient characteristics are summarized in Table 1. Eleven patients had a primary diagnosis of pneumonia while 3 developed ARDS as a complication of peritonitis. The study was approved by the institutional ethics committee of the University of Utah. For the LPS in vivo challenge, 8 healthy non-smoking males (age 23.2 ± 0.6 years) were recruited as described before [19]. Ethical approval was obtained at the University of Amsterdam. Informed consent was obtained from each subject prior to the study.

Bronchoalveolar lavage (BAL)
BAL was performed according to the guidelines of the American Thoracic Society, using a flexible fiberoptic videobronchoscope. Eight successive 20 ml aliquots of prewarmed 0.9% NaCl were instilled in a subsegment of the right middle lobe and each aspirated immediately with low suction. Average return of fluid was 52 ± 16% (83 ± 24 ml; range 55-105 ml). BALF was immediately centrifuged and supernatants stored at –80 °C.

Table 1. Characteristics of ARDS patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>ARDS survivors</th>
<th>ARDS non-survivors</th>
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<tbody>
<tr>
<td>Subjects</td>
<td></td>
<td></td>
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<tr>
<td>N</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>37 (29-48)</td>
<td>47 (32-68)</td>
<td>71 (55-83) *</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/f</td>
<td>7/1</td>
<td>1/7</td>
<td>3/3</td>
</tr>
<tr>
<td>Days of ARDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>7</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>BAL Cells x 10^3/ml</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>median (range)</td>
<td></td>
<td>200 (34-580)</td>
<td>290 (60-625)</td>
</tr>
<tr>
<td>S100A12 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (SEM)</td>
<td>13 (3)</td>
<td>69 (31) *</td>
<td>99 (51) *</td>
</tr>
<tr>
<td>sRAGE pg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (SEM)</td>
<td>2,160 (510)</td>
<td>3,405 (1,280)</td>
<td>4,240 (1,660)</td>
</tr>
</tbody>
</table>

* p<0.01 controls vs. ARDS
Antibodies and reagents
Expression of recombinant human S100A12 and production of specific polyclonal affinity-purified rabbit antibodies were achieved as described [12,14,20]. The recombinant protein was purified with Talon metal affinity columns (Clontech, Palo Alto, CA, USA). Subsequently, the His-tag was cleaved from the protein by thrombin cleavage and capture (Novagen, Madison WI, USA). S100A12 stock solutions were essentially free of endotoxin, as tested by a limulus lysate assay (E-Toxate Reagent Kit, Sigma, Deisenhofen, Germany).

Monoclonal mouse anti-CD68 (Dako, Hamburg, Germany) and monoclonal mouse anti-CD15 (Dako, Hamburg, Germany) were used to detect leukocyte subsets. In addition, goat anti-RAGE polyclonal antibodies (Chemicon International, Hampshire, UK) were used.

Immunohistochemistry
Paraffin embedded lung biopsies were collected at the University of Utah. Sections were from healthy controls (n=3), early ARDS (≤7 days after onset; n=3), and late ARDS (≥7 days after onset; n=3). Staining on serial sections was performed to detect co-expression of CD68, CD15, and S100A12 in infiltrates as described before [21,22]. The sections were analyzed by 3 independent investigators (HW, AS, DF) who were blinded to the patient data. The expression of different markers was scored semi-quantitatively on a four point scale (0=no expression; 1=little expression with <10 positive cells per high power field and/or only sparse tissue staining; 2=moderate expression with 10-50 positive cells and/or diffuse tissue staining; 3=strong expression with >50 positive cells and/or extensive tissue staining).

Measurements of S100A12 and sRAGE
S100A12 concentrations were analyzed by an ELISA as described before [14]. Levels of sRAGE were analyzed using a sandwich ELISA kit according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA).

Inhalation of LPS by healthy subject
Healthy volunteers inhaled either normal saline (n=8) or 100 µg LPS (n=8) as described in detail before [19].

LPS induced stimulation of granulocytes
Granulocytes were purified and cultured as reported [14]. After stimulation with 1 µg/ml LPS, S100A12 RNA expression was analyzed in granulocytes by reverse transcription–polymerase chain reaction (RT-PCR), compared to unstimulated controls and normalized to the expression of GAPDH as described [23].
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Expression of pro-inflammatory genes by endothelial cells
Human microvascular endothelial cells (HMEC-1) were cultivated as previously reported and stimulated with either 10 ng/ml TNF or 20 µg/ml S100A12 for 4 hours [24]. RNA expression of vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) was analyzed by RT-PCR [23].

FACS analyses
FACS-analysis of ICAM-1 surface expression on HMEC-cells was performed after 16 hours stimulation with 20 µg/ml S100A12 as reported before [23].

Transendothelial resistance (TER) and transendothelial migration (TEM)
TEM assays in transwell plates were performed as described elsewhere [25]. Briefly, HMECs (2.2 x 10^5/well) were grown to confluence on fibronectin-coated 6.5 mm Transwell filters (5 µm pore size). IL-8 was added as chemoattractant in the lower chamber. Endothelial cells were preincubated with either 5 or 20 µg/ml S100A12 for 4 hours. Isolated human granulocytes were allowed to migrate for 4 hours after the stimulus-containing medium was replaced by fresh assay medium. The number of migrated cells was calculated by analyzing the cells in the lower chamber using a cell counter (Coulter Counter ZM; Beckman Coulter, Miami, FL, USA).

TER of HMEC cell layers treated with S100A12 or left untreated at 37 °C was measured using a voltohmmeter (World Precision Instruments, Sarasota, Florida, USA). TER of the fibronectin-coated filters was determined as control and subtracted from TER-values of filter-grown endothelial monolayers. TER was expressed as Ohm x cm^2 [23].

Statistical analyses
Values are expressed as mean ± SEM or mean and data range except otherwise stated. All analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL). Comparisons between groups of patients or subjects were based on Mann-Whitney U-test. Non-parametric Spearman-Rho test was used for calculating correlations of parameters without normal distribution. Comparisons between sets of in vitro experiments were based on Student’s t-test.
RESULTS

S100A12 and RAGE are extensively expressed in pulmonary tissue from ALI patients
The expression of S100A12 and RAGE were analyzed in lung biopsies from healthy controls and patients with inflammatory lung diseases. Extensive RAGE staining was found in non-inflamed tissue from healthy controls (Figure 1B). This staining resembled the broad RAGE expression pattern seen in inflamed lung tissue. In contrast, only scattered S100A12 expression in single granulocytes was found in normal lungs (Figure 1A). S100A12 staining was significantly enhanced in early ARDS, with S100A12-positive neutrophils infiltrating a thickened and cell-rich interstitial tissue and accumulating in the subepithelial layer (Figure 1C-F and 2A,B). On serial sections, S100A12-staining was restricted to CD15-positive granulocytes (not shown).

While S100A12 was differentially expressed, the counter-receptor RAGE was massively expressed in both healthy and inflamed lungs (Table 2). RAGE was mainly present on alveolar and bronchial epithelial cells, but also on endothelial cells and infiltrating mononuclear cells (Figure 2C,D). The expression pattern of RAGE was different from that of CD68, a marker of monocytes/macrophages (Figure 2E,F).

<table>
<thead>
<tr>
<th>Table 2. Expression of S100A12 and RAGE in lung biopsies</th>
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<tbody>
<tr>
<td>Normal Lung (n=3)</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td><strong>S100A12</strong></td>
</tr>
<tr>
<td>mean (range)</td>
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<tr>
<td><strong>RAGE</strong></td>
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<td>mean (range)</td>
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</table>

Microscopic score: 0=no expression; 1=little expression (<10 positive cells, only sparse tissue staining); 2=moderate expression (10-50 positive cells, diffuse tissue staining); 3=strong expression (>50 positive cells, extensive tissue staining)

S100A12 and sRAGE concentrations are elevated in BALF from ARDS patients
Next we analyzed concentrations of S100A12 and soluble RAGE in BALF of patients with ARDS and compared them to healthy controls. S100A12 levels found in BALF from patients with ARDS were significantly higher than in controls (82 ± 29 vs. 13 ± 3 ng/ml; p=0.002; Figure 3A). Patients who died from ARDS had higher BALF concentrations of
**Figure 1.** S100A12 and RAGE staining in lung biopsies from healthy controls and patients with ARDS. S100A12 expression was only found in single granulocytes in normal lungs (A). In contrast, there was extensive staining (in red color) for RAGE in normal lung tissue (B). S100A12 staining was more prominent in early stages of ARDS, with S100A12-positive neutrophils infiltrating a thickened and cell-rich interstitial tissue (C). S100A12-positive neutrophils were present in the subepithelial tissue (black arrow; D). Later stages of ARDS (E,F) were characterized by more severe tissue destruction. Bronchiectasis and loss of alveolar structures were a hallmark of late ARDS, as were interstitial fibrosis and fibrinous exsudates. S100A12-expression was less prominent in these stages compared to early ARDS. The substrate aminoethylcarbazole (AEC) was used for the red colour reaction. Sections were counterstained with Mayer’s haematoxylin. Scale bars indicate 100 µm.

**Figure 2.** Expression of S100A12, RAGE, and CD68 in early ARDS. S100A12 was stained in neutrophils, which were found in small vessels and also infiltrating the subepithelial layer. Extracellular staining indicated the presence of secreted S100A12 (A,B). RAGE was massively expressed in inflamed lungs. RAGE was mainly present on alveolar and bronchial epithelial cells as well as on endothelial cells. In inflamed lungs, RAGE was also expressed on infiltrating mononuclear cells (black arrows; C,D). The expression pattern of RAGE differed from that of CD68 (E,F). Scale bars indicate 100 µm.
S100A12 then those who survived (Table 1), but this difference was not significant in our small cohort. Patients with ARDS also had higher BALF concentrations of sRAGE than healthy controls (3,750 ± 990 vs. 2,160 ± 510 pg/ml; p=0.73). Unlike S100A12, the differences for RAGE between these groups did not reach statistical significance (Figure 3B). Rather than being linked to the outcome of patients, the S100A12 concentrations were associated with the disease phase of ARDS, with higher levels during early stages. There was no significant correlation of S100A12 to BALF cell counts (r=0.11; p=0.71). The concentrations of S100A12 correlated negatively to the days after onset, with highest levels within first week after onset (Figure 4). These findings confirm our observation from immunohistochemical analyses which revealed strongest expression of S100A12 in early ARDS, while in later stages S100A12-positive cells were less abundant.

Figure 3. S100A12 and sRAGE concentrations in BALF from ARDS patients compared to healthy controls. A) S100A12 levels found in BALF from patients with ARDS were significantly higher than in controls (**p<0.01). B) Patients with ARDS also had higher BALF concentrations of sRAGE than healthy controls, but this difference did not reach statistical significance.

Figure 4. Correlation of S100A12 in BALF to the duration of the disease. S100A12 correlated negatively with the number of days after onset, with highest levels within the first four days after onset (semi-logarithmic scale). The negative exponential regression analysis (black graph) reaches an asymptote at approximately day 6. The Spearman-Rho correlation of S100A12 to days after onset was r = -0.74 (p<0.01).
S100A12 concentrations increase in BALF from healthy subjects after challenge with LPS *in vivo*

Inhalation of 100 µg LPS or saline did not induce any clinical signs or symptoms and was not associated with significant changes in FVC and FEV1 (data not shown). LPS induced a significant increase of S100A12 in BALF after 6 hours (about 20-fold, Figure 5A) which is comparable to other cytokines [19]. Instead, sRAGE levels did not differ significantly between the groups (3,846 ± 950 vs. 3,328 ± 690 pg/ml).

![Figure 5. LPS induces an upregulation of S100A12 *in vivo and in vitro*. S100A12 concentrations increased in BALF from healthy subjects 6 hours after inhaling 100 µg LPS (A). S100A12 transcription was augmented in granulocytes upon stimulation with LPS (1 µg/ml) *in vitro*. This increased RNA expression of S100A12 was more pronounced than that of IL-8. It was a sustained response which reached a maximum after 12 hours (B). Data of 3 independent experiments represent n-fold increase compared to unstimulated controls and normalized to GAPDH expression (**p<0.01).](image)

**LPS induces an upregulation of S100A12 expression by human granulocytes *in vitro***

S100A12 transcription was augmented in granulocytes upon stimulation with LPS. This increased RNA expression of S100A12 was a sustained response which reached a maximum after 12 hours (Figure 5B). The induction of S100A12 RNA expression was more prominent than induction of IL-8.

**S100A12 induces a pro-inflammatory response in lung endothelial cells**

Finally, we analyzed the effects of S100A12 on endothelium *in vitro*. S100A12 increased PCR transcripts for VCAM-1, ICAM-1, IL-8, and MCP-1 in HMECs. The enhanced expression of adhesion molecules was confirmed on the protein level for ICAM-1 by FACS analyses of HMECs (Figure 6). S100A12 also reduced TER of HMEC monolayers. The
result is an enhanced TEM of granulocytes through the endothelial monolayer (Figure 7), which could also contribute to the pathophysiology of ALI.

**Figure 6.** Effects of S100A12 on endothelium in vitro. S100A12 stimulation (20 µg/ml) for 4 hours resulted in an increased RNA expression of VCAM-1, ICAM-1, IL-8, and MCP-1 on HMECs. Stimulation with TNF (10 ng/ml) served as a positive control. Relative gene expression was normalized to the endogenous housekeeping control gene GAPDH (A). The enhanced expression of ICAM-1 on HMECs after stimulation with S100A12 for 16 hours was confirmed by FACS analyses (B). Data are representative of 3 independent experiments.

**Figure 7.** S100A12 effects on endothelial integrity. Transendothelial resistance of HMEC monolayers grown to confluence on transwell filter membranes in a two-chamber model was reduced in response to S100A12 stimulation (A). As endothelial permeability as well as expression of chemokines and adhesion molecules are relevant for transendothelial migration of granulocytes and influenced by S100A12, the migration of granulocytes from the upper to the lower chamber is enhanced when the endothelium is treated with S100A12 (B). Data represent mean ± SEM of 3 independent experiments (**p<0.01).
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Discussion

High concentrations of the pro-inflammatory protein S100A12 have previously been found in serum of patients suffering from pneumonia, peritonitis, and sepsis [14,17]. Here we report elevated S100A12 concentrations in BALF during inflammatory processes in ALI, which are characterized by a diffuse alveolar damage emerging from severe injury to the alveolar-capillary unit. Activated neutrophils are especially involved in the exudative and proliferative phases of ALI, while the late fibrotic phase is rather characterized by chronic remodeling and fibrosis [26]. In experimental models of ALI, the neutrophils that accumulate in the lungs demonstrate increased activation of nuclear factor (NF)-κB and produce large amounts of pro-inflammatory cytokines [15,16,19]. Accordingly, we found massive neutrophil infiltration in early stages of ALI. Since activated neutrophils are known to secrete S100A12, there is also extracellular S100A12 disposition at sites of neutrophil extravasation [12-14]. This accumulation of S100A12 in inflamed tissue has previously been found in other diseases including infectious exacerbations of cystic fibrosis [14,17,27]. S100A12 contributes to inflammation via activation of RAGE which is abundantly expressed in the lung [7,28].

Endothelial cell injury within the lung is one of the earliest events in ARDS [29-31]. Several etiologies for endothelial cell damage have been implicated in the pathophysiology of ALI. Interestingly, fragments of soluble cadherins can be detected in the serum of patients with ARDS, suggesting disruption of endothelial cell junctions [32]. On the other hand, circulating endothelial progenitor cells are associated with endothelial repair [33]. The affected endothelial barrier function promotes neutrophil accumulation and pulmonary edema formation, both hallmarks of ALI [34]. Activated neutrophils contribute to these processes by releasing S100A12 which activates endothelial cells. The consequences include enhanced expression of adhesion molecules, production of chemokines, and down-regulation of endothelial junction proteins, all together leading to augmented transmigration of leukocytes from the vasculature into inflamed tissue [7].

Our findings of elevated S100A12 in pulmonary tissue and BAL fluid during early stages of ARDS support the hypothesis that this neutrophilic protein may contribute to some key elements of ALI. As a novel finding, we confirmed pro-inflammatory effects of human S100A12 on endothelial cells in vitro. The augmented expression of adhesion molecules and chemokines confirms previous findings obtained by stimulating endothelial cells with bovine S100A12 [7]. We now add evidence for effects of human S100A12 on endothelial layer integrity which has functional consequences on transendothelial resistance and migration.

A strong expression of RAGE in lungs has been noted by various observers. RAGE was indeed first purified from pulmonary tissue. However, there is some controversy about
the expression of RAGE on alveolar cell types I and II [28,35]. In our staining we found a broad expression in type I alveolar cells, lung endothelial cells, and infiltrating monocytes-macrophages. It is hypothesized that inflammatory stimuli, such as LPS, can increase transcription of RAGE via NF-κB resulting in increased expression of full length RAGE [36]. However, this mechanism cannot sufficiently explain the increase of the soluble isoform in the alveolar space. Possibly a certain equilibrium between membrane bound and soluble RAGE forms a regulatory system in innate immunity in which sRAGE may function as a decoy receptor for pro-inflammatory ligands like S100A12. There is evidence that, at least in the murine lung, soluble isoforms of RAGE are produced by carboxyl terminal truncation, not by alternative splicing [37]. Murine pulmonary epithelial cells shed the soluble isoform into the culture medium. Thus, proteolysis of full length RAGE may be a mechanism for the increase of sRAGE in the alveolar space [38].

In contrast to S100A12, we found RAGE also constitutively expressed in non-inflamed lungs, and sRAGE was not significantly increased in BALF from ARDS patients compared to healthy controls. On the other hand, elevated S100A12 levels in BALF correlate to the local S100A12 expression at different time points (Table 2). S100A12 is most dramatically elevated during early stages of ALI, both in affected tissue and in exudates detected in BALF. To test whether S100A12 was directly increased after acute lung injury, we analyzed S100A12 in BALF from subjects challenged with LPS. S100A12 levels significantly increased in response to LPS. Our in vitro experiments on LPS-induced S100A12 RNA expression confirm that activated granulocytes are also capable of upregulating S100A12 RNA transcription, thus leading to even more augmented S100A12 release at sites of pulmonary inflammation.

**Conclusions**

Taken together, neutrophil-derived S100A12 is strongly expressed during early ALI. The S100A12-receptor RAGE is abundant in lung tissue. S100A12 lung expression is most prominent in early stages of ARDS. Our findings indicate that S100A12 may contribute to early RAGE-mediated events of ALI characterized by activation of pulmonary endothelium, leukocyte extravasation, and neutrophil accumulation. Although our patient cohort is too small to draw definite conclusions on the usefulness of S100A12 as a marker of ALI, it is conceivable that this protein may be an indicator of inflammatory activity in BALF of patients with ARDS. While S100A12 and sRAGE represent a regulatory system, the differential expression of the pro-inflammatory ligand S100A12 seems to be more important than that of its receptor. The pro-inflammatory axis of S100A12 and RAGE could eventually serve as a target for future treatment strategies in ARDS.


