Experimental strategies directed at inflammation and coagulation in ARDS and TRALI
Tuinman, P.R.

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Damage-associated molecular patterns in transfusion-related acute lung injury

Marcella C.A. Müller
Pieter R. Tuinman
Alexander P. Vlaar
Anita M. Tuip
Kelly Maijoor
Achmed Achouiti
Cornelis van t Veer
Margreeth B. Vroom
Nicole P. Juffermans

Submitted for publication
Abstract

Background: Transfusion related acute lung injury (TRALI) has a high incidence in cardiac surgery patients and contributes to an adverse outcome. Damage-associated molecular pattern molecules (DAMPs) HMGB1 and S100A12, are thought to mediate inflammatory changes in ARDS. We aimed to determine whether DAMPs are involved in the pathogenesis of TRALI in cardiac surgery patients.

Methods: Secondary analysis of a trial in cardiac surgery patients; 14 TRALI cases were randomly matched with transfused and non-transfused controls. Pulmonary levels of HMGB1, S100A12 and sRAGE were determined.

Results: HMGB1 expression and levels of sRAGE in TRALI patients did not differ from controls. There was a trend towards higher S100A12 levels in TRALI patients compared to the controls. Furthermore, S100A12 levels were associated with increased levels of markers of pulmonary inflammation, prolonged cardiopulmonary bypass, hypoxemia and prolonged mechanical ventilation.

Conclusion: HMGB1 and sRAGE do not contribute to the development of TRALI. S100A12 is associated with prolonged duration of cardiopulmonary bypass, pulmonary inflammation, hypoxia and prolonged mechanical ventilation and may be involved in the priming phase of acute lung injury in cardiac surgery patients.
Introduction

Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion related morbidity and mortality. Incidence of TRALI is much higher in patients undergoing cardiac surgery compared to the general hospital population, significantly contributing to an adverse outcome [1]. TRALI is thought to result from the interaction of activated neutrophils with endothelial cells, thereby inducing endothelial damage, vascular leakage and pulmonary edema. However, precise pathways of TRALI are largely unknown.

The receptor for advanced glycation end products (RAGE), which is abundantly expressed in the lung, is pivotal in the pro-inflammatory response in acute lung injury. RAGE is a multi-ligand receptor expressed on type I alveolar cells, endothelium and neutrophils. Ligation of the RAGE receptor with damage associated molecular pattern molecules (DAMPs), including soluble RAGE (sRAGE), high-mobility group box 1 (HMGB1) and S100A12 (also known as EN-RAGE) induces NF-κB activation, leading to a pro-inflammatory response [2,3]. DAMPs have been shown to contribute to the inflammatory response in infectious conditions, but also in sterile inflammatory conditions such as acute pancreatitis, trauma and hemorrhagic shock [2,4].

In lung injury inflicted by mechanical ventilation or by trauma, increased levels of HMGB1 have been demonstrated and are associated with adverse outcome [5-8]. HMGB1 plays a role in neutrophil trafficking, as demonstrated by a study in which intratracheally instilled HMGB1 resulted in neutrophil accumulation, increased cytokine release and pulmonary edema [9]. Thereby, HMGB1 may play an important role in neutrophil mediated acute lung injury (ALI). S100A12 is thought to mediate the early phase of acute lung injury. In vitro experiments showed a direct pro-inflammatory effect of S100A12 on lung endothelial cells [10-12]. sRAGE is a marker of alveolar cell injury in ALI/ARDS [13] and increased levels have been demonstrated in ALI following trauma [14], injurious mechanical ventilation [15] and lung transplantation [16]. Interestingly, sRAGE levels were associated with both blood transfusion as well as with the use of cardiopulmonary bypass [16]. Of note, AGE formed in red blood cells was found to ligate to endothelial bound RAGE, resulting in endothelial damage [17]. Thereby, RAGE ligands may play a role in the neutrophil-endothelial interactions in the pathogenesis of TRALI.

We performed a secondary analysis of a previous clinical trial in cardiac surgery patients, pulmonary levels of S100A12, HMGB1 and soluble RAGE were compared between patients developing TRALI and transfused and non-transfused controls. Furthermore we correlated these pulmonary levels of DAMPs to markers of lung injury.
Materials and Methods

Setting
The study is a secondary analysis of a trial in cardiac surgery patients performed in 2006-2009 in a university hospital in the Netherlands [1]. With approval from the ethical committee, patients 18 years or older were asked written informed consent for participation in the study prior to valvular and/or coronary artery surgery. Exclusion criteria were off-pump surgery and emergency surgery.

Design
Patients were prospectively screened for the onset of TRALI up to 30 hours post surgery. Using the Canadian Consensus Conference definition [18], TRALI was defined as new onset hypoxemia or deterioration demonstrated by a PaO2/FiO2 < 300, occurring within 6 hours after transfusion, with bilateral pulmonary changes, in the absence of cardiac pulmonary edema [18-20]. Cardiogenic pulmonary edema was identified when pulmonary arterial occlusion pressure was >18 mmHg (during the study period all patients were peri-operatively monitored with a pulmonary artery catheter). Chest radiographs routinely taken before surgery and on arrival at the ICU were scored for the presence of new onset bilateral interstitial abnormalities by two independent physicians who were blinded to the predictor variables.

Sixteen TRALI cases were identified, of which non-directed bronchoalveolar lavage (NBL) fluid and plasma was available from 14 patients for analysis. TRALI cases were randomly matched with controls (transfused patients not developing ALI and patients not transfused not developing ALI, n=16 per group).

Cardiothoracic surgery, anesthesia procedures and ICU management
Patients were anesthetized according to local protocol, with lorazepam, etomidate, sufentanil and rocuronium for induction of anesthesia and sevoflurane plus propofol for maintenance of anesthesia. As part of standard care, a pulmonary artery catheter was inserted for peri-operative monitoring. In all patients, cardiopulmonary bypass was performed under mild to moderate hypothermia (28ºC-34ºC), using a membrane oxygenator and a non-pulsatile blood flow. During the procedure, lungs were deflated. After the procedure, all patients were transferred to the ICU with mechanical ventilation. The postoperative ICU protocol involved fluid infusions with normal saline and starch solutions and transfusion of leuko-depleted erythrocytes to maintain hemoglobin level above 8.5 g/dL. If indicated, norepinephrine was used to maintain a mean arterial blood pressure of 65 mmHg and dobutamine and/or milrinone were used to achieve a cardiac index of ≥2.5 L/min/m².
Patient data collection
Preoperative European System for Cardiac Operative Risk Evaluation (EuroSCORE), the physical status according American Society of Anesthesiologists (ASA score), predicted vital capacity and forced expiratory volume in 1 second were determined. Left ventricular function was categorized in good (ejection fraction (EF) >45%), moderate (EF<45% but >30%) or bad (EF ≤ 30%). Data on operation-time, clamp-time and time on cardiopulmonary bypass were recorded in the electronic patient data system. Furthermore, the duration of mechanical ventilation, partial pressure of oxygen in arterial blood were registered.

Plasma
Arterial blood samples, collected in EDTA tubes before cardiopulmonary bypass and after arrival at the ICU, were centrifuged at 1500 x g for 15 minutes at 4°C. The supernatant was stored at -80°C until sRAGE was measurement was performed.

Analysis in plasma
Levels of sRAGE were determined by an enzyme-linked immunosorbent assay developed in our laboratory [21]. In short, 96-well plates were coated overnight with mouse anti-human RAGE antibody (R&D systems, Minneapolis, Minnesota, USA). Samples diluted as appropriate were added and incubated for 2 hours. Next, biotinylated goat anti-human RAGE antibody (R&D Systems) was added and incubated for another 2 hours. Streptavidin poly-HRP was added for 30 minutes. Finally, sodium-acetate buffer (pH 5.5) containing 100 microg/ml tetramethylbenzidine and 0.003% H₂O₂ was added and the color reaction was stopped by 1 N H₂SO₄. All measurements were made in duplicate.

Non-directed bronchoalveolar lavage technique
At onset of TRALI, a non-directed bronchoalveolar lavage (NBL) was performed, controls were lavaged within 30 hours of ICU admission. No significant difference was observed concerning the timing of the bronchoalveolar lavage fluid (BALF) between the groups [1]. As described previously, a standard 50 cm, 14 gauge tracheal suction catheter was introduced via the endotracheal tube and advanced until significant resistance was encountered. Immediately after instillation of 20 ml of sterile 0.9% saline over 10-15 seconds, fluid was aspirated before withdrawal of the catheter. Generally, 4-8 ml of fluid was recovered. NBL samples were centrifuged at 1500 x g for 10 minutes at 4°C and stored at -80°C until assays were performed [22].

Analyses in NBL fluid
HMGB1 release was evaluated by Western blotting. BALF samples were mixed with 3-fold concentrated Sodium Dodecyl Sulphate (SDS) sample buffer containing 6% β-mercaptoethanol in a 2:1 ratio and denatured for 5 minutes at 95°C. Twenty μl of
Each sample was run on a 15% polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Pharmacia, Piscataway, NJ). Cell extracts of 293T cells were included as positive control. After blocking with 5% non-fat dry milk in phosphate-buffered saline + 0.05% Tween-20 (PBS-T), the membrane was incubated overnight at room temperature with a rabbit polyclonal antibody directed against human HMGB1 (ab18256, Abcam, Cambridge, MA) in PBS-T with 1% non-fat dry milk followed by secondary labeling with a HRP-labeled goat-anti-rabbit IgG polyclonal antibody (Bioké, Leiden, the Netherlands) in PBS-T with 1% non-fat dry milk. PVDF membranes were developed using Lumilight plus ECL substrate (Roche, Darmstadt, Germany) and a chemoluminescence detector with a cooled CCD camera (Syngene, Cambridge, UK). The density of HMGB1 bands at 35 kD were measured using AIDA image analysis software (Raytest, Straubenhardt, Germany). HMGB1 levels were compared to a standard curve prepared by serial dilution of 293T cell lysates which was run on the same gel with a lower detection limit of 500 cells. NBL fluid HMGB1 was then expressed in cell units (band densitometry correlated to the number of lysated cells).

Levels of sRAGE were determined by an enzyme-linked immunosorbent assay developed in our laboratory as described above [21].

Levels of S100A12 were analyzed by an ELISA (CircuLex™, Nagano, Japan). Diluted samples were added to a microplate pre-coated with a monoclonal antibody specific for S100A12/EN-RAGE and incubated for 1 hour. After washing, HRP conjugated anti-S100A12/EN-RAGE polyclonal antibody was added and incubated for 1 hour. Next, the substrate reagent tetra-methylbenzidine was added for 20 minutes. The reaction was stopped with 1 N H₂SO₄. All measurements were made in duplicate. Lower detection limit was 61 pg/ml.

As described previously, interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor α (TNFα) were measured using specific commercially available ELISAs, according to the instructions of the manufacturer (IL-1β, IL-6, IL-8 and TNFα from PeliKine-compact™ kit, Sanquin, Amsterdam, the Netherlands) [22,23].

**Statistical analysis**

Continuous data are expressed as mean and standard deviation (SD) or as medians and interquartile ranges (IQR) according to their distribution. Categorical variables are expressed as n (%). TRALI patients were compared with the control groups using one-way ANOVA and Dunnett post test for normally distributed data. Kruskall Wallis test and Mann-Whitney U test were used for non-normally distributed data. To compare categorical variables Chi-square test is used. Correlations were determined by Spearman’s Rho. Because of the small size of the individual groups (TRALI and controls), associations were determined for the total group of patients.

Statistical significance was defined as p<0.05. Statistical analysis was performed with SPSS 18.0.
Results

Of a 1000 evaluated patients who underwent cardiac surgery, 16 patients developed TRALI. Of these, samples from 14 patients were available for analysis. Transfused patients who did not develop lung injury and non-transfused patients (n=16 for both) served as controls. Patient characteristics are shown in table 1. Patients in the TRALI group were older, had higher ASA scores and lower FEV1 values prior to surgery when compared to controls. There were no differences in cardiac function, nor in other risk factors for ALI, alcohol abuse, smoking and myocardial infarction (data not shown). TRALI patients more often underwent combined bypass and valve replacement surgery compared to controls. Operation time and cardiopulmonary bypass time were significantly longer in TRALI patients compared to controls. Patients developing TRALI received more red blood cells, fresh frozen plasma and platelets compared to transfused controls. Cases had a lower PaO2/FiO2 ratio compared to controls as well as a longer duration of mechanical ventilation.

Table 1 Patient characteristics

<table>
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<th>Patient data</th>
<th>Non transfused</th>
<th>Transfused</th>
<th>P value</th>
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<td>No ALI n=16</td>
<td>No ALI n=16</td>
<td>TRALI n=14</td>
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<td>Age, years*</td>
<td>63±13</td>
<td>65±10</td>
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<td>Sex, male (%)</td>
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<td>81±19</td>
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<td>Valve replacement (%)</td>
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<td>Other (%)</td>
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<td>Pump time, min*</td>
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<td>PaO2/FiO2 ratio*</td>
<td>223±108</td>
<td>170±78</td>
<td>118±44</td>
</tr>
</tbody>
</table>

*normally distributed data, expressed as means ± standard deviation (SD)
#not normally distributed data, expressed as median and interquartile range (IQR)
NA: not applicable
Levels of cytokines in NBL fluid

Pulmonary levels of pro-inflammatory cytokines IL-6 and IL-8 in NBL fluid were elevated in TRALI patients compared to controls, as described previously [22]. Pulmonary levels of IL-1β did not differ among TRALI patients (29.5 (4.1-204.2) pg/ml) compared to transfused (14.9 (10.5-55.2) pg/ml) and non-transfused controls (10.1 (1.4-20.8) pg/ml), (median and IQR), (p=0.14). Median levels of TNF-α did not differ among the groups, with 29 (4-174) pg/ml in TRALI patients compared to 40 (13-72) pg/ml in transfused and 34 (13-44) pg/ml in non-transfused controls (p=0.29).

Levels of sRAGE in plasma and NBL fluid of cardiac surgery patients with TRALI and controls

In all patients, cardiac surgery resulted in a modest increase in plasma sRAGE levels compared to baseline values (figure 1). Following surgery, levels of sRAGE in NBL fluid were low, with 34.1 (14.7-135.9) pg/ml in TRALI patients, 97.1 (19.3-150.4) pg/ml in transfused controls and 71.8 (27.3-200) pg/ml in non-transfused controls, p=0.57 (median and IQR) and were not elevated compared to plasma levels. Both pulmonary and plasma levels of sRAGE did not differ between TRALI patients and controls (figure 1).

Figure 1: sRAGE levels in plasma before and after cardiac surgery and sRAGE levels in NBL fluid after surgery.

Levels of HMGB1 in NBL fluid of cardiac surgery patients with TRALI and controls

No differences were found in pulmonary HMGB1 expression between patients with TRALI (712 ± 379 [cell units], mean and SD) and in transfused (605 ± 205 [cell units]) and non-transfused controls (556 ± 123 [cell units]), p=0.86 (figure 2). There was a wide variation in expression of HMGB1. If dichotomized (detection versus no detection), no differences were found among the three groups (data not shown).
Figure 2: Levels of HMGB1 in NBL fluid after cardiac surgery.

TRALI: TRALI cases Transfusion: transfused patients who did not develop lung injury. No transfusion: patients not transfused and without lung injury. Data expressed as mean and standard deviation. Western blot is representative for HMGB1 from a patient with TRALI (A) and two control patients (B and C).

Levels of S100A12 in NBL fluid of cardiac surgery patients with TRALI and controls

S100A12 could be detected at substantial levels in NBL fluid of all patients, with the highest median levels measured in TRALI patients (702 (77-1661) ng/ml compared to 388 (217-1199) ng/ml in transfused and 119 (71-349) ng/ml in non-transfused controls (figure 3), however this was a non-significant trend (p=0.15).

Figure 3: Levels of S100A12 in NBL fluid after cardiac surgery.

TRALI: TRALI cases Transfusion: transfused patients who did not develop lung injury. No transfusion: patients not transfused and without lung injury. Data expressed as median and interquartile range.

There was a negative correlation between S100A12 levels and pO₂ six hours post-operatively (ρ = -0.584, p = 0.014) and a positive correlation between levels of S100A12 in NBL fluid and
duration of mechanical ventilation ($\rho = 0.445, p = 0.005$). Furthermore, duration of ventilation was positively associated with the total amount of transfused units ($\rho = 0.537, p = 0.002$). Then, we determined whether there was a correlation between the degree of pulmonary inflammation and pulmonary levels of S100A12. Patients with higher NBL levels of S100A12, had higher NBL cell count ($\rho = 0.762, p < 0.001$) and higher NBL levels of TNF ($\rho = 0.481, p = 0.002$), IL-1B ($\rho = 0.777, p < 0.001$), IL-6 ($\rho = 0.866, p < 0.001$) and IL-8 ($\rho = 0.893, p < 0.001$) (figure 4).

**Figure 4:** Pulmonary levels of inflammatory markers grouped by S100A12 in all patients.

Cell count in NBL fluid was significantly higher in patients with higher S100A12 levels (a). Percentage of neutrophils in NBL fluid (b). Levels of IL-1β (c), IL-6 (d), IL-8 (e) and TNFα (f) were significantly higher in patients with high levels of S100A12. Data presented in quartiles. *$P < 0.001$ or **$p=0.002$ based on Spearman's rho.
Levels of S100A12 were not significantly associated with EUROscore, ASA score and amount of transfusion. Levels of S100A12 did show a positive correlation with time on cardiopulmonary bypass ($\rho = 0.337$, $p < 0.05$).

**Discussion**

We found that cardiac surgery patients had strongly elevated pulmonary levels of S100A12 than previously reported [10]. Levels were associated with longer time on cardiopulmonary bypass, hypoxemia, prolonged mechanical ventilation and pulmonary inflammation. S100A12 levels were highest in cardiac surgery patients developing TRALI and may therefore play a role in TRALI pathogenesis.

S100A12 has been demonstrated to increase early in the course of acute lung injury [10]. In surgical patients who developed lung injury postoperatively, S100A12 serum levels peaked directly postoperative compared to controls that did not develop lung injury, showing a decline in the following postoperative day [11]. We found very high levels of S100A12 following cardiac surgery compared to previous studies in other postoperative patients [11,24]. As time on cardiopulmonary bypass was significantly associated with increased S100A12 levels in this study, use of the bypass machine may have contributed to increased pulmonary levels of S100A12. This is in line with the recent report of increased levels of S100A12 after cardiopulmonary bypass in children, which were found to correlate with severity of acute lung injury [24]. We also found that levels of S100A12 correlated with decreased oxygenation and increased time on mechanical ventilation and expand on these findings by demonstrating an association with an increase of pulmonary levels of pro-inflammatory cytokines. Interestingly, the pulmonary S100A12 levels we measured were about 10-fold higher than those reported in ARDS [10]. In addition, levels in TRALI patients were clearly higher compared to patients with acute lung injury after cardiopulmonary bypass [24]. Given that S100A2 is involved early in the course of lung injury and that TRALI syndrome classically is a rapid pulmonary reaction to a blood transfusion, we speculate that the increase in S100A12 contributes to priming of neutrophils and pulmonary vascular endothelium in cardiac surgery patients, making them more susceptible for lung injury. However, as statistical significance was not met, possibly due to small sample size, this hypothesis remains to be determined in future studies.

In this study, expression of HMGB1 was not increased in TRALI patients compared to controls. HMGB1 has been demonstrated to be an important mediator of inflammation in ARDS due to various causes [5,6,9]. Its relation to outcome has however not unequivocally been demonstrated. In trauma-induced ARDS, HMGB1 was associated with a poor outcome [8]. However, in another group of ARDS patients, HMGB1 levels did not differ between survivors and non-survivors, nor did they correlate with lung injury score and hypoxemia [6]. These conflicting results may be related to the time course of lung injury. In a mice model of LPS-induced endotoxemia, as well as in sepsis patients, HMGB1 was found to be a late mediator of the inflammatory response [25,26]. A late increase in HMGB1 was also found
in patients with ARDS, with peak levels occurring between day 1 and 7, but not at onset of disease [6]. Also, pulmonary HMGB1 release during mechanical ventilation could only be demonstrated after days and not within hours of mechanical ventilation [7]. An alternative explanation for the absence of HMGB1 expression in TRALI could be the method used to detect HMGB1. It has been demonstrated that there is a poor correlation between HMGB1 concentration detected by ELISA and immunoblot [27]. Since HMGB1 and HMGB2 show high homology there may be simultaneous determination by ELISA [28]. Western blot, as used in this study, is more specific for HMGB1. Taken together, although HMGB1 has been suggested as a late mediator of inflammation during acute lung injury, it does not seem to play a role in TRALI.

Plasma levels of sRAGE increased modestly in all patients following cardiac surgery, as shown before [24,29]. In addition, NBL fluid levels after surgery were not elevated and did not differ among TRALI patients and their controls. In line with this finding, sRAGE levels in BALF were not elevated in an experimental model of TRALI [30]. Plasma sRAGE has been postulated as a marker of lung injury [13]. Of note, sRAGE concentrations do not necessarily represent cellular RAGE expression in the lung, as was demonstrated by comparison of RAGE isoforms in BALF and lung homogenate [13]. In addition, the mechanisms that account for the release of sRAGE in the alveolus are still unclear. Therefore it is doubtful whether pulmonary sRAGE levels can be used as a marker of lung injury.

Taken together, we did not find a clear association between DAMPs and TRALI. However, we did find an association of S100A12 and pulmonary inflammation, suggesting that this early DAMP may be a strong driver of the inflammatory response in acute lung injury following cardiac surgery. Although the increase in pulmonary S100A12 levels in TRALI patients was not statistically different from controls, the observed trend suggests that the study was underpowered to establish a clear association. Therefore, whether S100A12 is a mediator in TRALI remains to be established.

There are limitations to our study. First, as noted, the number of patients is limited, which may have underpowered the study. Second, NBL was only collected once, precluding conclusion about the time course of the role of DAMPs in the maintenance and amplification of the inflammatory response in TRALI. However, this cohort of TRALI patients in whom lavage samples were obtained is the largest to date.

**Conclusion**

HMGB1 and sRAGE do not contribute to the development of TRALI. Early DAMP S100A12 is associated with prolonged cardiopulmonary bypass, pulmonary inflammation, prolonged ventilation and hypoxemia after cardiac surgery and may mediate the priming phase of acute lung injury in cardiac surgery patients who develop acute lung injury. Further research is warranted to establish the role of DAMPs in the inflammatory response in TRALI.
Reference List


