Experimental strategies directed at inflammation and coagulation in ARDS and TRALI
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Lack of evidence of CD40Ligand involvement in Transfusion-Related Acute Lung Injury

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Abstract

Background: Activated platelets have been implicated to play a major role in transfusion-related hypothesized that binding of platelet CD40L(-igand) to endothelial CD40 is essential in the onset of TRALI.

Methods: Mice were challenged with monoclonal MHC-1 antibody which induced TRALI, evidenced by pulmonary edema, accompanied by significantly elevated BALF levels of total protein and elevated plasma levels of KC and MIP-2 compared to infusion of isotype Ab (p < 0.05 to all).

Results: Treatment with Ciglitazone, which inhibits platelet CD40L expression, had no effect on pulmonary and systemic inflammation compared to controls. In addition, also treatment with anti-CD40L antibody, which antagonizes all CD40-CD40L interactions, did not abrogate the TRALI reaction. Furthermore, levels of soluble CD40L were measured in a cohort of cardiac surgery patients, who were prospectively followed for the onset of TRALI after transfusion. Plasma levels of sCD40L at baseline and at time of developing TRALI did not differ between TRALI patients and controls (transfused cardiac surgery patients not developing acute lung injury) (275±192 vs 258±346 and 93±82 vs 93±123 pg/ml resp., ns).

Conclusion: These results do not support the idea that the CD40-CD40L interaction is involved in mediating TRALI.
Introduction

Transfusion-related acute lung injury (TRALI) is a major cause of transfusion-related morbidity and mortality [1]. Activation of lung endothelium induced by the underlying condition of the patient (e.g. cardiac surgery or sepsis) primes polymorphic neutrophils (PMNs) in the lung. Transfusion of blood products can result in activation of these primed PMNs and enhance microvascular permeability with subsequent pulmonary leakage [2,3]. The pathogenesis of activation of PMNs during TRALI is not fully understood. Donor-derived leukocyte antibodies (Abs) appear to be a key stimulus of PMNs in TRALI. Of these, leukocyte Abs directed against the human leukocyte antigen class (HLA) II, human neutrophil alloantigen-3a and HLA-A2 antigens are associated with the most severe TRALI cases [4]. Other factors that have been implicated in TRALI are bioactive substances which accumulate during storage of blood products [5,6].

Recently, platelets were identified as important mediators of vascular damage in TRALI [7,8]. Both platelet depletion and aspirin protected against lung injury and reduced mortality in a two-event mouse model of TRALI and aspirin was coined as a novel therapy [9]. However, the molecular mechanism of the protective effect of blocking platelet activation in TRALI still needs to be unraveled.

CD40 ligand (CD40L; CD154) is a pro-inflammatory mediator found in soluble (sCD40L) and cell-associated forms [10]. CD40L is primarily platelet derived but is also expressed on T cells and binds to CD40, expressed on endothelial and epithelial cells, neutrophils, B cells, dendritic cells and macrophages [11-13]. The CD40-CD40L system plays a pivotal role in inflammation and thrombosis in various clinical settings, including cancer [14], auto-immune diseases [15] and atherothrombosis [16,17]. Recombinant sCD40L was found to serve as the second event in an in vitro model of PMN-mediated endothelial damage [13,18]. In line with this, blocking of CD40-CD40L has been found to be protective in various models of acute lung injury (ALI) [19-21].

In TRALI, the CD40-CD40L system is also postulated to play a role. sCD40L accumulates during storage of red blood cells and platelet concentrates which are implicated in TRALI reactions [22]. In addition, increased post transfusion levels of sCD40L were found in patients with TRALI [13]. Thereby, CD40L on platelets may induce lung injury trough binding of CD40 with the endothelium in the lung, or with neutrophils or other immune cells involved in TRALI.

In this manuscript, we investigated the role of CD40L as mediator of lung injury in a murine model of antibody-mediated TRALI. Furthermore, plasma sCD40L levels were measured in patients who developed TRALI after cardiac surgery. Cardiac surgery patients were chosen because cardiac surgery is a risk factor for TRALI, these patients are often transfused and sCD40L is released during cardiopulmonary bypass [23].
Materials and Methods

Experiments were performed with healthy male BALB/c mice (Charles River, Someren, The Netherlands), aged 10-12 weeks and weighing 22-25 g, randomly assigned to 6 groups (n=8 per group). Animal studies were approved by the Animal Care and Use Committee of the Academic Medical Center at the University of Amsterdam, The Netherlands (#102033). Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

Interventions

Two interventions were performed with appropriate control groups. First, 24 hours before induction of TRALI, mice were pretreated with Ciglitazone (Ciglitazone 5-[4-(1-methylcyclohexylmethoxy) benzyl]-thiazolidine-2,4-dione (5 mg/kg) (Enzo Life Science, Zandhoven, Belgium) intraperitoneal (i.p.) as described before [24]. Ciglitazone, an anti-diabetic drug with anti-inflammatory capacity, inhibits the expression of CD40L on platelets [25] and lowers the serum level of sCD40L [26]. Controls received vehicle (2.5% Ethanol in 200 μl saline) i.p. Secondly, immediately prior to infusion of TRALI-inducing Abs, mice were pretreated with anti-CD40L Ab i.p. (10 mg/kg diluted in PBS in a volume-weight depending dose of 180-220 μL). Controls received isotype Ab (hamster-anti-rat CD40L, both from Bioceros, Utrecht, The Netherlands). Anti-CD40L Ab is capable of antagonizing all CD40-CD40L interactions, thereby making no distinction between CD40L from platelets and T cells [27,28].

Experimental Study protocol

After pre-hydration with 1 mL NaCl 0.9% i.p., mice were anesthetized with 0.075 mL/10 gram of a mix intraperitoneally (i.p.) containing Ketamine (EurovetAnimal Health B.V., Bladel, the Netherlands), Medetomidine (Pfizer Animal Health B.V., Capelle a/d Ijssel, the Netherlands) and Atropine (Pharmachemie, Haarlem, The Netherlands) in a ratio of 1.26 mL 100 mg/mL Ketamine, 0.2 mL 1mg/mL Medetomidine and 1 mL 0.5 mg/mL Atropine in 5 mL NaCl 0.9%. Then, mice were placed supine on a warming blanket and the jugular vein was isolated. Using a 30-gauge sterile needle attached to PE-tubing, venous blood was aspirated from the jugular vein to verify intravascular placement of the needle. Mice were infused with either MHC-1 Ab (IgG2a, κ, 4.5 mg/kg), which has previously been shown to induce TRALI [8,29] or matched isotype Ab (IgG2a, CRL-1908) (both from the American Type Culture Collection). The skin was closed with Prolene 5-0. The mice were kept under a heating lamp until recovery from anesthesia and then placed back in their cages. After 2 hours, mice were exsanguinated by drawing blood from the carotic artery. The left lung was ligated and the right lung was lavaged three times with 0.5 mL of normal saline. Approximately 1.0 mL of lavage fluid was retrieved per mouse. Left lungs were weighed and homogenized in 4 x lung weight (mg) in
0.9% saline using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA) and 1:1 diluted with Greenberger Lysis Buffer. Supernatant was stored at -20°C for total protein level and cytokine measurement. The left lung was used to calculate wet lung to body weight ratio.

**Clinical Study**

Blood samples were derived from a larger trial on TRALI incidence performed in the mixed medical-surgical intensive care unit of a university hospital in The Netherlands [30]. The study was approved by the Institutional Review Board (06/201 # 06.17.1506). Prior to valvular and/or coronary artery surgery, patients of 18 years or older were asked informed consent for participation in the study. Exclusion criteria were off-pump surgery, emergency surgery and use of immunosuppressive drugs. Patients were prospectively followed for the development of TRALI using the consensus definition (new onset hypoxemia or deterioration demonstrated by a PaO₂/FiO₂ ratio < 300, occurring within 6 hours after transfusion, with bilateral pulmonary changes on the chest radiograph and a pulmonary arterial occlusion pressure of ≤ 18 mmHg) [31]. Cardiogenic pulmonary edema was identified when pulmonary arterial occlusion pressure was > 18 mmHg, or by the presence of at least two of the following; central venous pressure > 15 mmHg, a history of heart failure or valve dysfunction, ejection fraction < 45% as estimated by echocardiogram and a positive fluid balance [32]. Sixteen cardiac surgery patients were identified as having suspected TRALI. Cases were randomly matched with controls in a 1:2 ratio. Controls were transfused cardiac surgery patients not developing acute lung injury. All transfused RBCs were leukoreduced (buffy coat removed and the erythrocyte suspension was filtered to remove the leukocytes (< 1x10⁶)), which is the standard of practice in the Netherlands. Blood for analysis was drawn before and 6 hours after surgery.

**Assays**

Total protein levels (Bradford Protein Assay Kit, OZ Bioscience, Marseille, France) were measured in BALF. Keratinocyte-derived Chemokine (KC) and Macrophage inflammatory protein-2 (MIP-2) were measured in BALF and plasma using Enzyme-Linked Immuno Sorbent Assay (ELISA) according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN). We choose to measure KC and MIP-2 since CD40L on activated platelets triggers an inflammatory reaction of endothelial cells [33]. Human sCD40L/TNFSF5 was measured in plasma according to the instructions of the manufacturer using an ELISA (Quantikine, R&D Systems, Minneapolis, MN). Detection limit was 4.2 pg/mL. To minimize postvenapuncture CD40L hydrolysis, EDTA anticoagulation was used [23].

**Donor antibody analysis**

Donor antibody analysis was performed described before [30]. In short, in donor samples of PLTs and FFP products, leukocyte reactive antibodies were examined using a standard complement-dependent cytotoxicity (CDC) assay with an HLA-typed donor
panel (to detect complement-fixing antibodies to HLA class I and II) and a Luminex screening assay. Leukocyte agglutinating antibodies were examined using a Leukocyte Agglutination Technique. Granulocyte-reactive antibodies were examined by the Granulocyte Immunofluorescence Test.

**Statistical analysis**
Data were expressed as mean ± standard deviation (SD) or median (IQR) when appropriate. Comparisons between experimental groups were performed using student's T-test or Mann-Whitney test depending on data distribution. On clinical data, we performed a secondary analysis using patients from a case-control study. For comparison of sCD40L levels, a Mann-Whitney test was used. A p-value <0.05 is considered statistically significant. Statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, IL) and Prism 5.0 (GraphPad Software, San Diego, CA).

**Results**
All mice in the isotype MHC-1 Ab control group survived. In contrast, 26% of the mice challenged with TRALI Abs were sacrificed because of respiratory distress. All mice were used for analysis.

**Induction of TRALI with MHC-1 Antibody**
Infusion with MHC-1 Abs resulted in an increase in lung-to-body weight ratio and total protein concentration in the BALF compared to controls, indicating a decrease in alveolar fluid clearance and an increased lung vascular permeability (Fig. 1), with a non-significant elevation of the level of KC in the BALF (Fig. 2). The level of MIP-2 in BALF did not differ between TRALI and control mice. Plasma levels of KC and MIP-2 were elevated after MHC-1 Ab injection compared to controls (Fig. 2).

**The effect of blocking CD40L in a TRALI model**
Pretreatment with Ciglitazone did not reduce pulmonary edema nor BALF protein concentration compared to the TRALI group that had received vehicle (Fig. 1). Also, Ciglitazone did not modulate local or systemic inflammation compared to the TRALI group and vehicle controls (Fig. 2).

To investigate the effect of antagonizing CD40L on all cell types, mice were treated i.p. with anti-CD40L Ab. Antagonizing of CD40L did not reduce lung edema nor protein leakage compared to the TRALI group (Fig. 1). Also, local and systemic levels of chemo-attractants were not different between the anti-CD40L antibody treated group and controls (Fig. 2).
Figure 1. Pulmonary edema and protein leakage after induction of transfusion-related acute lung injury and treatment with anti-CD40L antibody or Ciglitazone and controls.

* p < 0.05; ** p < 0.01

Figure 2. Levels of chemo-attractants in broncoalveolar lavage (BALF, upper panels) and plasma (lower panels) after induction of transfusion-related acute lung injury and treatment with anti-CD40L antibody or Ciglitazone and controls.

KC: Keratinocyte-derived Chemokine, MIP-2: Macrophage-Inflammatory Protein-2
Levels of sCD40L in TRALI patients and controls before and after cardiac surgery

Patients that had developed TRALI after cardiac surgery were compared to transfused cardiac surgery patients that did not develop ALI [30]. Baseline characteristics are presented in the Table. There was no difference in factors associated with an increase in sCD40L levels, including diabetes mellitus (DM) and vascular disease, or a decrease in sCD40L levels, such as use of statins and aspirin in type 2 DM [34]. None of the diabetic patients used a thiazolidinedione derivate. Patients developing TRALI had received more RBCs, fresh frozen plasma and platelets than controls (Table). In 63% of these patients, a leukocyte antibody was detected in an associated blood product. Patients developing TRALI received significantly more blood products containing HLA-I, HLA-II and HNA antibodies compared to controls (38 vs 3, 44 vs 6 and 19 vs 3 %, resp., p = 0.005). Of the HLA/HNA antibodies positive products, of which 80% originated from female donors [30].

Table 1. Baseline characteristics, transfusion data and pre-operative medication of patients developing TRALI compared to transfused control subjects.

<table>
<thead>
<tr>
<th></th>
<th>TRALI (n=16)</th>
<th>Controls (n=32)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>74 (29)</td>
<td>68 (13)</td>
<td>0.19</td>
</tr>
<tr>
<td>Gender, male</td>
<td>12 (75%)</td>
<td>20 (63%)</td>
<td>0.52</td>
</tr>
<tr>
<td>EuroSCORE*</td>
<td>6.0 (5.5)</td>
<td>5.0 (3.8)</td>
<td>0.94</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>2 (13%)</td>
<td>11 (34%)</td>
<td>0.17</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8 (50%)</td>
<td>24 (75%)</td>
<td>0.18</td>
</tr>
<tr>
<td>PVD</td>
<td>3 (19%)</td>
<td>10 (31%)</td>
<td>0.20</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4 (25%)</td>
<td>12 (63%)</td>
<td>0.40</td>
</tr>
<tr>
<td>RBCs, units°</td>
<td>3.2 (±2.3)</td>
<td>2.0 (±1.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>Fresh frozen plasma, units°</td>
<td>3.3 (±3.5)</td>
<td>1.2 (±1.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Platelets, units°</td>
<td>0.8 (±0.8)</td>
<td>0.3 (±0.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Thiazolidinedione</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Aspirin</td>
<td>9 (56%)</td>
<td>22 (69%)</td>
<td>0.52</td>
</tr>
<tr>
<td>Statin</td>
<td>11 (69%)</td>
<td>26 (81%)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

TRALI, transfusion-related acute lung injury; ALI, acute lung injury; EuroSCORE: European System for Cardiac Operative Risk Evaluation; PVD: peripheral vascular disease; Data are presented as or number (%), ° mean (±SD) or * median (IQR).

Prior to surgery, baseline levels of sCD40L did not differ between TRALI patients and controls. Post surgery, at onset of TRALI, levels of sCD40L were significantly lower in both groups compared to baseline values (p ≤ 0.01 to both) but there were no differences between cases and controls (Fig. 3). Because a drop in platelet counts may account for lower sCD40L concentrations, levels of platelets between groups were compared. Platelet counts correlated with sCD40L levels, showing a decrease in platelet count after surgery in both groups (Fig. 3), but no difference was observed between TRALI and controls.
Figure 3. Plasma levels of sCD40L and platelet count before and after cardiac surgery in transfusion-related acute lung injury (TRALI) patients and transfused controls.

Discussion

In this study on the role of CD40L in TRALI, we found that blocking of CD40L-CD40 is not protective in an antibody-mediated murine model of TRALI, suggesting there is no role for CD40L in the onset of immune-mediated TRALI. Furthermore and confirmative to this observation, the levels of sCD40L in patients developing TRALI were not different from transfused controls.

Infusion of MHC-1 Abs resulted in a decrease in alveolar fluid clearance and an increased lung vascular permeability, as found before [8]. The CD40-CD40L interaction was inhibited in two different ways. As platelets were found to be pivotal in mediating lung injury in this TRALI model, Ciglitazone was given, which inhibits CD40L expression on platelets. A dose was used which previously was found to ameliorate lung injury during murine pneumonia [24]. However, Ciglitazone did not reduce lung injury in our model. As immune cells other then platelets may also express CD40L and contribute to development of TRALI, animals were then infused with anti-CD40L Ab, which antagonizes binding of CD40L with all immune cells and with the endothelium [35], and completely blocks any CD40–CD40L interaction [27,28]. In this model however, anti-CD40L Ab also had no effect on pulmonary leakage and inflammation. Therefore, these results indicate that CD40-CD40L interaction does not play a major role in this model of TRALI.

A possible explanation for contrasting results with previous experimental findings may be a difference in the models used. In a previous study, sCD40L has been implicated in a ‘two event’ in vitro TRALI model after LPS priming [13], whereas an immune-mediated Ab model was used in this study. As sCD40L accumulates during storage of blood [13,22], it can be hypothesized that blocking of CD40-CD40L interaction may be protective in transfusion models using stored blood products. However, in a rat transfusion model of lung injury induced by stored PLT products after LPS priming, we previously found that pulmonary expression of CD40L was not enhanced [36].
To further examine the role of CD40-CD40L pathway in TRALI, sCD40L was measured in transfused cardiac surgery patients developing TRALI. Cardiac surgery is recognized as a risk factor for TRALI [3,32], possibly due to PMN priming during cardiopulmonary bypass [37,38]. As recombinant sCD40L was found to activate primed PMNs in vitro [13], we hypothesized that sCD40L activates primed PMNs following cardiac surgery, increasing susceptibility for a TRALI reaction. However, levels of sCD40L in TRALI cases and controls in this study were not different, suggesting that sCD40L is not implicated in the onset of TRALI in cardiac surgery patients.

These findings do not accord with previous observations suggesting that sCD40L is implicated in TRALI [13]. However, the finding of an accumulation of sCD40L in stored platelets is indirect evidence and may not have a functional consequence for a mediating role of sCD40L. In accordance, the increase in sCD40L found in platelet products implicated in transfusion reactions, was accompanied by a concomitant increase in levels of several other inflammatory mediators, suggesting that any single mediator is unlikely to account for a transfusion reaction [39]. Of note, the increase in sCD40L previously found was not univocal, occurring in only 8 out of 12 patients with TRALI, and was not statistically different from sCD40L concentrations in the pre-transfusion samples [13]. Thereby, increased levels of sCD40L may have been associated, but not causal in TRALI.

An alternative explanation for an absence of elevated levels of sCD40L in TRALI patients may have been the timing of sampling. However, as levels of sCD40L were measured before and at the onset of TRALI, it is less likely that the timing of sCD40L measurement was too late in the pathophysiological process of TRALI, allowing for CD40 to internalize or ligate sCD40L [40,41]. Moreover, it should be noted that transfused RBCs in this clinical study were leukoreduced, which has previously been shown to result in lower sCD40L levels compared to non-leukoreduced RBCs, possibly due to a reduction in contaminating platelets [13]. However, TRALI continues to occur, also after introduction of leukoreduction [42,43]. In line with this, 16 TRALI cases were detected in our prospective study using leukoreduced blood [44].

Of note, sCD40L levels were evidently lower in both groups after cardiac surgery. This drop in sCD40L probably reflects the concomitant decrease in platelet counts in both groups, as a tight correlation between sCD40L and platelet count has been found before [23,45]. We can not exclude that the decrease in platelets observed after cardiac surgery may have influenced results. Therefore, our clinical data can not be generalized to a non-cardiac surgery population.

The molecular mechanism by which aspirin was found to protect against TRALI in a murine model is still not known. Of note, the use of aspirin did not different between TRALI patients and controls in this study, which does not support the findings in the animal model [9]. However, patient numbers may have been too small in this study to comment on the effect of aspirin use.

In conclusion, antagonizing CD40-CD40L does not ameliorate lung injury in a murine model of antibody mediated TRALI. Furthermore, in the reported clinical setting, TRALI is not associated with increased sCD40L levels compared to transfused controls. Therefore, these results do not underline an important role for CD40-CD40L as a mediating pathway in TRALI.
Reference List


