Coagulopathy and plasma transfusion in critically ill patients
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Chapter 16

The effect of C1-inhibitor in a murine model of transfusion-related acute lung injury

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Abstract

Background and objective: Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related morbidity and mortality. Specific therapy is lacking. We assessed whether C1-inhibitor attenuates lung injury in a ‘two-hit’ TRALI model.

Methods: Mice were primed with lipopolysaccharide, subsequently TRALI was induced by MHC-I antibodies. In the intervention group, C1-inhibitor was infused concomitantly. Mice were supported with mechanical ventilation. After 2 hours, mice were killed, lungs were removed and bronchoalveolar lavage fluid (BALF) was obtained.

Results: Injection of MHC-I antibodies induced TRALI, illustrated by an increase in wet-to-dry ratio of the lungs, in BALF protein levels and in lung injury scores. TRALI was further characterized by complement activation, demonstrated by increased BALF levels of C3a and C5a. Administration of C1-inhibitor resulted in increased pulmonary C1-inhibitor levels with high activity. C1-inhibitor reduced pulmonary levels of complement C3a associated with improved lung injury scores. However, levels of pro-inflammatory mediators were unaffected.

Conclusion: In a murine model of TRALI, C1-inhibitor attenuated pulmonary levels of C3a associated with improved lung injury scores, but with persistent high levels of inflammatory cytokines.
Introduction

Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related mortality and morbidity [1]. TRALI can result in fulminant edema and ensuing hypoxia, associated with prolonged mechanical ventilation, increased hospital mortality and decreased survival in critically ill patient populations [2]. Therapy is only supportive, including mechanical ventilation and hemodynamic support, while curative therapy is lacking. While neutrophils and antileucocyte antibodies are considered key players, the exact pathophysiology of TRALI is still under debate. Animal studies indicate that complement activation is required for development of TRALI [3,4]. Also, in clinical TRALI cases, complement activation contributes to the inflammatory cascade [5,6]. Antibodies in transfused components may result in activation of the complement system [5,6]. Possibly, the formation of immune complexes activates the complement system via the classical pathway; however, the exact route of complement activation in TRALI remains to be established. Activation of the classical pathway of complement system induces a downstream response with formation of anaphylatoxins C3a and C5a. C1-inhibitor blocks activation of the classical as well as the mannan-binding lectine (MBL) pathway of the complement system [7]. Administration of extra C1-inhibitor was beneficial in other neutrophil-mediated inflammatory diseases including sepsis and myocardial infarction [7]. We hypothesized that lung injury in TRALI may be attenuated by blocking complement activation of the classical pathway. Therefore, we investigated the effect of administration of C1-inhibitor in murine “two-hit” model of antibody-mediated TRALI.

Materials and methods

Experiments were performed with male BALB/c mice (n=48) (Charles River, Someren, the Netherlands), aged 10-12 weeks and weighing 22-27 g. Mice were randomly assigned to 3 groups (n=16 per group). The study was approved by the Animal Care and Use Committee of the Academic Medical Center at the University of Amsterdam, the Netherlands. Animal procedures were carried out in compliance with Institutional Standards for Use of Laboratory Animals.
Experimental study protocol
Mice were primed with lipopolysaccharide (LPS, from E. Coli 0111:B4, 0.1 mg/kg) intraperitoneally (i.p.). Twenty-four hour later, mice were anesthetized, a tracheostomy was inserted and all mice were mechanically ventilated in a pressure-controlled mode, with an inspiratory pressure of 12 cm H₂O, PEEP of 2 cm H₂O (resulting in Vₚ ~ 7.5 mL/kg), respiratory rate 100 breaths/min and FiO₂ of 0.5 (Servo 900 C, Siemens, Sweden). Mice were mechanically ventilated because this TRALI model has a high mortality when unsupported. TRALI was induced by injecting MHC-I antibody (2 mg/kg) against H2Kd (IgG2a, k) into the jugular vein. Controls received phosphate-buffered saline (PBS). Immediately prior to infusion of antibodies, C1-inhibitor (400 U/kg) was infused (Cetor®, Sanquin, Amsterdam, the Netherlands). In previous experiments, we found that injection of control isotype antibody (IgG2a, (CRL-1908), American Type Culture Collection) did not induce an inflammatory reaction and was equivalent to PBS control [8]. Also, priming of LPS did not induce an inflammatory reaction. Therefore, isotype antibody and LPS control groups were omitted in these experiments. Two hours after injection, mice were bled from the carotid artery. Bronchoalveolar lavage fluid (BALF) was obtained from the right lung and centrifuged at 2000 g. Supernatant was stored at -80°C for cytokine measurement. The left lung was weighed and dried in an oven at 65°C. The ratio of wet-to-dry weight represents tissue edema. In a second set of experiments, right lungs were used for preparation of homogenate after dilution in saline and Greenberger lysis buffer using a tissue homogenizer (Biospec products, Bartlesville, OK, USA). Supernatant was stored at -80°C for cytokine measurements. Left lungs were fixed in 4% formalin and embedded in paraffin for histopathology.

Assays
Total protein levels (Bradford Protein Assay Kit, OZ Bioscience, Marseille, France) were measured in BALF. Keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2) and interleukin-6 (IL-6) were measured in BALF, homogenate and plasma using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Complement activation in BALF was determined by C3a and C5a ELISAs. Purified rat anti-mouse C3a (clone I87-1162) or purified rat anti-mouse C5a (clone I52-1486)
were used as capture antibody. Biotinylated rat anti-mouse C3a (clone I87-419) or biotinylated rat anti-mouse C5a (clone I52-278) was used as detection antibody for C3a and C5a, respectively (all from BD Biosciences). A standard curve for C3a and C5a was generated by serial dilutions of an in-house standard of maximal activated mouse serum, by incubating normal mouse serum (Sanquin Reagents) at 37°C for 1 week in the presence of sodium azide. Purified mouse C3a protein (native) and purified recombinant mouse C5a (both from BD Biosciences) were used to determine concentration of C3a and C5a, respectively, in maximal activated mouse serum. C1-inhibitor levels in BALF were assessed by ELISA using monoclonal mouse anti human C1-inhibitor (clone RII, Sanquin) as capture antibody and biotinylated rabbit anti-human C1-inhibitor (Sanquin) for detection. Normal human plasma with known C1-inhibitor concentration was used as standard.

**Lung injury score**

Four-micrometre sections were stained with hematoxylin-eosin (H&E) and analyzed by a pathologist who was blinded for group identity. Four pathologic parameters were scored on a scale of 0–4: (i) alveolar congestion, (ii) hemorrhage, (iii) leukocyte infiltration, and (iv) 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; 4, very severe, >75% lung involvement. The total histology score was expressed as the sum of the score for all parameters.

**Statistics**

Data are expressed as mean ± standard deviation (SD) or median [interquartile range] when appropriate. Comparisons between groups were performed using one-way ANOVA followed by Student’s t-test or Kruskall-Wallis followed by Mann Whitney U-test, depending on data distribution. A p value of <0.05 was considered statistically significant. Statistical analyses were performed with Prism version 5.0 (GraphPad Software, San Diego, CA, USA).
Chapter 16

Results

**Induction of TRALI with MHC-I antibody**

Injection of MHC-I antibodies induced TRALI, illustrated by an increase in wet-to-dry ratio of the lungs and elevated BALF protein levels (figure 1). Levels of chemokines MIP-2 and KC and pro-inflammatory cytokine IL-6 were all significantly increased in both the lung homogenate and BALF (table 1). Also, TRALI was characterized by complement activation, as demonstrated by increased levels of C3a and C5a in BALF compared to controls (figure 1). The inflammatory reaction resulted in lower oxygenation of mice ($pO_2$ 156 (±57) mmHg in controls vs. 133 (±67) mmHg in TRALI, p=0.41), which was statistically non-significant. Lung injury scores were higher in TRALI mice compared to controls (4.9 (±0.7) vs. (1.9 (±0.8), p<0.0001). TRALI induced a systemic inflammatory response reflected by increased plasma levels of KC and IL-6 (table 1).

**The effect of C1-inhibitor**

Administration of C1-inhibitor resulted in pulmonary C1-inhibitor levels of 12 ug/ml (± 6.4 ug/ml), with an activity of 99% (±20%), demonstrating effective levels locally. C1-inhibitor reduced C3a levels in BALF, but did not affect C5a levels compared to TRALI mice that were not treated with C1-inhibitor (figure 1). Injection of C1-inhibitor did not improve oxygenation ($pO_2$ 133 (±67) mmHg in TRALI vs. 93 (±30) mmHg in mice treated with C1-inhibitor, p=0.09), but tended to reduce pulmonary oedema and total protein levels; however, this was only a trend (figure 1). BALF levels of MIP-2, KC and IL-6 were unaffected. In lung homogenates, C1-inhibitor increased chemokine levels, although IL-6 was unaffected compared to untreated mice experiencing TRALI (table 1). Of note, treatment with C1-inhibitor improved lung injury scores from 4.9 (±0.7) in untreated mice to 3.5 (±0.8), (p=0.02) (figure 2). Systemic levels of KC and IL-6 were unaffected (table 1).
Figure 1: Wet-to-dry ratio of the lungs, BALF levels of total protein and complement levels in mice injected with antibodies that induce TRALI and those treated with C1-inhibitor.

Data expressed as mean, whiskers indicate minimum and maximum.

*p<0.05
**p<0.01
***p<0.001

TRALI = transfusion related acute lung injury
PBS = phosphate buffered saline
C1-inh = C1-esterase inhibitor
BALF = broncho-alveolar lavage fluid.
Table 1: Pulmonary and systemic levels of pro-inflammatory cytokine interleukin-6 (IL-6) and chemokines macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC) in mice with TRALI and those treated with C1-inhibitor.

<table>
<thead>
<tr>
<th></th>
<th>PBS controls</th>
<th>TRALI</th>
<th>TRALI + C1-inh</th>
<th>p value</th>
</tr>
</thead>
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<tr>
<td><strong>BALF (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td>39 (7)</td>
<td>164 (74)</td>
<td>127 (22)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KC</td>
<td>56 (12)</td>
<td>245 (145)</td>
<td>153 (79)</td>
<td>&lt;0.01</td>
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<tr>
<td>IL-6</td>
<td>24 (9)</td>
<td>188 (97)</td>
<td>114 (44)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Lung homogenate (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td>94 (14)</td>
<td>2038 (473)</td>
<td>3748 (645)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KC</td>
<td>339 (145)</td>
<td>7337 (3451)</td>
<td>31727 (12007)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IL-6</td>
<td>31 (9)</td>
<td>1629 (1188)</td>
<td>957 (284)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Plasma (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>559 [285-957]</td>
<td>2103 [1476-6557]</td>
<td>5064 [2939-5389]</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6</td>
<td>220 [164-321]</td>
<td>2440 [1404-3890]</td>
<td>1894 [832-1947]</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Pulmonary levels are expressed as mean (± standard deviation) and plasma levels are expressed as median [interquartile ranges].

PBS = phosphate buffered saline
TRALI = transfusion related acute lung injury
C1-inh = C1-inhibitor

* p<0.01 compared to PBS controls
** p<0.01 compared to TRALI

Figure 2: Representative histological sections of hematoxylin and eosin stained lungs of control mice infused with PBS

(A), mice experiencing TRALI (B) and mice with TRALI who were treated with C1-inhibitor (C).
Discussion

In a ‘two-hit’ murine TRALI model, complement activation was demonstrated, reflected by elevated BALF levels of C3a and C5a. This is line with findings in other experimental models, which found that complement was involved in the inflammatory reaction or even a prerequisite for the development of TRALI [3,4]. In contrast, C5a receptors were not essential to develop an inflammatory reaction in a single hit model of antibody mediated TRALI [9]. Also in humans, data on the role of complement activation in TRALI are conflicting [6,10].

Administration of C1-inhibitor resulted in a reduction of C3a levels in BALF, whereas C5a levels were unaffected. The C1-inhibitor detected in the BALF was highly active (99%). Since C1-inhibitor is a highly effective inhibitor of the classical and the MBL activation pathway and the current TRALI model uses anti-MHC-I antibodies, the reduction of C3a levels suggests strong activation of the classical pathway of complement in this model. Activation of the classical pathway might also be an explanation for the persistent high C5a levels, since C5a can be generated by the activation of alternative pathway, which is not inhibited by C1-inhibitor. Moreover, coagulation factors generate C5a independent of the presence of C3 [11]. As markers of coagulation are increased in murine TRALI models as well as in TRALI patients [12], enhanced coagulation may have resulted in persistent high C5a levels.

C1-inhibitor reduced C3a complement levels in the lung associated with an improvement in lung pathology scores. Also, vascular leakage in the lung tended to decrease, as reflected by a decrease in pulmonary oedema and pulmonary protein levels. In contrast, systemic and pulmonary levels of inflammatory cytokines were unaffected or even enhanced. A possible explanation for these opposing effects may be the systemic and pulmonary pro-coagulant state in TRALI, with increased levels of thrombin-antithrombin complexes as well as a decrease in fibrinolysis [12], which may have contributed to inflammation. Furthermore, a relatively high dose of C1-esterase inhibitor was used, which resulted in BALF levels of C1-inhibitor with high activity. Interestingly, high doses of C1-inhibitor have been shown to induce a pro-coagulant state [13], which might have further contributed to the enhanced levels of inflammatory parameters.
The absence of systemic C1-inhibitor levels and pharmacokinetic data are a limitation to our study. However, the complete inhibition of C3a in BALF shows the presence of active C1-inhibitor in the lungs. We do not think that use of LPS accounts for the absence of an effect of C1-inhibitor in our model, because we used a low priming dose and C1-inhibitor is beneficial in LPS-induced endotoxemia [7]. Another limitation of our study is the absence of data on activation of coagulation and degree of fibrinolysis, as previous reports found an association between the effect of C1-inhibitor and thrombin-antithrombin levels [13].

In conclusion, C1-inhibitor in murine TRALI attenuates complement activation associated with improved lung injury scores. However, these effects are accompanied by persistent inflammation, possibly due to a high dose of C1-inhibitor, which may have aggravated inflammation. Further research is warranted to elucidate the role of complement activation in TRALI before more targeted therapeutic strategies can be developed.
C1-inhibitor in a murine model of TRALI

References