Transfusion-related acute lung injury in the critically ill: a translational approach
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Onset of transfusion-related lung injury is threshold dependent in a “two hit” murine ventilation model
Abstract

Background: Transfusion-related acute lung injury (TRALI) is suggested to be a threshold model in which a threshold must be overcome to induce a TRALI reaction. Factors that determine the threshold are the predisposition of the patient which primes the neutrophils and the ability of the mediators in the transfusion product to cause activation of primed neutrophils in the pulmonary compartment. Patient related factors for the onset of TRALI may include mechanical ventilation (MV). We determined whether onset of TRALI is dependent on the titer of MHC-I antibodies infused in a combined model of ventilator-induced lung injury and antibody-induced TRALI.

Methods: BALB/c mice (n=96) were ventilated for five hours with low (7.5 ml/kg) or high (15 ml/kg) tidal volume, a positive end-expiratory pressure of 2 cmH₂O and a fraction of inspired oxygen of 50%. After three hours of MV, TRALI was induced by infusion of 0.5 mg/kg, 2.0 mg/kg or 4.5 mg/kg MHC-I antibodies. Controls animals received vehicle. After five hours of MV, animals were sacrificed.

Results: MV with high tidal volumes resulted in increased levels of all markers of lung injury compared to animals ventilated with low tidal MV. Ventilator-induced lung injury was further enhanced after infusion of 4.5 mg/kg of MHC-I antibodies, but not after infusion of lower dose of MHC-I antibodies, as evidenced by an increased pulmonary wet-to-dry ratio, pulmonary neutrophil influx and pulmonary KC levels. In contrast, mice ventilated with low tidal volumes, did not develop lung injury, irrespective of the dose of antibody used.

Conclusions: In the presence of injurious mechanical ventilation, onset of TRALI depends on the titer of MHC-I antibodies infused. Results may suggest that decreasing the concentration of MHC-I antibodies in blood products may prevent or decrease the severity of a TRALI reaction in mechanically ventilated patients.
Introduction

Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion related morbidity and mortality.\textsuperscript{1-3} The pathogenesis of TRALI has not been fully elucidated. A two hit event has been postulated.\textsuperscript{4} The first event is an inflammatory condition of the patient (e.g. sepsis, recent surgery) causing sequestration and priming of neutrophils in the pulmonary compartment. The second event is the transfusion, containing either antibodies or bioactive lipids that have accumulated during blood storage, stimulating the primed neutrophils to release proteases. The result is endothelial damage, capillary leak and extravasation of neutrophils, finally resulting in pulmonary edema i.e. TRALI.

The association between HLA antibodies in donor plasma and TRALI is not always apparent.\textsuperscript{5-7} A threshold model has been suggested,\textsuperscript{8} in which a threshold must be overcome to induce a TRALI reaction. Factors that determine the threshold are the predisposition of the patient that determines priming of the lung neutrophils and the ability of the mediators in the transfusion to cause activation of primed neutrophils. A strong antibody-mediated response can cause severe TRALI in an otherwise “healthy” recipient. When activation status is too low, it is possible that priming factors in the transfusion are not strong enough to overcome the threshold. The threshold model may explain why the estimated incidence of TRALI is higher in critically ill patients.\textsuperscript{9-11} These patients often suffer from an inflammatory condition, which may contribute to neutrophil priming, after which transfusion of mediators with low activating capacity may be sufficient to overcome the threshold to induce a TRALI reaction.\textsuperscript{11} Indeed, LPS priming allowed for lower amounts of antibody needed to induce TRALI.\textsuperscript{12} In line with this, sepsis was found to be a risk factor for TRALI in critically ill patients.\textsuperscript{9,11} In addition, we recently showed that mechanical ventilation (MV) aggravates the course of a TRALI reaction in a murine model.\textsuperscript{13} Although data on patient related risk factors is increasing,\textsuperscript{9-11,14} data on the threshold titer of antibodies needed to induce TRALI is sparse.

Blood transfusion significantly contributes to morbidity and mortality in critically ill patients.\textsuperscript{15} Understanding the interaction between the underlying condition of the patient on the one hand and the concentration of antibodies needed to cause TRALI on the other, may help define interventions that diminish the risk of TRALI. To investigate whether onset of TRALI depends on the titer of MHC-I antibodies infused, we tested three concentrations of MHC-I antibodies in a combined model of TRALI and ventilator-induced lung injury.\textsuperscript{16,17}
Materials and Methods

The study was approved by the Animal Care And Use Committee of the Academic Medical Center at the University of Amsterdam, Amsterdam, The Netherlands. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

**MHC I mAb production**

A hybridoma (34-1-2S) was purchased from the American Type Culture Collection that produces a mAb against H2K\(^d\) (IgG\(_{2a}\), κ). The hybridoma was grown in tissue culture medium containing 1% fetal bovine serum and incubated at 37 °C and 5% CO\(_2\). Hybridoma supernatant was collected and filtered through a 0.2-µm filter. The MHC I mAb was purified using protein A sepharose affinity chromatography and dialyzed overnight in PBS (pH 7.4). The protein concentration of the mAb was spectrophotometrically determined using Bio-Rad protein reagent. The mAb stock solution (2.0–2.5 mg/ml) was frozen at –80°C until the time of the experiments.

**Mice**

Experiments were performed with healthy male BALB/c mice (n = 96, Charles River, Someren, the Netherlands), aged 8 to 10 weeks, with weights ranging from 19 to 25 g. Animals were mechanically ventilated with two different MV-strategies for 5 hours and received 0.5, 2.0 or 4.5 mg/kg MHC-I class antibodies infusion after 3 hours of ventilation. In a previous study using the same model, no differences between markers of lung injury were found between animals receiving ISO-type antibody and animals receiving vehicle infusion. Therefore, only controls receiving vehicle infusion were used.

**Instrumentation and anesthesia**

Anesthesia was achieved with intraperitoneal injection (i.p.) of a mix of ketamine (Eurovet Animal Health B.V., Bladel, the Netherlands), medetomidine (Pfizer Animal Health B.V., Capelle a/d IJssel, the Netherlands), and atropine (Pharmachemie, Haarlem, the Netherlands) (KMA). Induction anesthesia consisted of injection of KMA “induction”–mix: 7.5 µl per gram of body weight of 1.26 ml 100 mg/ml ketamine, 0.2 ml 1 mg/ml medetomidine, and 1 mL 0.5 mg/ml atropine in 5 ml normal saline. Throughout the experiments rectal temperature was maintained between 36.0 – 37.5 °C using a warming path. Maintenance anesthesia consisted of 10 µl per gram body weight of a mix of 0.72 ml 100 mg/ml ketamine, 0.08 ml 1 mg/ml medetomidine and 0.3 ml 0.5 mg/ml atropine in 20 mL normal saline.
saline administered via an intraperitoneal catheter (PE 10 tubing, BD, Breda, the Netherlands) every hour.

**Mechanical ventilation strategies**

A Y–tube connector, 1.0 mm outer diameter and 0.6 mm inner diameter (VBM Medizintechnik GmbH, Sulz am Neckar, Germany) was surgically inserted into the trachea under general anesthesia. Mice were placed in a supine position and connected to a ventilator (Servo 900 C, Siemens, Sweden). Mice were pressure controlled ventilated with either an inspiratory pressure of 10 cm H₂O (resulting in lung–protective Vₜ ~ 7.5 mL/kg; low Vₜ, LVₜ) or an inspiratory pressure of 18 cm H₂O (resulting in injurious Vₜ ~ 15 mL/kg; high Vₜ, HVₜ). Respiratory rate was set at 110 breaths/min and 50 breaths/min with LVₜ and HVₜ, respectively. These respiratory settings resulted in normal PaCO₂–values after 5 h of MV. PEEP was set at 2 cm H₂O with both MV–strategies. The fraction of inspired oxygen was kept at 0.5 and inspiration to expiration ratio was set at 1:1. A sigh (sustained inflation with 30 cm H₂O) for 5 breaths was performed every 30 minutes. Mice received an intraperitoneal bolus of 1 ml normal saline 1 hour before start of anesthesia and initiation of MV, followed by 0.2 ml sodium carbonate (200 mmol/L NaHCO₃) administered via the intraperitoneal catheter every 30 minutes until the end of MV. After 3 hours of mechanical ventilation the jugular vein was isolated. Using a 30-gauge sterile needle attached to PE-10 tubing, venous blood was aspirated from the jugular vein to verify intravascular placement of the needle and to remove a sample of blood (~200 µl). Mice were given an i.v. volume-matched injection (150–250 µl) of either 0.5, 2.0 or 4.5 mg/kg MHC-I class antibodies. Controls received vehicle. The skin was sutured with 6-0 silk suture, and the mice were sacrificed after two more hours of mechanical ventilation.

**Hemodynamic monitoring**

Systolic blood pressure and heart rate were non–invasively monitored using a murine tail–cuff system (AD Instruments, Spenbach, Germany). The data were recorded on a data acquisition system (PowerLab/4SP, AD Instruments). Systolic blood pressure and heart rate were averaged from three consecutive measurements.

**Study groups and sampling**

At the end of the experiment animals were sacrificed, bronchoalveolar lavage fluid (BALF) was obtained from the right lung (n=6), by instilling three times 0.5 mL aliquots of saline by a 22-gauge Abbocath–T catheter (Abbott, Sligo, Ireland) into the trachea. Approximately, 1.0 mL of lavage fluid was retrieved per mouse and cell counts were determined using a hemacytometer (Beckman Coulter, Fullerton, CA).
Differential counts were done (up to 100 cells per slide) on cytospin preparations stained with a modified Giemsa stain, Diff–Quick (Dade Behring AG, Düdingen, Switzerland). Supernatant was stored at -80° C for cytokine measurement. The left lung was weighed and dried for three days in an oven at 65°C. The ratio of wet weight to dry weight represents tissue edema. Another 6 mice were used for blood gas analysis from blood sampled from the carotid artery. The lungs of these mice were fixed in 4% formalin and embedded in paraffin for histopathology. 4 µm sections were stained with hematoxylin–eosin (H&E) and analyzed by a pathologist who was blinded for group identity. To score lung injury, we used a modified VILI histology scoring system as previously described 18. In short, four pathologic parameters were scored on a scale of 0 – 4: (a) alveolar congestion, (b) hemorrhage, (c) leukocyte infiltration, and (d) thickness of alveolar wall/hyaline membranes. A score of 0 represents normal lungs; 1, mild, < 25% lung involvement; 2, moderate, 25 – 50% lung involvement; 3, severe, 50 – 75% lung involvement and 4, very severe, > 75% lung involvement. The total histology score was expressed as the sum of the score for all parameters.

Assays
Cytokine and chemokine levels in the BALF were measured by enzyme–linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. Interleukin (IL)–6, macrophage inflammatory protein (MIP)–2 and keratinocyte–derived chemokine (KC) assays were all obtained from R&D Systems (Abingdon, UK).

Statistical analysis
All data in the results are expressed as means ± SEM or median ± interquartile range, where appropriate. To detect differences between groups, the Dunnett method was used, in conjunction with two–way analysis of variance or Mann Withney-U test. A p value of < 0.05 was considered significant. All statistical analyses were carried out using SPSS 12.0.2 (SPSS, Chicago, IL).

<table>
<thead>
<tr>
<th>Table 1. Cell counts in bronchoalveolar lavage fluid and histopathological examination of lung tissue</th>
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<tbody>
<tr>
<td>Control LowVt</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Total cells (x 10^4/ml BALF)</td>
</tr>
<tr>
<td>Neutrophil (x 10^4/ml BALF)</td>
</tr>
<tr>
<td>Lung Injury Score</td>
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</tbody>
</table>

Data are mean (SEM) or median [IQR]. Low VT = mice ventilated for five hours with a tidal of 7.5 ml/kg; High VT = mice ventilated for five hours with a tidal of 15 ml/kg. Dose of MHC-I antibodies infused 0.5, 2.0 and 4.5 mg/kg. BALF= bronchoalveolar lavage fluid. Controls received vehicle. n = 6 per group. *p<0.05 compared to control LowVt. **p<0.01 compared to 4.5 mg/kg LowVt and control HighVt.
Results

Hemodynamic and ventilatory monitoring
All ventilated animals survived the 5 hours of MV. Blood gas analysis showed adequate gas exchange as previously shown.\textsuperscript{13} Arterial pressure and heart rate remained stable in all animals throughout the experiment, with no differences noted between pre- and post-infusion nor between groups.

Mechanical ventilation with high tidal volume induces lung injury
Animals receiving high tidal MV showed pulmonary neutrophil sequestration with an increased wet to dry ratio compared to animals receiving low tidal MV (table 1, figure 1 and 2, p<0.01), consistent with previous results.\textsuperscript{13,17,19} Also, high tidal MV resulted in increased pulmonary levels of KC and IL-6 compared to low tidal MV (figure 3 a-b, p<0.01).

Onset of MHC-I antibody induced lung injury is threshold dependent in mice ventilated with injurious tidal volumes.
The increased wet dry ratio of the lungs induced by high tidal MV was further increased using a high dose of MHC-I antibodies (4.5 mg/kg) (figure 2, p<0.01). When lower dose of MHC-I antibodies were administered, the increased wet dry ratio of the lungs was absent. Animals ventilated with high tidal MV and receiving high dose of MHC-I antibodies had increased pulmonary levels of KC and increased neutrophil influx in the BALF compared to animals receiving lower titers of antibody (figure 3, and table 1, p<0.01). This effect was also seen for pulmonary levels of IL-6 and MIP-2, although not reaching statistical significance (figure 3, ns). Together, results suggest a threshold-dependent onset of TRALI in the presence of injurious mechanical ventilation.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Tidal Volume</th>
<th>Data</th>
<th>Neutrophil</th>
<th>Lung Injury Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/kg</td>
<td>HighV\textsubscript{t}</td>
<td>0.5 [76-153]</td>
<td>11.2 [0.8-23.3]</td>
<td>0.8 (0.3)</td>
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<td>2.0 mg/kg</td>
<td>LowV\textsubscript{t}</td>
<td>153 [85-176]</td>
<td>24.6 [9.8-46.7]</td>
<td>1.7 (0.4)</td>
</tr>
<tr>
<td>2.0 mg/kg</td>
<td>HighV\textsubscript{t}</td>
<td>152 [140-193]</td>
<td>24.6 [8.1-46.7]</td>
<td>1.7 (0.4)</td>
</tr>
<tr>
<td>4.5 mg/kg</td>
<td>LowV\textsubscript{t}</td>
<td>153 [85-176]</td>
<td>36.5 [13.9-46.7]</td>
<td>3.4 (0.5)</td>
</tr>
<tr>
<td>4.5 mg/kg</td>
<td>HighV\textsubscript{t}</td>
<td>152 [140-193]</td>
<td>46.2 [40.3-82.9]</td>
<td>4.0 (1.0)</td>
</tr>
</tbody>
</table>

Data are mean (SEM) or median [IQR]. Low VT = mice ventilated for five hours with a tidal of 7.5 ml/kg; High VT = mice ventilated for five hours with a tidal of 15 ml/kg. Dose of MHC-I antibodies infused 0.5, 2.0 and 4.5 mg/kg. BALF= bronchoalveolar lavage fluid. Controls received vehicle. n = 6 per group. *p<0.05 compared to control LowV\textsubscript{t}. **p<0.01 compared to 4.5 mg/kg LowV\textsubscript{t} and control HighV\textsubscript{t}.
Figure 1. Transfusion of MHC-I antibodies aggravates ventilator induced lung injury. Histologic sections of hematoxylin and eosin stained mice lungs at 100x magnification. Ventilated animals receiving 0.5 mg/kg, 2.0 mg/kg or 4.5 mg/kg MHC-I antibody. Animals were mechanical ventilated using low ventilation tidal (VT) (7.5 ml/kg) or High VT (15 ml/kg). (a): Low VT control (b): High VT control (c): 0.5 mg/kg Low VT (d): 0.5 mg/kg High VT (e): 2.0 mg/kg Low VT (f): 2.0 mg/kg High VT (g): 4.5 mg/kg Low VT (h): 4.5 mg/kg High VT. Neutrophils sequestrated in the vasculature (arrow) (a-h). Increased pulmonary edema and neutrophil extravasation (d, f-h). For color figure see page 355
Figure 2. Wet to dry ratio of the lungs. Ventilated animals receiving 0.5 mg/kg, 2.0 mg/kg or 4.5 mg/kg MHC-I antibody. Animals were Low VT (mice ventilated for 5 hours with a tidal of 7.5 ml/kg) and High VT (mice ventilated for 5 hours with a tidal of 15 ml/kg). Controls received vehicle. **p<0.01

Figure 3. (a) keratinocyte-derived chemokin (KC), (b) Interleukin (IL)-6 and (c) MIP-2 concentrations in the bronchoalveolar lavage fluid (BALF). Ventilated animals receiving 0.5 mg/kg, 2.0 mg/kg or 4.5 mg/kg MHC-I antibody. Animals were Low VT (mice ventilated for 5 hours with a tidal of 7.5 ml/kg) and High VT (mice ventilated for 5 hours with a tidal of 15 ml/kg). Controls received vehicle. *p<0.05, **p<0.01

Onset of TRALI is threshold dependent

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Effect of MHC-I antibody infusion in mice ventilated with protective tidal volumes.

In animals ventilated with low tidal volume, infusion of MHC-I antibodies did not increase wet to dry ratio, irrespective of the concentration used. Also, pulmonary levels of IL-6, KC and MIP-2 did not differ between groups infused with different doses of MHC-I antibodies (figure 3). Although not reaching statistical difference, infusion of high dose (4.5 mg/kg) MHC-I antibodies in animals ventilated with protective ventilator settings tended to induce pulmonary neutrophil sequestration and pulmonary edema, compared to animals receiving lower dose of MHC-I antibodies or vehicle, as evidenced on histopathologic examination (table 1, figure 1).

Discussion

We describe the effect of different doses of antibodies in inducing TRALI in a clinically relevant model of mechanically ventilated animals. In the presence of injurious MV (“first hit”), but not during protective MV, infusion of antibodies aggravated parameters of lung injury in a threshold manner. These findings suggest that onset of TRALI depends both on the “first hit” as well as on the amount of MHC-I antibodies infused, supporting a threshold model. Furthermore, the data suggest that immune-mediated TRALI is a “two hit” event.

Our results support the threshold model of TRALI. The concept that the presence of a “first hit” is required for a TRALI reaction, has been shown in several non-immune-mediated TRALI models. Infusion of stored blood products or bioactive lipids require LPS priming before inducing a TRALI reaction.20-24 Recently, it was shown that onset of TRALI induced by antibodies in the presence of priming with LPS is threshold dependent.12 Here, we confirm the finding of a threshold-dependency with a clinically relevant “first hit” of mechanical ventilation. In the presence of injurious MV, additional injury inflicted by TRALI occurred only following infusion of a high dose (4.5 mg/kg) of MHC-I antibodies, an effect that was not observed after infusion of lower concentrations of MHC-I antibodies. Of note, not all endpoints of lung injury showed a significant difference between groups infused with high and low doses of MHC-I antibodies, which may be have been due to a type II error. However, these data suggest that below a certain concentration of antibodies, a TRALI reaction does not occur, or is at least abrogated.

Clinical data on the amount of antibodies sufficient to induce TRALI are absent. It was found that plasma volumes in red blood cell units as small as 10-20 mL containing donor-derived antibodies or a single buffy coat donor from a pooled
platelet product, are sufficient to cause TRALI. However, the practice of pooling of plasma from a high number of donors, which results in dilution of any antibody that may be present, was found to essentially eliminate TRALI reactions. Also, we previously showed that deferring women from plasma donation resulted in less TRALI in a critically ill patient population. Further research is needed to establish which titer of antibodies can be safely transfused and whether this concentration differs between relative healthy patients and patients suffering from an underlying condition.

Our findings suggest that mechanically ventilated patients may be at risk for the onset of TRALI. This accords with the finding that mechanically ventilated critically ill patients are at risk for onset of ALI after transfusion with fresh frozen plasma. The mechanism of the synergistic effect of injurious MV and TRALI may be recruitment of neutrophils to the pulmonary compartment induced by MV, resulting in a pro-inflammatory response, (i.e. priming), resembling a “first hit” in TRALI models. The primed pulmonary neutrophils may be prone to activation after a second hit, which results in TRALI. In the absence of the primed neutrophils (i.e. first hit), the second hit may not overcome the threshold and TRALI will not occur. In support of this view, resting neutrophils express HLA class II antigens at very low levels, whereas cytokine activated neutrophils express increased HLA class II antigens. The finding that MV predisposes patients to TRALI, may account, at least in part, for the high incidence of TRALI among the critically ill.

Originally, TRALI was thought to be a single hit antibody-mediated reaction, in which antibodies in the blood product react with a matching antigen in the recipient, leading to pulmonary neutrophil activation and increased pulmonary capillary permeability and subsequent pulmonary edema. Then, evidence emerged that TRALI may also be the result of biological response modifiers, which were able to cause TRALI after a priming event. Recent studies show that the “two event model” also holds true for immune-mediated TRALI using LPS as a “first hit”. Here, we confirm this finding, using a clinically relevant “first hit” of mechanical ventilation. The two-event model may explain why TRALI does not always develop in a transfused patient even when an antibody-antigen match is present.

Injurious mechanical ventilation settings can facilitate the onset and course of a TRALI reaction, allowing for a lower titer of MHC-I antibodies to induce TRALI. This may have relevance for the prevention of TRALI. Plasma from multi-parous donors contain higher levels of leukocyte and/or neutrophil antibodies due to sensitization during labour compared to male donors. Excluding female donors for high volume plasma components in the UK and The Netherlands reduced the
onset of TRALI in several critically ill patient populations.\textsuperscript{29,35,36} However, deferring women from plasma donation has a strong impact on blood supply. When blood supply is an issue, our results may support a policy of plasma transfusions from male donors only to the critically ill, or mechanically ventilated patients, instead of to all patient populations. Alternatively, screening of donors with a history of pregnancy or transfusion may be a logical and cost-effective TRALI prevention strategy.\textsuperscript{37} In addition, pooling of plasma may prevent or reduce the severity of a TRALI reaction.\textsuperscript{26-28} Another implication of our results is the relevance of low tidal volume ventilation in critically ill patients exposed to a blood transfusion. Although low tidal volume is now strongly recommended,\textsuperscript{38} it is still not widely implemented in ALI patients.\textsuperscript{39} Our data clearly show that the application of low tidal volumes in patients exposed to the risk of a blood transfusion is recommendable.

**Conclusion**

In the presence of injurious mechanical ventilation, onset of TRALI is dependent on the amount of MHC-I antibodies infused, supporting the threshold model. Results suggest that decreasing the concentration of MHC-I antibodies in blood products may abrogate or at least decrease the severity of a TRALI reaction in mechanically ventilated patients.
Reference List


