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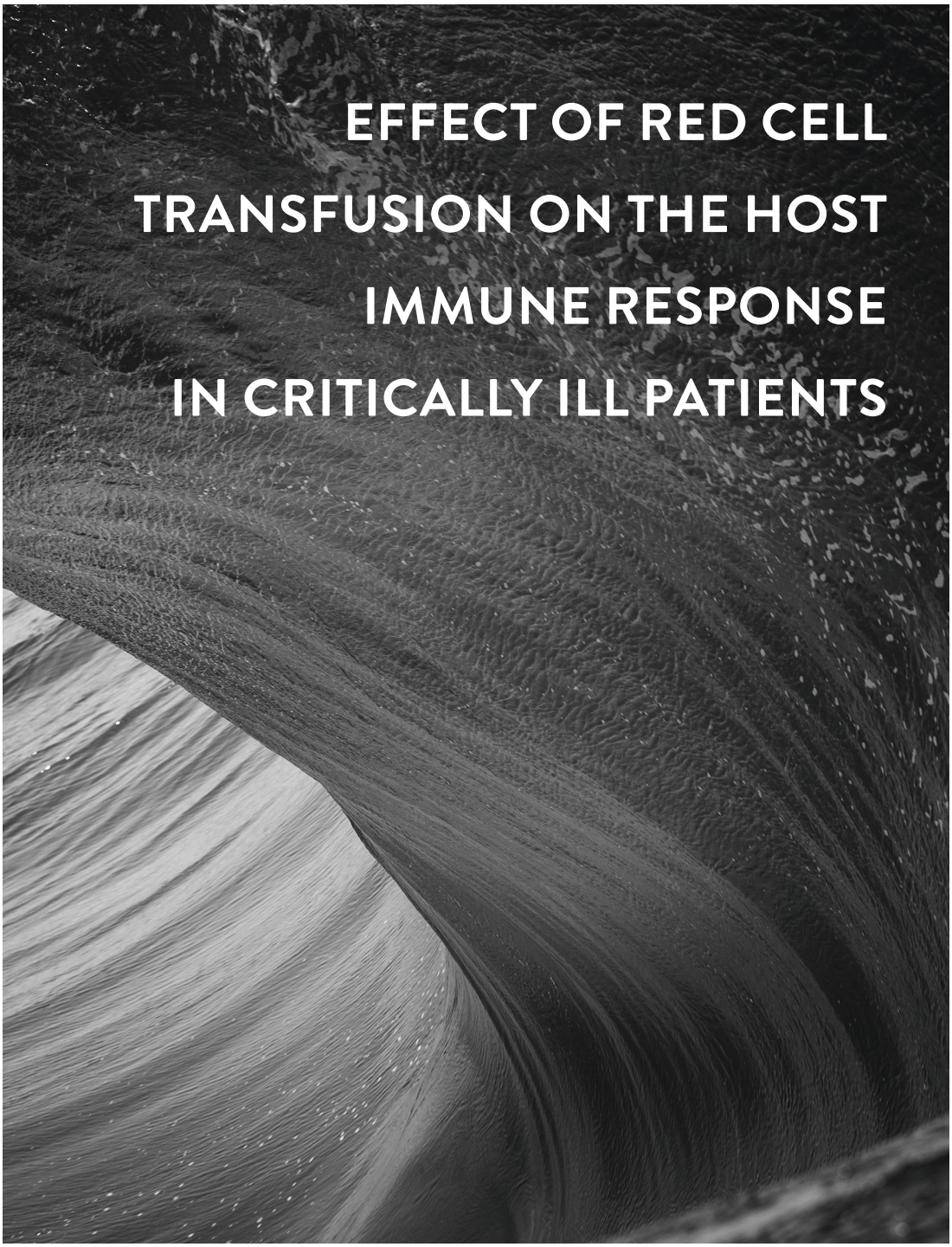

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Maike Eline van Hezel

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Effect of Red Cell Transfusion on the Host Immune Response in Critically Ill Patients

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CONTENTS

CHAPTER 1

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General introduction and outline of this thesis

INTRODUCTION

Red blood cell transfusion on the ICU

Red blood cell (RBC) transfusions are frequently administered to critically ill patients on the Intensive Care Unit (ICU). Up to 26% of the critically ill patients receive a RBC transfusion during their admission [1]. The main goal of a RBC transfusion is to increase oxygen delivery to the tissues by restoring the oxygen transport capacity [2, 3]. However, RBC transfusions are associated with adverse outcome. Adverse events following transfusion include transfusion- related acute lung injury (TRALI) and transfusion- related circulatory overload (TACO). These syndromes are the leading cause of transfusion related morbidity and mortality [4]. TRALI is defined as onset of acute lung injury within 6 hours of blood transfusion without any additional risk factors for acute lung injury [5]. Also in TACO respiratory distress can occur, with or without hypertension, which is due to circulatory volume overload [6]. The 'two hit' model is generally assumed in the pathophysiology of TRALI; the first hit is the underlying inflammatory condition of the recipient, followed by the transfusion which is the second hit, resulting in activation of the endothelium with enhanced permeability and edema [7]. Also in TACO, there are indications that the two hit model applies. This is in line with the fact that these syndromes are primarily diagnosed in critically ill patients, in whom an inflammatory condition is often present [4].

Besides TACO and TRALI, RBC transfusions can also directly influence the host immune response, termed transfusion related immunomodulation (TRIM) [8]. An impaired host response is thought to underlie the association between RBC transfusion and nosocomial infections [9], whereas an exaggerated immune response following transfusion may contribute to organ injury [10]. Again, as in TRALI and TACO, TRIM effects seem most obvious in the critically ill or injured patients [2, 11]. Additionally, an adverse outcome of RBC transfusion that hitherto has not received much attention is the fact that RBC transfusion is also associated with thromboembolic events [12, 13]. Also in this event, it is conceivable that an inflammatory host condition plays a role in mediating thromboembolic events following RBC transfusion, in which activation of the endothelium and subsequent shedding of coagulation factors and activation of platelets all may contribute to this risk. In short, many effects of transfusion on the host response are described, so the risks of RBC transfusion are abundant.

At the same time, the efficacy of RBC transfusion to improve outcomes of critically ill patients has been questioned [11]. In line with the observational studies described in this systematic review from Marik et al [11], RBC transfusions do not always show benefits on tissue oxygenation [14-17]. In recent years, a restrictive hemoglobin level of 7 g/dL has been generally adopted as a trigger for RBC transfusion in ICU practice [18]. However, this restrictive strategy may not be tolerated by all patients. It has been shown that patients with acute coronary syndromes as well as patients with oncologic disease benefit from a more liberal strategy [19, 20]. Thereby, 7 g/dL is probably on the verge of anemia tolerance. Also, anemia is associated with adverse outcome [21]. Together, this means that RBC transfusion will remain a common therapy on ICU, calling for the need to optimize transfusion strategies.

Many questions remain about the underlying mechanisms of the relation between transfusion and adverse outcome. However, activation of the host immune response may be a key factor and has been linked to adverse outcome, which is reviewed in detail below. The mechanisms by which RBC transfusion activates the host immune response are not fully understood, probably many aspects of the host's immune response play a role, such as inflammation, coagulation, endothelial activation etc. Improving our understanding of which factors influence the host immune response following RBC transfusion is important, for several reasons. Firstly, this knowledge may help to improve the product, informing blood banks as to whether expensive and elaborate procedures such as washing or filtering may help to diminish host immune effects. Secondly, knowledge on the effects of RBC transfusion on the immune system may help to identify the patient who is at risk of developing an adverse host immune response. This may improve the risk-benefit assessment of a decision to transfuse. Thirdly, knowledge of effects of RBC transfusion may generate ideas about adjunctive therapies, which can be administered alongside a RBC transfusion with the aim to counteract effects on the host immune response.

It is with these hopes and aims that this PhD project was designed. This introductory chapter summarizes the current knowledge on the host immune response following RBC transfusion, based on which the scientific chapters of this project have been designed.

Transfusion related immunomodulation (TRIM)

As already pointed out, RBC transfusion can modulate the recipient immune response, also referred to as transfusion related immunomodulation (TRIM). TRIM is a reservoir of effects, containing associations between RBC transfusion

and fewer episodes of organ rejection [22, 23], a higher recurrence rate of cancer [24] and the decreased recurrence of auto- immune diseases [9]. As noted, in this thesis we do not focus on those effects, but rather we focus on the inflammatory host immune response following RBC transfusion. TRIM is thought to include both pro-inflammatory as well as immunosuppressive effects. The pro- inflammatory propensity may contribute to organ failure by enhancing the underlying inflammation [25-27]. In a murine transfusion model, RBC transfusion results in an inflammatory response in the spleen induced by pro- inflammatory cytokines [25]. The pro- inflammatory cytokine response after RBC transfusion is also found in dogs [26, 27]. In an experimental pneumonia canine model, massive transfusion results in lung injury and mortality [28]. Also in clinical studies, RBC transfusions are associated with organ failure, a phenomenon that occurs in particular in the critically ill [11, 29-31]. In a systematic review, RBC transfusion was associated with an increased risk for multiple organ failure [11]. In observational cohort studies in septic patients, RBC transfusion was associated with an increased risk for acute lung injury [32, 33] and acute kidney injury [34]. In patients with cardiovascular diseases, RBC transfusion is associated with higher risk of myocardial infarction [35-37] and acute kidney injury [36]. In patients who undergo cardiac surgery, transfusion of RBCs as well as RBC supernatant are related to a higher incidence of postoperative kidney injury [38-40] and pulmonary morbidity [41]. In trauma patients RBC transfusion is an independent risk factor for multiple organ failure [31] and associated with pulmonary morbidity [42]. Altogether, in several critically ill and injured patient populations, an association can be found between RBC transfusion and organ injury. This risk of organ failure is suggested to be dose- dependent [12, 43]. Of note, most studies were observational studies and did not correct for confounders [44]. However, after correction by logistic regression analysis, RBC transfusion is independently associated with organ failure as well [31, 42, 43].

Besides pro-inflammatory effects, there is also a link between transfusion and increased infection risk in the ICU, which suggest that RBC transfusion is associated with immune suppression [9, 45-49]. In meta- analyses, restrictive transfusion strategies in critically ill patients are associated with a reduced risk of infections compared to a liberal transfusion policy [47, 50]. In both patients who undergo cardiac surgery as well as trauma patients, the infection rate after transfusion seems to be dose dependent [45, 51-58].

The factors that mediate TRIM are unknown, residual leukocytes/ platelets, soluble mediators in the supernatant and the RBCs themselves may be involved.

Leukoreduction of the RBC units, which involves removal of the donor leukocytes and platelets, could decrease organ failure [59-62] and postoperative infections [63-65] and is now the standard for RBC products in many countries. However, it was demonstrated that leukoreduced RBCs still contribute to organ failure and infection rates after transfusion [66-69].

There are indications in the literature that mediators in the supernatant of the RBCs may be detrimental to the recipient. In an animal model, where rats were primed with LPS first, transfusion of supernatant from RBC units resulted in lung inflammation and coagulopathy [70]. Also in cardiac surgery patients, it was found that transfusion of RBC supernatant was associated with a reduced pulmonary function resulting in longer need of mechanical ventilation, kidney injury [71] and heart failure [72], suggesting a role for soluble mediators in the purported TRIM effects. So far, lots of work has been done to identify potential mediators of TRIM in the transfusion product [8, 9, 73].

This thesis elaborates on potential mediators and focuses on the effects of RBC transfusion on the host immune response following transfusion. Whether an unfavorable host immune response will develop (either a pro-inflammatory response associated with organ failure, or an anti-inflammatory response associated with nosocomial infections), probably depends on both the type and strength of the mediator in the transfusion bag as well as on host factors of the recipients of the transfusion.

Mediators in the transfusion product implicated in immune modulating effects of an RBC transfusion

Extracellular vesicles (EVs). EVs are small phospholipid-rich vesicles that are released by different cells in response to shear stress, complement activation, oxidative stress and apoptotic stimuli [74]. EVs can interact with other cells by transferring receptors or their contents [75], which includes membrane proteins, cytoplasmic components or genetic information of the cells from which the EVs were derived [76]. During storage of RBC units, EVs are released in the supernatant and the amount increases over storage time [77-79]. These vesicles are procoagulant. In fact, the expression of tissue factor on EVs is abundant [74]. Thereby, EVs have been found to induce thrombin formation in vitro [80, 81]. Most EVs in the RBC units are from RBCs, but EVs from platelets and leukocytes are found as well [81]. Studies in vitro have shown that EVs from stored RBC units can be both inflammatory [79] as anti- inflammatory [82], measured by the production of pro- inflammatory cytokines. However, pro

inflammatory effects are more abundant. EVs can also trigger the production of pro- inflammatory cytokines by promoting the adhesion of leukocytes and platelets to the endothelium [83, 84]. Besides that, RBC derived EVs can bind complement fragments and activate the complement cascade [82] and can induce T- cell proliferation *in vitro* as well [85]. Injection of EVs from RBCs in a murine endotoxemia model results in leukocyte recruitment and the production of pro-inflammatory cytokines, an effect that was not found in the complement deficient mice, suggesting that complement activation plays a role in these pro- inflammatory effects of RBC- EVs [84]. Thus, EVs probably have the ability to modulate the immune system. However, most studies were performed in vitro.

Cell free hemoglobin (fHb). In trauma patients who received a massive blood transfusion, the fHb increases in the circulation [86]. The high clearance of the RBCs in the circulation may be responsible for an increase in the plasma fHb in the recipient, because of the excess is haptoglobin not able to regulate it anymore [87]. During storage, a limited amount of cell free hemoglobin will be released in the bag due to lysis of the RBCs [88, 89]. In a guinea pig transfusion model, transfusion of stored RBCs older than 28 days result in an increased level of plasma heme compared to transfusion with fresh RBCs [90]. In healthy volunteers, after transfusion of RBCs the plasma fHb increases, and this effect was more pronounced after transfusion of longer stored RBCs [91]. Also in a clinical trial with septic patients, an increase in the plasma fHb after transfusion with stored RBCs compared to pre- transfusion levels was found [92]. However, no effect of storage time is found in the increase of fHb after transfusion of 2 units in patients with hematological malignancy [88]. Whether storage time plays a role in the level of fHb in the supernatant is thereby not always clear [88, 91, 92]

Free hemoglobin can react with nitric oxide (NO) [93], a potent vasodilatator, resulting in lower NO bioavailability. Also the NO production is disturbed, due to the release of red cell arginase-1 by hemolysis, which converts a substrate for the NO synthase [94]. In vitro, even low levels of hemolysis decreased the bioavaibility of NO [89]. In a rat transfusion model, transfusion of fHb containing supernatant resulted in vasoconstriction [95]. A higher level of free hemoglobin is associated with vascular inflammation [96, 97] and organ damage in animal models [90, 98]. Also after administration of haptoglobin, a cell free hemoglobin scavenger, the endothelial damage decreased [90].

Heme could also play a role in inflammation. Normally, the heme-degrading enzyme heme-oxygenase 1 can neutralize the effect of heme on inflammation, but after RBC transfusion the enzyme may not be able to handle the amount of heme anymore [99]. In vitro, heme increases the expression of adhesion molecules, the permeability of endothelium and recruitment of leukocytes [100, 101]. Also, it is shown that heme can induce activation of neutrophils via Toll- like receptor 4 [102], resulting in both an increase in in vitro ROS production [103] as well as in formation of neutrophil extracellular traps (NETs) [104].

Iron. One RBC unit contains 220- 250mg iron. Thereby, RBC transfusion, in particular repeated transfusion, can result in increases of plasma iron that exceeds the iron binding capacity of transferrin, which is the major iron transport protein in the circulation [105]. The monocyte/ macrophage system is able to clear a limited amount of iron but that system can be saturated, which results in freely circulating non- transferrin bound iron (NTBI) [106]. The formation of non-transferrin bound iron (NTBI) then results in reactive oxygen species (ROS) that contribute to inflammation and organ injury [106]. Increased NTBI levels was found to promote the bacterial growth in vitro [107, 108], suggesting an increased risk of nosocomial infections. In both an animal study and in healthy volunteers, RBC transfusion was associated with an elevation of the NTBI level and a concomitant pro- inflammatory response [25, 107]. Also in preterm infants the NTBI level was significant higher after RBC transfusion compared to pre- transfusion levels [109]. The increase of NTBI in this study was associated with an increase in oxidative stress [110]. Other studies also showed that free iron is able to induce reactive oxygen species (ROS), which can result in inflammasome and NFkB activation [111] and may cause organ injury [112, 113]. In this thesis we focus on the effect of RBC transfusion on iron metabolism in critically ill patients. Of note, during storage of RBCs, the amount of NTBI in the RBC product increases over time [114], which suggests that storage duration is a risk factor for free iron and subsequent immunologic effects. Thereby, RBC storage duration is also studied.

Bioactive lipids. During storage of RBCs, both non- polar as polar bioactive lipids accumulate. Arachidonic acid, 5- hydroxeicosatetranoic (HETE), 12-HETE, 15-HETE have been identified as non- polar lipids in stored RBCs [115]. Non- polar lipids from stored RBCs are able to induce acute lung injury in an *in vivo* rat model after priming with LPS [115]. Filtration of the RBCs before storage may reduce the accumulation of non- polar lipids [116]. Polar lipids, such as lyophosphatidylcholines (PCs), are less present in stored RBCs compared to stored platelets [117]. In addition, the presence

of LysoPCs is not always found in stored RBCs [70]. In vitro, LysoPCs can affect the neutrophil function by increasing the adhesion and reducing the chemotaxis [118]. LysoPCs can also cause TRALI after priming with LPS in *ex vivo* and *in vivo* animal models [117, 119]. Also in patients an association between in an increased level of LysoPCs and transfusion related acute lung injury is found [120].

Soluble CD40 ligand (sCD40L). CD40L is a transmembrane protein and a member of the tumor necrosis factor (TNF) receptor family. During storage of RBCs, an increase in soluble CD40L from residual platelets in the plasma can be measured [121, 122]. This effect is less pronounced after leukoreduction of the RBCs [121]. Soluble CD40L is able to induce the respiratory burst of neutrophils and may promote endothelial damage in TRALI l [121]. In a sheep model, CD40L levels increased following RBC transfusion, but to lower levels than expected based on earlier trials. This finding is thought to be due to the heat- inactivation treatment of the supernatant [122]. In a murine TRALI model, no role for CD40L in the involvement of TRALI was found [123]. So probably, CD40L plays only a minor role in immune modulation after RBC transfusion.

The effect of storage duration on immune modulating effects of an RBC transfusion

Mediators that may be involved in the RBC- induced host response increases during storage in the bag due to oxidative stress [124]. The RBCs undergo morphological and functional changes during storage, known as the 'storage lesion', which reduces the efficacy by declining the post- transfusion survival and function [125, 126]. Therefor the maximum storage of RBC is restricted to 35 days in the Netherlands or 42 days in other countries. Changes during storage of RBCs include changes in rheology, like reduced deformability [127-129], increased RBC aggregability [130] and increased adherence to endothelium, which can impede the microcirculation and inhibit the tissue oxygenation [92, 131]. Also the depletion of 2,3 DPG, which shifts oxyhemoglobin dissociation curve to the left, and the reduction in nitric oxide (potential vasodilator) may reduce the oxygen delivery [2]. So both the accumulation of bioactive substances and the function of the stored red cells as an oxygen carrier, may change during storage.

In critically ill patients, lots of research has been done on the effects of storage time on outcome. Recently published large randomized controlled trials did not show any differences in mortality after transfusion with fresh compared to standard/ stored RBCs [132-134]. Thereby, it seems there is enough evidence to dispute any negative

effects of prolonged RBC storage duration on outcome. However, parameters such as infections were never the primary outcome in these studies. Despite the fact that it is convincing that the storage duration does not have a significant influence on mortality, it may be relevant to include storage time in studies on RBC- related host immune response. In addition to that, it is still informative to include storage duration in more mechanistically oriented studies such as in this thesis, as a number of mediators accumulate during storage, including compounds already discussed such as free heme.

It is not clear whether storage time influences the immune response of the host after RBC transfusion. Experimental studies have described both immunosuppressive as pro- inflammatory effects related to storage time. In *in vitro* RBC transfusion models, both longer stored leukoreduced RBCs [135-137] and supernatant from stored RBCs [138] have an increased immunosuppressive effect by increasing the anti- inflammatory cytokine response and reducing the pro- inflammatory response after *ex vivo* stimulation with lipopolysaccharide (LPS). In a murine transfusion model, transfusion of stored RBCs results in an exacerbation of gram- negative bacterial infection [139]. Also inflammatory effects of storage are described, like activation toll- like receptor (TLR4) after incubation with stored RBCs in whole blood in vitro [140]. The TLR4 is a pattern recognition receptor (PRR), especially well known in the recognition of lipopolysaccharide (LPS), and is important in the activation of the innate immune system. In a murine transfusion model, transfusion of stored non- leukoreduced RBCs increased the pro- inflammatory cytokine response compared to fresh RBCs, this effect was reduced after washing [141]. Leukoreduced stored RBCs induced the release of pro- inflammatory cytokines in a murine model as well, this effect was not found after transfusion with fresh RBCs [142]. In an observational clinical trial in critically injured children, transfusion with older prestorage leukoreduced RBCs was associated with impaired recovery of the innate immune response, measured by LPS- induced TNFα response ex vivo, compared to those who received fresh RBCs [143, 144] However, also some clinical studies in adults showed no significant differences in pro- inflammatory cytokines after transfusion with stored RBCs compared to fresh RBCs [145, 146] Thus, the role of time dependent changes during storage of RBCs in the development of immune dysfunction or nosocomial infections is still unclear. In this thesis, we tried to include the storage factor and the relation between storage duration and the host response.

The effect of RBC transfusion on different cell types of the recipient

Effect of RBC transfusion on neutrophils. Neutrophils are essential in the defense against pathogens [147]. After adhesion to the activated endothelium, neutrophils can migrate to the site of the infection, followed by extravasation and chemotaxis into the tissues. Neutrophils encapsulate microorganisms in phagosomes and kill them by the release of antibacterial granular proteases or by the production of reactive oxygen species (ROS) by the NADPH oxidase system [148]. Experimental studies show evidence that RBC transfusion may alter neutrophil function. Supernatant of stored RBC can induce neutrophil priming (defined as higher ROS production after stimulation) *in vitro*, an effect that increases with prolonged storage time [149] and is abrogated by leukocyte depletion, but not by RBC washing [150]. Contrary, RBC transfusion was also found to inhibit neutrophil chemotaxis [151, 152]. The effect on chemotaxis or other neutrophil functions may be different when there is an underlying inflammatory disease state where the chemotaxis may be increased due to the release of pro- inflammatory cytokines [153]. Of note, inappropriate activation and migration of neutrophils has been associated with organ failure [154, 155]. Hemin, derived from hemolysis in stored RBC units, induces the ROS production by neutrophils and the release of neutrophil extracellular traps (NETs) *in vitro* [104]. NETs are a meshwork of chromatin fibers coated with histones and antibacterial proteins, released by activated neutrophils [156] and can be beneficial in trapping pathogens [156], but also can contribute to tissue damage [157]. LysoPCs, which are lipid mediators that accumulate during RBC storage, are also able to activate neutrophils in vitro [150, 158]. The respiratory burst of neutrophils can also be induced by EVs from RBCs [159], which is a dose dependent effect [160]. Via the Fc receptors, the exposition of complement and immunoglobulins on EVs from RBCs can play a role in neutrophil activation [161]. Also in a mice model of hemorrhagic shock and resuscitation, injection of EVs from RBC results in neutrophil priming and neutrophil activation [162]. Enhanced neutrophil priming was found to be associated with the development of organ failure as well as with an increased risk of mortality [163-165]. In a two hit murine model, after LPS infusion the mice were transfused with plasma from stored RBCs or MHC class I antibodies, is demonstrated that acute lung injury is neutrophil dependent [117] and that NETs will be released [166, 167]. NETs nucleosomes were also found in the circulation of TRALI patients [167]. Neutrophils may play a role in the association between RBC transfusion and organ failure. However, the exact mechanism is unknown.

Effect of RBC transfusion on monocytes and macrophages. Monocytes and macrophages are able to release proteins and cytokines that initiate the immune response

[168, 169]. In critically ill patients, a reduced ability of monocytes to produce cytokines is correlated with mortality [170]. RBCs and supernatant from stored RBC units reduced the *ex- vivo* LPS induced TNFα production from peripheral blood monocytes and TNFα mRNA was also decreased [135, 171]. This suggests that RBC transfusion has immune suppressing effects on monocytes, an effect that was mediated by storage solution and storage time of the RBC units [135]. However, there is also an *in vitro* study that showed that packed RBCs or supernatant from RBCs potentiates the *ex- vivo* LPS induced IL-8 [150] and TNFα [172] production by monocytes, an effect that was attenuated by shorter storage time and leukocyte filtration.

Effect of RBC transfusion on platelets. Platelets maintain the hemostasis by formation of blood clots that repair vascular endothelium injury. To manage this process, platelets become activated after adhesion to the injured endothelium, resulting in conformational changes in glycoprotein GPIIa/IIIa on the platelet surface and secretion of granule proteins, that cause binding of platelets to coagulation factors or other platelets (platelet aggregation). RBC transfusion is associated with thromboembolic events, such as myocard infarction [12, 173] or reinfarction [13, 174], postoperative venous thromboembolic events [29, 175-177] and strokes [178]. These data suggest that RBC transfusion contributes to activation of the coagulation cascade. *In vitro*, RBC can induce platelet activation and aggregation [179, 180]. Supernatant from stored RBCs can induce thrombin generation [181]. RBC- derived EVs can interact with platelets to increase inflammatory chemokine bioavailability [182]. In addition, EVs from RBC can express phosphatidylserine (PS), which can activate tissue factor (TF) [183] or coagulation factors [80, 184] and induce thrombin generation *in vitro* [84, 185]. During storage of RBCs the amount of PS expressed EVs increases [79, 81], whereby the procoagulant effect could be even more pronounced [80]. In an observational, prospective study an enhanced activation of platelets after transfusion with RBCs was found [186]. Addition of EVs from RBCs can also induce interaction between neutrophils and platelets *in vitro* [183]. In a murine transfusion model, complement activation in LPS- primed mice can be prevented by inhibition of thrombin [84]. Therefore platelet activation and coagulation may be involved in the inflammatory response after transfusion as well [187, 188].

Effect of RBC transfusion on endothelial cells. Endothelial cells line the vasculature and have multiple functions, including regulating local blood flow, inflammation and the coagulant response and interaction with circulating blood [189, 190]. Stored RBCs can have an effect on the vasculature by reducing the NO bioavailability [191, 192], which results in hypoperfusion of the tissues and organ failure. *In vitro*, packed

RBCs and supernatant from RBCs can induce an inflammatory response in endothelial cells [193]. After incubation with supernatant the expression of intercellular adhesion molecule (ICAM)-1, an endothelial activation marker, was increased and resulted in PMN adherence [194]. *In vitro* under flow, RBC can adhere to endothelial cells as well [195]. This effect was also found in a rat model, where the adhesion was further enhanced after transfusion. However, the adhesion of donor RBCs in the capillaries was reduced after leukoreduction, suggesting that leukocytes and/or their byproducts are probably involved [196]. The adhesive capacity of endothelial cells for RBCs may even be further enhanced when the endothelium is activated by inflammation. LPS [197, 198] and oxidative stress [199] promote the adherence of RBCs to the endothelium *in vitro* under flow. During inflammation, leukocytes and platelets are also able to bind to endothelial cells [200]. In pediatric critically ill patients, the levels of soluble ICAM-1 increases after RBC transfusion [201]. Thus RBC transfusion may impede the blood flow circulation, especially in inflammatory recipients. Even though the exact mechanism of interaction between RBCs and endothelial cells is unclear.

The effect of an underlying inflammatory status of the recipient on immune modulating effects of an RBC transfusion

As already alluded to in the first paragraph of this introduction, an underlying inflammatory condition of the recipient may contribute to an increased risk of adverse outcome of RBC transfusion. In critically ill patients, the immune response to RBC transfusion is very complex and dynamic [202]. As sepsis is the most common cause of admission on the ICU [203] and anemia is a hallmark of sepsis, many critically ill patients receive a RBC transfusion [11, 204]. Sepsis is defined as a systemic response to infection with the presence of organ dysfunction [205]. Sepsis is characterized by a dysregulation of the host response, leading to hyper-inflammation as well as immune suppression. These conditions may occur simultaneously [206]. Also a dynamic host response can be found in conditions of severe sterile inflammation, such as systemic inflammatory response syndrome [207]. After severe trauma or following a major surgery, also both a hyper-inflammatory as well as immunosuppressive state can occur [208-210]. Both hyper-inflammation and immune suppression are associated with poor outcome in ICU patients [211, 212]. Also, anemia is frequently present in these sterile conditions of hyper-inflammation. To prevent organ failure, these patients frequently receive a RBC transfusion to improve the tissue oxygenation [18].

There may be distinct differences in the host immune response to RBC transfusion between a recipient with an inflammatory status versus a recipient who does not have severe inflammation at the time of transfusion. In a murine sepsis model, transfusion of RBCs result in both exaggeration of the cytokine production and induction of immune-suppressive phenotypes of leukocytes, compared to non-septic mice [213]. In a retrospective study in patients with sepsis, RBC transfusion is a risk factor for secondary bacterial infection, suggesting that during sepsis the immunomodulatory effects of a blood transfusion contributes to an adverse outcome [67]. Of note, the observation that the association between RBC transfusion and nosocomial infections and organ injury is particularly relevant in critically ill patients, may suggest that the underlying immune status of the recipient may play a role. This notion is underlined by the pathophysiology of TRALI [214, 215]. Critically ill patients have an increased risk to develop TRALI when compared to the general patient population, with an incidence varying between 0.08- 15.1% per patient transfused [215]. Thus in TRALI, an underlying disease primes the immune system (sepsis, surgery, cancer, trauma ed) and is conditional for this syndrome to develop after transfusion [216-219]. Thereby, ICU patients may be more sensitive to immunomodulatory mediators in blood products [202] compared to recipients who do not have an ongoing inflammatory state.

THE AIM OF THIS THESIS

In summary of the above, this thesis aims to study the effect of RBC transfusion on the host immune response in critically ill patients.

Specific research questions are:

1. Which mediators in the RBC transfusion bag are associated with a host immune response after transfusion?

2. Which effect does RBC transfusion have on different immune cells in the host immune response following transfusion?

3. What is the role of the underlying immune status in the recipient, by making a distinction between recipients with and without sepsis, on the host immune response following transfusion?

4. What is the effect of storage time of the RBC unit on the host immune response after transfusion?

OUTLINE OF THIS THESIS

The research in this thesis first describes the effect of RBC transfusion, including investigation of the possible mediators on the host immune response.

Chapter 2: we reviewed the knowledge of the ability of EVs to induce a pro-inflammatory immune responses.

Chapter 3: we reviewed the mediators in TRALI.

Chapter 4: we investigated the hypothesis that RBC- derived EVs can activate endothelial cells though activation of monocytes or granulocytes.

Chapter 5: we investigated the effect of RBC transfusion on iron metabolism in critically ill patients.

Besides mediators, we then investigated which cells are target cell in host immune response following RBC transfusion.

Chapter 6: we investigated the effect of RBC transfusion on neutrophil function in an endotoxemia model as well in critically ill patients.

Chapter 7: we investigated the effect of RBC transfusion on the platelet function in critically ill patients.

Finally, we go deeper into the mechanism behind immune suppression after transfusion.

Chapter 8: we investigated the effect of RBC transfusion on cytokine mRNA levels or induced tolerance of the TLR2 or TLR4 for stimulation in an endotoxemia model.

REFERENCES

1. Vincent, J.L., et al., *Worldwide audit of blood transfusion practice in critically ill patients.* Crit Care, 2018. **22**(1): p. 102.
2. Tinmouth, A., et al., *Clinical consequences of red cell storage in the critically ill.* Transfusion, 2006. **46**(11): p. 2014-27.
3. Raat, N.J. and C. Ince, *Oxygenating the microcirculation: the perspective from blood transfusion and blood storage.* Vox Sang, 2007. **93**(1): p. 12-8.
4. Li, G., et al., *Long-term survival and quality of life after transfusion-associated pulmonary edema in critically ill medical patients.* Chest, 2010. **137**(4): p. 783-9.
5. Kleinman, S., *A perspective on transfusion-related acute lung injury two years after the Canadian Consensus Conference.* Transfusion, 2006. **46**(9): p. 1465-8.
6. Gajic, O., M.A. Gropper, and R.D. Hubmayr, *Pulmonary edema after transfusion: how to differentiate transfusion-associated circulatory overload from transfusion-related acute lung injury.* Crit Care Med, 2006. **34**(5 Suppl): p. S109-13.
7. Semple, J.W., J. Rebetz, and R. Kapur, *Transfusion-associated circulatory overload and transfusion-related acute lung injury.* Blood, 2019. **133**(17): p. 1840-1853.
8. Remy, K.E., et al., *Mechanisms of red blood cell transfusion-related immunomodulation.* Transfusion, 2018. **58**(3): p. 804-815.
9. Vamvakas, E.C. and M.A. Blajchman, *Transfusion-related immunomodulation (TRIM): an update.* Blood Rev, 2007. **21**(6): p. 327-48.
10. Dunne, J.R., et al., *Allogenic blood transfusion in the first 24 hours after trauma is associated with increased systemic inflammatory response syndrome (SIRS) and death.* Surg Infect (Larchmt), 2004. **5**(4): p. 395-404.
11. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
12. Rao, S.V., et al., *Relationship of blood transfusion and clinical outcomes in patients with acute coronary syndromes.* JAMA, 2004. **292**(13): p. 1555-62.
13. Chatterjee, S., et al., *Association of blood transfusion with increased mortality in myocardial infarction: a meta-analysis and diversity-adjusted study sequential analysis.* JAMA Intern Med, 2013. **173**(2): p. 132-9.
14. Sadaka, F., et al., *The effect of red blood cell transfusion on tissue oxygenation and microcirculation in severe septic patients.* Ann Intensive Care, 2011. **1**(1): p. 46.
15. Sakr, Y., et al., *Microvascular response to red blood cell transfusion in patients with severe sepsis.* Crit Care Med, 2007. **35**(7): p. 1639-44.
16. Frenzel, T. and M. Westphal, *Role of the microcirculation in intensive care and resuscitation: the devil is in the details.* Curr Opin Anaesthesiol, 2009. **22**(2): p. 141-2.
17. Creteur, J., A.P. Neves, and J.L. Vincent, *Near-infrared spectroscopy technique to evaluate the effects of red blood cell transfusion on tissue oxygenation.* Crit Care, 2009. **13 Suppl 5**: p. S11.
18. Holst, L.B., et al., *Lower versus higher hemoglobin threshold for transfusion in septic shock.* N Engl J Med, 2014. **371**(15): p. 1381-91.
19. Docherty, A.B. and T.S. Walsh, *Anemia and blood transfusion in the critically ill patient with cardiovascular disease.* Crit Care, 2017. **21**(1): p. 61.

20. Bergamin, F.S., et al., *Liberal Versus Restrictive Transfusion Strategy in Critically Ill Oncologic Patients: The Transfusion Requirements in Critically Ill Oncologic Patients Randomized Controlled Trial.* Crit Care Med, 2017. **45**(5): p. 766-773.
21. Hayden, S.J., et al., *Anemia in critical illness: insights into etiology, consequences, and management.* Am J Respir Crit Care Med, 2012. **185**(10): p. 1049-57.
22. Opelz, G., et al., *Effect of blood transfusions on subsequent kidney transplants.* Transplant Proc, 1973. **5**(1): p. 253-9.
23. Keown, P.A. and B. Descamps, *Improved renal allograft survival after blood transfusion: a nonspecific, erythrocyte-mediated immunoregulatory process?* Lancet, 1979. **1**(8106): p. 20-2.
24. Atzil, S., et al., *Blood transfusion promotes cancer progression: a critical role for aged erythrocytes.* Anesthesiology, 2008. **109**(6): p. 989-97.
25. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
26. Callan, M.B., et al., *Transfusion of 28-day-old leucoreduced or non-leucoreduced stored red blood cells induces an inflammatory response in healthy dogs.* Vox Sang, 2013. **105**(4): p. 319-27.
27. Cortes-Puch, I., et al., *Washing older blood units before transfusion reduces plasma iron and improves outcomes in experimental canine pneumonia.* Blood, 2014. **123**(9): p. 1403-11.
28. Solomon, S.B., et al., *Mortality increases after massive exchange transfusion with older stored blood in canines with experimental pneumonia.* Blood, 2013. **121**(9): p. 1663-72.
29. Spinella, P.C., et al., *Duration of red blood cell storage is associated with increased incidence of deep vein thrombosis and in hospital mortality in patients with traumatic injuries.* Crit Care, 2009. **13**(5): p. R151.
30. Zallen, G., et al., *Age of transfused blood is an independent risk factor for postinjury multiple organ failure.* Am J Surg, 1999. **178**(6): p. 570-2.
31. Moore, F.A., E.E. Moore, and A. Sauaia, *Blood transfusion. An independent risk factor for postinjury multiple organ failure.* Arch Surg, 1997. **132**(6): p. 620-4; discussion 624-5.
32. Janz, D.R., et al., *Longer storage duration of red blood cells is associated with an increased risk of acute lung injury in patients with sepsis.* Ann Intensive Care, 2013. **3**(1): p. 33.
33. Iscimen, R., et al., *Risk factors for the development of acute lung injury in patients with septic shock: an observational cohort study.* Crit Care Med, 2008. **36**(5): p. 1518-22.
34. Plataki, M., et al., *Predictors of acute kidney injury in septic shock patients: an observational cohort study.* Clin J Am Soc Nephrol, 2011. **6**(7): p. 1744-51.
35. Singla, I., et al., *Impact of blood transfusions in patients presenting with anemia and suspected acute coronary syndrome.* Am J Cardiol, 2007. **99**(8): p. 1119-21.
36. Yang, X., et al., *The implications of blood transfusions for patients with non-ST-segment elevation acute coronary syndromes: results from the CRUSADE National Quality Improvement Initiative.* J Am Coll Cardiol, 2005. **46**(8): p. 1490-5.

37. Nikolsky, E., et al., *Prognostic impact of blood transfusion after primary angioplasty for acute myocardial infarction: analysis from the CADILLAC (Controlled Abciximab and Device Investigation to Lower Late Angioplasty Complications) Trial.* JACC Cardiovasc Interv, 2009. **2**(7): p. 624-32.
38. Habib, R.H., et al., *Role of hemodilutional anemia and transfusion during cardiopulmonary bypass in renal injury after coronary revascularization: implications on operative outcome.* Crit Care Med, 2005. **33**(8): p. 1749-56.
39. Benedetto, U., et al., *Miniaturized cardiopulmonary bypass and acute kidney injury in coronary artery bypass graft surgery.* Ann Thorac Surg, 2009. **88**(2): p. 529-35.
40. Karkouti, K., et al., *Acute kidney injury after cardiac surgery: focus on modifiable risk factors.* Circulation, 2009. **119**(4): p. 495-502.
41. Koch, C., et al., *Transfusion and pulmonary morbidity after cardiac surgery.* Ann Thorac Surg, 2009. **88**(5): p. 1410-8.
42. Croce, M.A., et al., *Transfusions result in pulmonary morbidity and death after a moderate degree of injury.* J Trauma, 2005. **59**(1): p. 19-23; discussion 23-4.
43. Koch, C.G., et al., *Morbidity and mortality risk associated with red blood cell and blood-component transfusion in isolated coronary artery bypass grafting.* Crit Care Med, 2006. **34**(6): p. 1608-16.
44. Middelburg, R.A., L.M. van de Watering, and J.G. van der Bom, *Blood transfusions: good or bad? Confounding by indication, an underestimated problem in clinical transfusion research.* Transfusion, 2010. **50**(6): p. 1181-3.
45. Claridge, J.A., et al., *Blood transfusions correlate with infections in trauma patients in a dose-dependent manner.* Am Surg, 2002. **68**(7): p. 566-72.
46. Taylor, R.W., et al., *Impact of allogenic packed red blood cell transfusion on nosocomial infection rates in the critically ill patient.* Crit Care Med, 2002. **30**(10): p. 2249-54.
47. Rohde, J.M., et al., *Health care-associated infection after red blood cell transfusion: a systematic review and meta-analysis.* JAMA, 2014. **311**(13): p. 1317-26.
48. Blajchman, M.A., *Immunomodulation and blood transfusion.* Am J Ther, 2002. **9**(5): p. 389-95.
49. Shorr, A.F. and W.L. Jackson, *Transfusion practice and nosocomial infection: assessing the evidence.* Curr Opin Crit Care, 2005. **11**(5): p. 468-72.
50. Salpeter, S.R., J.S. Buckley, and S. Chatterjee, *Impact of more restrictive blood transfusion strategies on clinical outcomes: a meta-analysis and systematic review.* Am J Med, 2014. **127**(2): p. 124-131 e3.
51. Leal-Noval, S.R., et al., *Transfusion of blood components and postoperative infection in patients undergoing cardiac surgery.* Chest, 2001. **119**(5): p. 1461-8.
52. Chelemer, S.B., et al., *Association of bacterial infection and red blood cell transfusion after coronary artery bypass surgery.* Ann Thorac Surg, 2002. **73**(1): p. 138-42.
53. Horvath, K.A., et al., *Blood transfusion and infection after cardiac surgery.* Ann Thorac Surg, 2013. **95**(6): p. 2194-201.
54. Agarwal, N., et al., *Blood transfusion increases the risk of infection after trauma.* Arch Surg, 1993. **128**(2): p. 171-6; discussion 176-7.
55. Carson, J.L., et al., *Risk of bacterial infection associated with allogeneic blood transfusion among patients undergoing hip fracture repair.* Transfusion, 1999. **39**(7): p. 694-700.

56. Hill, G.E., et al., *Allogeneic blood transfusion increases the risk of postoperative bacterial infection: a meta-analysis.* J Trauma, 2003. **54**(5): p. 908-14.
57. Offner, P.J., et al., *Increased rate of infection associated with transfusion of old blood after severe injury.* Arch Surg, 2002. **137**(6): p. 711-6; discussion 716-7.
58. Palmieri, T.L., et al., *Effect of blood transfusion on outcome after major burn injury: a multicenter study.* Crit Care Med, 2006. **34**(6): p. 1602-7.
59. Romano, G., et al., *Leukoreduction program for red blood cell transfusions in coronary surgery: association with reduced acute kidney injury and in-hospital mortality.* J Thorac Cardiovasc Surg, 2010. **140**(1): p. 188-95.
60. Plurad, D., et al., *Leukoreduction is associated with a decreased incidence of late onset acute respiratory distress syndrome after injury.* Am Surg, 2008. **74**(2): p. 117-23.
61. Netzer, G., et al., *Association of RBC transfusion with mortality in patients with acute lung injury.* Chest, 2007. **132**(4): p. 1116-23.
62. Miyaji, K., et al., *The effectiveness of prestorage leukocyte-reduced red blood cell transfusion on perioperative inflammatory response with a miniaturized biocompatible bypass system.* J Thorac Cardiovasc Surg, 2010. **139**(6): p. 1561-7.
63. Fergusson, D., et al., *Transfusion of leukoreduced red blood cells may decrease postoperative infections: two meta-analyses of randomized controlled trials.* Can J Anaesth, 2004. **51**(5): p. 417-24.
64. Hebert, P.C., et al., *Clinical outcomes following institution of the Canadian universal leukoreduction program for red blood cell transfusions.* JAMA, 2003. **289**(15): p. 1941-9.
65. Blumberg, N., et al., *The intention-to-treat principle in clinical trials and meta-analyses of leukoreduced blood transfusions in surgical patients.* Transfusion, 2007. **47**(4): p. 573-81.
66. Englehart, M.S., et al., *Use of leukoreduced blood does not reduce infection, organ failure, or mortality following trauma.* World J Surg, 2009. **33**(8): p. 1626-32.
67. Juffermans, N.P., et al., *Transfusion-related risk of secondary bacterial infections in sepsis patients: a retrospective cohort study.* Shock, 2011. **35**(4): p. 355-9.
68. Weinberg, J.A., et al., *Transfusions in the less severely injured: does age of transfused blood affect outcomes?* J Trauma, 2008. **65**(4): p. 794-8.
69. Vamvakas, E.C., *Meta-analysis of randomized controlled trials investigating the risk of postoperative infection in association with white blood cell-containing allogeneic blood transfusion: the effects of the type of transfused red blood cell product and surgical setting.* Transfus Med Rev, 2002. **16**(4): p. 304-14.
70. Vlaar, A.P., et al., *Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model.* Anesthesiology, 2010. **113**(1): p. 92-103.
71. Vamvakas, E.C. and J.H. Carven, *Allogeneic blood transfusion and postoperative duration of mechanical ventilation: effects of red cell supernatant, platelet supernatant, plasma components and total transfused fluid.* Vox Sang, 2002. **82**(3): p. 141-9.
72. Kachhy, R.G., et al., *Long-term outcomes of the symmetry vein graft anastomosis device: a matched case-control analysis.* Circulation, 2006. **114**(1 Suppl): p. I425-9.
73. Vamvakas, E.C., *Possible mechanisms of allogeneic blood transfusion-associated postoperative infection.* Transfus Med Rev, 2002. **16**(2): p. 144-60.

74. Rubin, O., et al., *Microparticles in stored red blood cells: submicron clotting bombs?* Blood Transfus, 2010. **8 Suppl 3**: p. s31-8.
75. McVey, M., A. Tabuchi, and W.M. Kuebler, *Microparticles and acute lung injury.* Am J Physiol Lung Cell Mol Physiol, 2012. **303**(5): p. L364-81.
76. Mause, S.F. and C. Weber, *Microparticles: protagonists of a novel communication network for intercellular information exchange.* Circ Res, 2010. **107**(9): p. 1047-57.
77. Rubin, O., et al., *Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation.* Transfusion, 2013. **53**(8): p. 1744-54.
78. Kriebardis, A.G., et al., *RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components.* Transfusion, 2008. **48**(9): p. 1943-53.
79. Straat, M., et al., *Extracellular Vesicles from Red Blood Cell Products Induce a Strong Pro-Inflammatory Host Response, Dependent on Both Numbers and Storage Duration.* Transfus Med Hemother, 2016. **43**(4): p. 302-305.
80. Gao, Y., et al., *Elevated levels of thrombin-generating microparticles in stored red blood cells.* Vox Sang, 2013. **105**(1): p. 11-7.
81. Jy, W., et al., *Microparticles in stored red blood cells as potential mediators of transfusion complications.* Transfusion, 2011. **51**(4): p. 886-93.
82. Sadallah, S., C. Eken, and J.A. Schifferli, *Erythrocyte-derived ectosomes have immunosuppressive properties.* J Leukoc Biol, 2008. **84**(5): p. 1316-25.
83. Meziani, F., et al., *Bench-to-bedside review: circulating microparticles--a new player in sepsis?* Crit Care, 2010. **14**(5): p. 236.
84. Zecher, D., A. Cumpelik, and J.A. Schifferli, *Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement.* Arterioscler Thromb Vasc Biol, 2014. **34**(2): p. 313-20.
85. Danesh, A., et al., *Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses in vitro.* Blood, 2014. **123**(5): p. 687-96.
86. Nishiyama, T. and K. Hanaoka, *Free hemoglobin concentrations in patients receiving massive blood transfusion during emergency surgery for trauma.* Can J Anaesth, 2000. **47**(9): p. 881-5.
87. Buehler, P.W. and E. Karnaukhova, *When might transferrin, hemopexin or haptoglobin administration be of benefit following the transfusion of red blood cells?* Curr Opin Hematol, 2018. **25**(6): p. 452-458.
88. Vermeulen Windsant, I.C., et al., *Blood transfusions increase circulating plasma free hemoglobin levels and plasma nitric oxide consumption: a prospective observational pilot study.* Crit Care, 2012. **16**(3): p. R95.
89. Gladwin, M.T. and D.B. Kim-Shapiro, *Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis.* Curr Opin Hematol, 2009. **16**(6): p. 515-23.
90. Baek, J.H., et al., *Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy.* J Clin Invest, 2012. **122**(4): p. 1444-58.
91. Risbano, M.G., et al., *Effects of Aged Stored Autologous Red Blood Cells on Human Endothelial Function.* Am J Respir Crit Care Med, 2015. **192**(10): p. 1223-33.

92. Damiani, E., et al., *Plasma free hemoglobin and microcirculatory response to fresh or old blood transfusions in sepsis.* PLoS One, 2015. **10**(5): p. e0122655.
93. Herold, S., M. Exner, and T. Nauser, *Kinetic and mechanistic studies of the NO*-mediated oxidation of oxymyoglobin and oxyhemoglobin.* Biochemistry, 2001. **40**(11): p. 3385-95.
94. Rother, R.P., et al., *The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease.* JAMA, 2005. **293**(13): p. 1653-62.
95. Donadee, C., et al., *Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion.* Circulation, 2011. **124**(4): p. 465-76.
96. Conran, N. and C.B. Almeida, *Hemolytic vascular inflammation: an update.* Rev Bras Hematol Hemoter, 2016. **38**(1): p. 55-7.
97. Almeida, C.B., et al., *Acute hemolytic vascular inflammatory processes are prevented by nitric oxide replacement or a single dose of hydroxyurea.* Blood, 2015. **126**(6): p. 711-20.
98. Minneci, P.C., et al., *Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by decompartmentalized oxyhemoglobin.* J Clin Invest, 2005. **115**(12): p. 3409-17.
99. Yazdanbakhsh, K., W. Bao, and H. Zhong, *Immunoregulatory effects of stored red blood cells.* Hematology Am Soc Hematol Educ Program, 2011. **2011**: p. 466-9.
100. Wagener, F.A., et al., *Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase.* Blood, 2001. **98**(6): p. 1802-11.
101. Graca-Souza, A.V., et al., *Neutrophil activation by heme: implications for inflammatory processes.* Blood, 2002. **99**(11): p. 4160-5.
102. Figueiredo, R.T., et al., *Characterization of heme as activator of Toll-like receptor 4.* J Biol Chem, 2007. **282**(28): p. 20221-9.
103. Porto, B.N., et al., *Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors.* J Biol Chem, 2007. **282**(33): p. 24430-6.
104. Kono, M., et al., *Heme-related molecules induce rapid production of neutrophil extracellular traps.* Transfusion, 2014. **54**(11): p. 2811-9.
105. Ganz, T., *Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation.* Blood, 2003. **102**(3): p. 783-8.
106. Ozment, C.P. and J.L. Turi, *Iron overload following red blood cell transfusion and its impact on disease severity.* Biochim Biophys Acta, 2009. **1790**(7): p. 694-701.
107. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
108. Cross, J.H., et al., *Oral iron acutely elevates bacterial growth in human serum.* Sci Rep, 2015. **5**: p. 16670.
109. Hirano, K., et al., *Blood transfusion increases radical promoting non-transferrin bound iron in preterm infants.* Arch Dis Child Fetal Neonatal Ed, 2001. **84**(3): p. F188-93.

110. Stark, M.J., A.K. Keir, and C.C. Andersen, *Does non-transferrin bound iron contribute to transfusion related immune-modulation in preterms?* Arch Dis Child Fetal Neonatal Ed, 2013. **98**(5): p. F424-9.
111. Hod, E.A. and S.L. Spitalnik, *Stored red blood cell transfusions: Iron, inflammation, immunity, and infection.* Transfus Clin Biol, 2012. **19**(3): p. 84-9.
112. Brissot, P., et al., *Non-transferrin bound iron: a key role in iron overload and iron toxicity.* Biochim Biophys Acta, 2012. **1820**(3): p. 403-10.
113. Shander, A. and K. Sazama, *Clinical consequences of iron overload from chronic red blood cell transfusions, its diagnosis, and its management by chelation therapy.* Transfusion, 2010. **50**(5): p. 1144-55.
114. Marwah, S.S., et al., *Increased non-transferrin bound iron in plasma-depleted SAG-M red blood cell units.* Vox Sang, 2002. **82**(3): p. 122-6.
115. Silliman, C.C., et al., *Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury.* Transfusion, 2011. **51**(12): p. 2549-54.
116. Silliman, C.C., et al., *Experimental prestorage filtration removes antibodies and decreases lipids in RBC supernatants mitigating TRALI in vivo.* Blood, 2014. **123**(22): p. 3488-95.
117. Kelher, M.R., et al., *Plasma from stored packed red blood cells and MHC class I antibodies causes acute lung injury in a 2-event in vivo rat model.* Blood, 2009. **113**(9): p. 2079-87.
118. Silliman, C.C., et al., *Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium.* J Leukoc Biol, 2003. **73**(4): p. 511-24.
119. Silliman, C.C., et al., *Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model.* J Clin Invest, 1998. **101**(7): p. 1458-67.
120. Silliman, C.C., et al., *Transfusion-related acute lung injury: epidemiology and a prospective analysis of etiologic factors.* Blood, 2003. **101**(2): p. 454-62.
121. Khan, S.Y., et al., *Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury.* Blood, 2006. **108**(7): p. 2455-62.
122. Tung, J.P., et al., *Age of blood and recipient factors determine the severity of transfusion-related acute lung injury (TRALI).* Crit Care, 2012. **16**(1): p. R19.
123. Tuinman, P.R., et al., *Lack of evidence of CD40 ligand involvement in transfusion-related acute lung injury.* Clin Exp Immunol, 2011. **165**(2): p. 278-84.
124. Lee, J.S. and M.T. Gladwin, *Bad blood: the risks of red cell storage.* Nat Med, 2010. **16**(4): p. 381-2.
125. Hess, J.R., *Measures of stored red blood cell quality.* Vox Sang, 2014. **107**(1): p. 1-9.
126. Luten, M., et al., *Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods.* Transfusion, 2008. **48**(7): p. 1478-85.
127. Frank, S.M., et al., *Decreased erythrocyte deformability after transfusion and the effects of erythrocyte storage duration.* Anesth Analg, 2013. **116**(5): p. 975-81.
128. Relevy, H., et al., *Blood banking-induced alteration of red blood cell flow properties.* Transfusion, 2008. **48**(1): p. 136-46.

129. Berezina, T.L., et al., *Influence of storage on red blood cell rheological properties.* J Surg Res, 2002. **102**(1): p. 6-12.
130. Hovav, T., et al., *Alteration of red cell aggregability and shape during blood storage.* Transfusion, 1999. **39**(3): p. 277-81.
131. Ayhan, B., et al., *The effects of non-leukoreduced red blood cell transfusions on microcirculation in mixed surgical patients.* Transfus Apher Sci, 2013. **49**(2): p. 212-22.
132. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
133. Steiner, M.E., et al., *Effects of red-cell storage duration on patients undergoing cardiac surgery.* N Engl J Med, 2015. **372**(15): p. 1419-29.
134. Rygard, S.L., et al., *Effects of shorter versus longer storage time of transfused red blood cells in adult ICU patients: a systematic review with meta-analysis and Trial Sequential Analysis.* Intensive Care Med, 2018. **44**(2): p. 204-217.
135. Muszynski, J., et al., *Immunosuppressive effects of red blood cells on monocytes are related to both storage time and storage solution.* Transfusion, 2012. **52**(4): p. 794-802.
136. Biedler, A.E., et al., *Impact of alloantigens and storage-associated factors on stimulated cytokine response in an in vitro model of blood transfusion.* Anesthesiology, 2002. **97**(5): p. 1102-9.
137. Mynster, T., *Effects of red cell storage and lysis on in vitro cytokine release.* Transfus Apher Sci, 2001. **25**(1): p. 17-23.
138. Karam, O., et al., *Length of storage and in vitro immunomodulation induced by prestorage leukoreduced red blood cells.* Transfusion, 2009. **49**(11): p. 2326-34.
139. Prestia, K., et al., *Transfusion of stored blood impairs host defenses against Gram-negative pathogens in mice.* Transfusion, 2014. **54**(11): p. 2842-51.
140. Escobar, G.A., et al., *Stored packed red blood cell transfusion up-regulates inflammatory gene expression in circulating leukocytes.* Ann Surg, 2007. **246**(1): p. 129-34.
141. Belizaire, R.M., et al., *Resuscitation with washed aged packed red blood cell units decreases the proinflammatory response in mice after hemorrhage.* J Trauma Acute Care Surg, 2012. **73**(2 Suppl 1): p. S128-33.
142. Hendrickson, J.E., et al., *Transfusion of fresh murine red blood cells reverses adverse effects of older stored red blood cells.* Transfusion, 2011. **51**(12): p. 2695-702.
143. Muszynski, J.A., et al., *Red blood cell transfusion and immune function in critically ill children: a prospective observational study.* Transfusion, 2015. **55**(4): p. 766-74.
144. Muszynski, J.A., et al., *Innate immune function predicts the development of nosocomial infection in critically injured children.* Shock, 2014. **42**(4): p. 313-21.
145. Kor, D.J., et al., *Fresh red blood cell transfusion and short-term pulmonary, immunologic, and coagulation status: a randomized clinical trial.* Am J Respir Crit Care Med, 2012. **185**(8): p. 842-50.
146. Lebiedz, P., et al., *Influence of Red Blood Cell Storage Time on Clinical Course and Cytokine Profile in Septic Shock Patients.* Transfus Med Hemother, 2012. **39**(4): p. 271-276.
147. Segal, A.W., *How neutrophils kill microbes.* Annu Rev Immunol, 2005. **23**: p. 197-223.
148. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.

149. Sparrow, R.L. and K.A. Patton, *Supernatant from stored red blood cell primes inflammatory cells: influence of prestorage white cell reduction.* Transfusion, 2004. **44**(5): p. 722-30.
150. Cardo, L.J., D. Wilder, and J. Salata, *Neutrophil priming, caused by cell membranes and microvesicles in packed red blood cell units, is abrogated by leukocyte depletion at collection.* Transfus Apher Sci, 2008. **38**(2): p. 117-25.
151. Ghio, M., et al., *Transforming growth factor-beta1 in supernatants from stored red blood cells inhibits neutrophil locomotion.* Blood, 2003. **102**(3): p. 1100-7.
152. Ottonello, L., et al., *Nonleukoreduced red blood cell transfusion induces a sustained inhibition of neutrophil chemotaxis by stimulating in vivo production of transforming growth factor-beta1 by neutrophils: role of the immunoglobulinlike transcript 1, sFasL, and sHLA-I.* Transfusion, 2007. **47**(8): p. 1395-404.
153. Davidson, D. and H. Patel, *Cytokine-induced neutrophil chemotaxis assay.* Methods Mol Biol, 2014. **1172**: p. 107-13.
154. Brown, K.A., et al., *Neutrophils in development of multiple organ failure in sepsis.* Lancet, 2006. **368**(9530): p. 157-69.
155. Hoesel, L.M., et al., *Harmful and protective roles of neutrophils in sepsis.* Shock, 2005. **24**(1): p. 40-7.
156. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.
157. Xu, J., et al., *Extracellular histones are major mediators of death in sepsis.* Nat Med, 2009. **15**(11): p. 1318-21.
158. Khan, S.Y., et al., *Lysophosphatidylcholines activate G2A inducing G(alphai)(-)(1)-/G(alphaq/)(1)(1)- Ca(2)(+) flux, G(betagamma)-Hck activation and clathrin/beta-arrestin-1/GRK6 recruitment in PMNs.* Biochem J, 2010. **432**(1): p. 35-45.
159. Jank, H. and U. Salzer, *Vesicles generated during storage of red blood cells enhance the generation of radical oxygen species in activated neutrophils.* ScientificWorldJournal, 2011. **11**: p. 173-85.
160. Kent, M.W., et al., *The pro-inflammatory potential of microparticles in red blood cell units.* Transfus Med, 2014. **24**(3): p. 176-81.
161. Bosman, G.J., et al., *The proteome of red cell membranes and vesicles during storage in blood bank conditions.* Transfusion, 2008. **48**(5): p. 827-35.
162. Belizaire, R.M., et al., *Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation.* J Am Coll Surg, 2012. **214**(4): p. 648-55; discussion 656-7.
163. Hotchkiss, R.S. and I.E. Karl, *The pathophysiology and treatment of sepsis.* N Engl J Med, 2003. **348**(2): p. 138-50.
164. Yang, K.Y., J.J. Arcaroli, and E. Abraham, *Early alterations in neutrophil activation are associated with outcome in acute lung injury.* Am J Respir Crit Care Med, 2003. **167**(11): p. 1567-74.
165. Biffl, W.L., et al., *Neutrophils are primed for cytotoxicity and resist apoptosis in injured patients at risk for multiple organ failure.* Surgery, 1999. **126**(2): p. 198-202.
166. Caudrillier, A., et al., *Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury.* J Clin Invest, 2012. **122**(7): p. 2661-71.

167. Thomas, G.M., et al., *Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice.* Blood, 2012. **119**(26): p. 6335-43.
168. Arango Duque, G. and A. Descoteaux, *Macrophage cytokines: involvement in immunity and infectious diseases.* Front Immunol, 2014. **5**: p. 491.
169. Xing, L. and D.G. Remick, *Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils.* Shock, 2003. **20**(1): p. 10-6.
170. Ploder, M., et al., *Lipopolysaccharide-induced tumor necrosis factor alpha production and not monocyte human leukocyte antigen-DR expression is correlated with survival in septic trauma patients.* Shock, 2006. **25**(2): p. 129-34.
171. Muszynski, J.A., et al., *Supernatants from stored red blood cell (RBC) units, but not RBC-derived microvesicles, suppress monocyte function in vitro.* Transfusion, 2015. **55**(8): p. 1937-45.
172. Baumgartner, J.M., et al., *Red blood cell supernatant potentiates LPS-induced proinflammatory cytokine response from peripheral blood mononuclear cells.* J Interferon Cytokine Res, 2009. **29**(6): p. 333-8.
173. Wu, W.C., et al., *Blood transfusion in elderly patients with acute myocardial infarction.* N Engl J Med, 2001. **345**(17): p. 1230-6.
174. Mincu, R.I., T. Rassaf, and M. Totzeck, *Red blood cell transfusion in patients with ST-elevation myocardial infarction-a meta-analysis of more than 21,000 patients.* Neth Heart J, 2018. **26**(9): p. 454-460.
175. Goel, R., et al., *Association of Perioperative Red Blood Cell Transfusions With Venous Thromboembolism in a North American Registry.* JAMA Surg, 2018. **153**(9): p. 826-833.
176. Nilsson, K.R., et al., *Association between venous thromboembolism and perioperative allogeneic transfusion.* Arch Surg, 2007. **142**(2): p. 126-32; discussion 133.
177. Hendrickson, J.E., et al., *Coagulopathy is prevalent and associated with adverse outcomes in transfused pediatric trauma patients.* J Pediatr, 2012. **160**(2): p. 204-209 e3.
178. Brascia, D., et al., *Impact of transfusion on stroke after cardiovascular interventions: Meta-analysis of comparative studies.* J Crit Care, 2017. **38**: p. 157-163.
179. Silvain, J., et al., *Impact of red blood cell transfusion on platelet activation and aggregation in healthy volunteers: results of the TRANSFUSION study.* Eur Heart J, 2010. **31**(22): p. 2816-21.
180. Reimers, R.C., S.P. Sutera, and J.H. Joist, *Potentiation by red blood cells of shear-induced platelet aggregation: relative importance of chemical and physical mechanisms.* Blood, 1984. **64**(6): p. 1200-6.
181. Sweeney, J., N. Kouttab, and J. Kurtis, *Stored red blood cell supernatant facilitates thrombin generation.* Transfusion, 2009. **49**(8): p. 1569-79.
182. Xiong, Z., et al., *Red blood cell microparticles show altered inflammatory chemokine binding and release ligand upon interaction with platelets.* Transfusion, 2011. **51**(3): p. 610-21.
183. Fischer, D., et al., *Microparticles from stored red blood cells enhance procoagulant and proinflammatory activity.* Transfusion, 2017. **57**(11): p. 2701-2711.
184. Mackman, N., *Role of tissue factor in hemostasis and thrombosis.* Blood Cells Mol Dis, 2006. **36**(2): p. 104-7.

185. van der Pol, E., et al., *Classification, functions, and clinical relevance of extracellular vesicles.* Pharmacol Rev, 2012. **64**(3): p. 676-705.
186. Silvain, J., et al., *Impact of red blood cell transfusion on platelet aggregation and inflammatory response in anemic coronary and noncoronary patients: the TRANSFUSION-2 study (impact of transfusion of red blood cell on platelet activation and aggregation studied with flow cytometry use and light transmission aggregometry).* J Am Coll Cardiol, 2014. **63**(13): p. 1289-1296.
187. Cognasse, F., et al., *The Inflammatory Role of Platelets via Their TLRs and Siglec Receptors.* Front Immunol, 2015. **6**: p. 83.
188. Smyth, S.S., et al., *Platelet functions beyond hemostasis.* J Thromb Haemost, 2009. **7**(11): p. 1759-66.
189. Wagner, D.D. and P.S. Frenette, *The vessel wall and its interactions.* Blood, 2008. **111**(11): p. 5271-81.
190. Gross, P.L. and W.C. Aird, *The endothelium and thrombosis.* Semin Thromb Hemost, 2000. **26**(5): p. 463-78.
191. Alexander, J.T., et al., *Red blood cells stored for increasing periods produce progressive impairments in nitric oxide-mediated vasodilation.* Transfusion, 2013. **53**(11): p. 2619-2628.
192. Neuman, R., et al., *Effects of storage-aged red blood cell transfusions on endothelial function in hospitalized patients.* Transfusion, 2015. **55**(4): p. 782-90.
193. Urner, M., et al., *Effects of blood products on inflammatory response in endothelial cells in vitro.* PLoS One, 2012. **7**(3): p. e33403.
194. Silliman, C.C., et al., *Supernatants and lipids from stored red blood cells activate pulmonary microvascular endothelium through the BLT2 receptor and protein kinase C activation.* Transfusion, 2017. **57**(11): p. 2690-2700.
195. Anniss, A.M. and R.L. Sparrow, *Storage duration and white blood cell content of red blood cell (RBC) products increases adhesion of stored RBCs to endothelium under flow conditions.* Transfusion, 2006. **46**(9): p. 1561-7.
196. Chin-Yee, I.H., et al., *Transfusion of stored red blood cells adhere in the rat microvasculature.* Transfusion, 2009. **49**(11): p. 2304-10.
197. Anniss, A.M. and R.L. Sparrow, *Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions.* Am J Hematol, 2007. **82**(6): p. 439-45.
198. Eichelbronner, O., et al., *Endotoxin promotes adhesion of human erythrocytes to human vascular endothelial cells under conditions of flow.* Crit Care Med, 2000. **28**(6): p. 1865-70.
199. Mittag, D., et al., *Stored red blood cell susceptibility to in vitro transfusion-associated stress conditions is higher after longer storage and increased by storage in saline-adenine-glucose-mannitol compared to AS-1.* Transfusion, 2015. **55**(9): p. 2197-206.
200. Langer, H.F. and T. Chavakis, *Leukocyte-endothelial interactions in inflammation.* J Cell Mol Med, 2009. **13**(7): p. 1211-20.
201. Keir, A.K., et al., *Plasma cytokines and markers of endothelial activation increase after packed red blood cell transfusion in the preterm infant.* Pediatr Res, 2013. **73**(1): p. 75-9.

202. Muszynski, J.A., et al., *Transfusion-related immunomodulation: review of the literature and implications for pediatric critical illness.* Transfusion, 2017. **57**(1): p. 195-206.
203. Perner, A., et al., *Sepsis: frontiers in diagnosis, resuscitation and antibiotic therapy.* Intensive Care Med, 2016. **42**(12): p. 1958-1969.
204. Sihler, K.C. and L.M. Napolitano, *Anemia of inflammation in critically ill patients.* J Intensive Care Med, 2008. **23**(5): p. 295-302.
205. Vincent, J.L., et al., *Sepsis definitions: time for change.* Lancet, 2013. **381**(9868): p. 774-5.
206. Hotchkiss, R.S., G. Monneret, and D. Payen, *Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy.* Nat Rev Immunol, 2013. **13**(12): p. 862-74.
207. Rock, K.L., et al., *The sterile inflammatory response.* Annu Rev Immunol, 2010. **28**: p. 321-42.
208. Huber-Lang, M., J.D. Lambris, and P.A. Ward, *Innate immune responses to trauma.* Nat Immunol, 2018. **19**(4): p. 327-341.
209. Marik, P.E. and M. Flemmer, *The immune response to surgery and trauma: Implications for treatment.* J Trauma Acute Care Surg, 2012. **73**(4): p. 801-8.
210. Shankar Hari, M. and C. Summers, *Major surgery and the immune system: from pathophysiology to treatment.* Curr Opin Crit Care, 2018. **24**(6): p. 588-593.
211. Boomer, J.S., et al., *Immunosuppression in patients who die of sepsis and multiple organ failure.* JAMA, 2011. **306**(23): p. 2594-605.
212. Muszynski, J.A., R. Thakkar, and M.W. Hall, *Inflammation and innate immune function in critical illness.* Curr Opin Pediatr, 2016. **28**(3): p. 267-73.
213. Nacionales, D.C., et al., *The acute immunological response to blood transfusion is influenced by polymicrobial sepsis.* Shock, 2012. **38**(6): p. 598-606.
214. Gajic, O., et al., *Transfusion-related acute lung injury in the critically ill: prospective nested case-control study.* Am J Respir Crit Care Med, 2007. **176**(9): p. 886-91.
215. Vlaar, A.P. and N.P. Juffermans, *Transfusion-related acute lung injury: a clinical review.* Lancet, 2013. **382**(9896): p. 984-94.
216. Aiboshi, J., et al., *Blood transfusion and the two-insult model of post-injury multiple organ failure.* Shock, 2001. **15**(4): p. 302-6.
217. Sachs, U.J., *Pathophysiology of TRALI: current concepts.* Intensive Care Med, 2007. **33 Suppl 1**: p. S3-S11.
218. Silliman, C.C., *The two-event model of transfusion-related acute lung injury.* Crit Care Med, 2006. **34**(5 Suppl): p. S124-31.
219. Bux, J. and U.J. Sachs, *The pathogenesis of transfusion-related acute lung injury (TRALI).* Br J Haematol, 2007. **136**(6): p. 788-99.

CHAPTER 2

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The ability of extracellular vesicles to induce a pro-inflammatory host response

ABSTRACT

Extracellular vesicles (EVs) can modulate the host immune response, executing both pro- and anti-inflammatory effects. As EVs increasingly gain attention as potential carriers for targeted gene and drug delivery, knowledge on the effects of EVs on the host immune response is important. This review will focus on the ability of EVs to trigger a pro-inflammatory host response by activating target cells. The overall view is that EVs can augment an inflammatory response, thereby potentially contributing to organ injury. This pro-inflammatory potential of EVs may hamper its use for therapeutic drug delivery. Whether removal of EVs as a means to reduce a pro-inflammatory or pro-coagulant response during hyper-inflammatory conditions is beneficial remains to be determined. Prior to any proposed therapeutic application, there is a need for further studies on the role of EVs in physiology and pathophysiology using improved detection and characterization methods to elucidate the roles of EVs in inflammatory conditions.

INTRODUCTION

Extracellular vesicles (EVs), including microparticles and exosomes, are phospholipid-enclosed vesicles of less than 100 nm, diameter often 100-fold smaller than the smallest cells. EVs are released by red blood cells (RBCs), platelets, white blood cells and endothelial cells, and are involved in a broad spectrum of biological activities. Because all cells release EVs into the environment, all body fluids contain high concentrations of cell-derived EVs.

There is increasing evidence that EVs may have specialized functions and play a role in intercellular signaling, for example, by exchanging biomolecules as transmembrane receptors and genetic information [1]. Additionally, EVs may protect cells from the accumulation of intracellular waste. Furthermore, EVs may expose phosphatidyl serine, a negatively-charged phospholipid to which activated coagulation factors can bind, and tissue factor, the protein that initiates coagulation.

During disease states the concentration of EVs, their cellular origin, composition, and functional features may change, thereby affecting disease development and progression. At present, however, the reason why cells release EVs is unknown. As the majority of studies on EVs have been performed in vitro, we can only speculate about the clinical relevance. Furthermore, due to recent improvements in isolation methods of EVs, components previously associated with EVs may need to be reconsidered to be isolation artefacts [2]. Despite these considerable knowledge gaps, endogenously released EVs probably should not be regarded as having only “good” or “bad” effects. To further complicate the interpretation of the relevance of EVs, EVs themselves can also be used by micro-organisms to facilitate spreading and to escape from immune surveillance.

Given the ability of EVs to exchange information between cells, as well as between cells and their microenvironment, the use of EVs in cancer vaccines and drug delivery is increasingly gaining attention. As the natural carrier of signal molecules, EVs may be an attractive vehicle for therapeutic delivery, with a presumed low toxicity. Potential areas of application include cancer [3] and cardiovascular diseases [4]. However, EVs can also modulate the host immune response, executing both pro- and anti-inflammatory effects, which may offset any potential beneficial effects of EVs used as carriers. Thereby, knowledge on the effects of EVs on the host immune response is important. This review will focus on the ability of EVs to trigger a pro-inflammatory host response by activating target cells. In experimental models

of hyper-inflammatory disease states, e.g., following ischemia-reperfusion injury, the pro-inflammatory potential of EVs aggravates organ damage. Whether EVs modulate organ damage in patients remains to be determined.

Inflammatory effects of extracellular vesicles (EVs)

Extracellular vesicles (EV)-produced inflammatory mediators

EVs have the ability to modulate the immune system by transferring receptors and inflammatory mediators (Table 1). For example, EVs are essential for the release of interleukin (IL)-1β [5]. Interleukin-1-containing EVs from monocytes activate endothelial cells and stimulate the production of IL-1β from monocytes in an autocrine fashion [6]. In line with this, upon stimulation, macrophages and dendritic cells release vesicles containing IL-1β, caspase-1, and other components of the inflammasome [7]. Furthermore, EVs contain platelet-activating factor [8] and can expose (tumor necrosis factor) TNF receptor 1 [9]. To which extent these inflammatory mediators associated with EVs represent a major fraction of the total amount of such mediators released from cells, however, remains to be shown.

Table 1. Pro-inflammatory effects of endogenous extra-cellular vesicles from immune cells and endothelial cells.

Cellular EV origin	Target cell	Inflammatory effect	Type of study	Reference
RBC	Whole blood	Production of TNFα, IL-6, IL-8	Ex vivo	Straat [10]
RBC	Granulocytes	Respiratory burst	In vitro, in vivo	Belizaire [11]
RBC	-	Leukocyte homing	In vivo	Zecher [12]
RBC	Monocytes	Binding and phagocytosis	In vitro	Straat [13]
RBC	Endothelial cells	Expression of ICAM-1, E-selectin	In vitro	Straat [13]
Monocyte	Monocyte	IL-1b production	In vitro	McKenzie [5]
Monocyte	Endothelial cells	IL-1b production	In vitro	Wang [6]
Monocyte	Endothelial cells	Expression of ICAM-1, VCAM-1, E- selectin	In vitro	Wang [6], Halim [14]
Monocytes	Endothelial cell	Induction nitrosative stress	In vitro	Mastronardi [15]
Macrophages	Macrophages	Activate TLR-4, TNF production	In vitro	Thomas [16]
Macrophages	-	IL-1, caspase-1 production	In vitro	Qu [7]
Marcophages, Dendritic cells	-	Leukotrienes synthesis, granulocyte migration	In vitro	Esser [17]
Macrophages	Hepatocytes	TNF production	In vitro	Teoh [18]

Table 1. Continued.

Cellular EV origin	Target cell	Inflammatory effect	Type of study	Reference
Granulocytes	-	PAF production	In vitro	Watanabe [8] Mesri [19]
Granulocytes	Endothelial cells	TF and IL-6 production	In vitro	Mesri [19]
Granulocytes	Red blood cells	Complement activation	In vitro	Gasser [20]
T cells	Monocytes	TNFα, IL-6, sIL-1RA production	In vitro	Scanu [21]
T cells	Endothelial cells	NO synthase, COX-2 production,	In vitro, in vivo	Martin [22]
Platelets	Endothelial cells	COX-2 production	In vitro	Barry
Platelets	Endothelial cells	PAF production	In vitro	Wolf [23]
Platelets	Endothelial cells	CD11b expression	In vitro	Xie [24]
Platelets	-	Thrombin generation	In vivo	Mooberry [25]
Platelets	Whole blood	Production IL-6, TNFα	Ex vivo	Balvers [26]
Endothelial cells	Endothelial cells	Transfer miRNA	In vitro	Jansen [27]
Endothelial cells	Endothelial cells	Adherence monocytes, expression of ICAM-1	In vitro	Lee [28]
Endothelial cells	Endothelial cells	IP-10 production	In vitro	Liu [29]

EV: extracellular vesicle, TNF: tumor necrosis factor, TF: tissue factor, IL: inter-leukin, ICAM: intercellular adhesion molecule, VCAM: vascular cell adhesion molecule, TLR: Toll-like receptor, PAF: platelet activating factor.

EVs can contribute to an inflammatory response via their lipid fraction, as this fraction can activate the Toll-like receptor (TLR) 4 on macrophages [16]. The ability of EVs to activate TLR4 is impaired after incubation with an inhibitor of phospholipase D, confirming that the lipid component of EVs may contribute to the inflammatory response. In addition, EVs from dendritic cells, macrophages and plasma contain enzymes involved in synthesis of leukotrienes [17]. Additionally, RBC-derived EVs can promote the secretion of the von Willebrand factor by endothelial cells [13], which is a plasma ligand for cell adhesion. An increase in the von Willebrand factor may thereby promote cellular adhesion and augment inflammation. In vitro, it was shown that RBC-derived EVs can interact with platelets to increase inflammatory chemokine bioavailability [30]. Taken together, EVs can exert direct pro-inflammatory effects by the production of pro-inflammatory mediators, without the interaction of immune cells.

EV-mediated pro-inflammatory responses of effector cells in the circulation

EVs can directly interact with different cell types, inducing a functional inflammatory response from immune cells. Incubation of whole blood with RBC-derived EVs induced a dose-dependent production of (TNF)α, IL-6, and IL-8 [10]. The response of cells to EVs was very strong, equaling the response to incubation with TNFα. EVs can directly interact with various types of cells.

RBC-derived EVs can dose-dependently trigger human neutrophils to increase production of CD11b [31], induce a respiratory burst, and increase the ability of neutrophils to phagocytose. Similar results were obtained in a murine model, in which injection of RBC-derived EVs into healthy mice primed neutrophils, as reflected by an increased expression of neutrophil CD11b [11]. RBC-derived EVs can also have a direct interaction with monocytes. Using confocal microscopy, it was shown that RBC-derived EVs are phagocytized by monocytes, a process that is partially inhibited by incubation with antibodies directed against complement receptor 3.

Monocyte-derived EVs also have largely pro-inflammatory effects, mostly through interaction with endothelial cells, but also with other cells, including monocytes themselves, fibroblasts, and smooth muscle cells [14]. Monocyte-derived EVs interact with the endothelium, inducing expression of adhesion molecules [6] as well as nitrosative stress [15]. Monocyte-derived EVs can induce production of pro-inflammatory mediators MCP-1 and IL-6 from podocytes, associated with increased levels of a marker of glomerular permeability, indicating glomerular inflammation [32]. Additionally, monocyte-derived EVs containing caspases are capable of inducing apoptosis in endothelial cells [33] and in vascular smooth muscle cells [34].

Neutrophil-derived EVs are the least studied EVs to date. It is clear that levels of circulating neutrophil-derived EVs are increased under inflammatory conditions, which holds true for auto-immune conditions, asthma, and severe infections [35, 36]. Neutrophil-derived EVs can mediate binding of neutrophils to red blood cells following complement activation [20]. T and B cell-derived EVs have been mostly described for their interaction with tumor cells, although pro-inflammatory effects of T cells have been demonstrated [21, 22].

Platelet-derived EVs have mostly been implicated in coagulation, but can also exert pro-inflammatory effects. Platelet-derived EVs from synovial fluid from patients with rheumatoid arthritis increased production of inflammatory cytokines in fibroblast-like synoviocytes in an IL-1-dependent manner [37].

Staphylococcal-induced formations of platelet-EVs were also able to stimulate monocytes to produce IL-1β, TNFα, and MCP-1 [38]. However, platelet-derived EVs also have anti-inflammatory effects. T cells exposed to platelet-derived EVs had reduced the production of interferon γ (IFNγ), TNFα, and IL-6 secretion [39]. Other pro-inflammatory effects of platelet-derived EVs were recently summarized [40].

EV-mediated inflammatory responses of endothelial cells

EVs activate endothelial cells via different pathways. Neutrophil-derived EVs trigger endothelial cells to secrete IL-6 and to produce tissue factor (TF) [19]. Monocyte-derived EVs activate endothelial cells, reflected by an increase in the release of EVs and an increase in production of adhesion markers, thus amplifying the inflammatory process [14]. In contrast, RBC-derived EVs alone do not induce the production of endothelial adhesion markers, but when endothelial cells are incubated with RBC-derived EVs in the presence of monocytes, expression of ICAM-1 and E-selectin increase compared to endothelial cells incubated with EV-depleted RBCs [13]. RBC-derived EVs do not activate endothelial cells when co-incubated with neutrophils, showing that the effect is specific for monocytes.

Additionally, platelet-derived EVs activate endothelial cells. These EVs contain arachidonic acid, which is transferred to the endothelial cells and then promotes the production of cyclooxygenase-2 and adhesion molecules [23, 41]. In an in vitro model of lipopolysaccharide (LPS)-stimulated endothelial cells, the addition of platelet-derived EVs increased endothelial activation and induced the respiratory burst in neutrophils [24]. Furthermore, platelet-derived EVs also modulate interactions between monocytes and endothelial cells. The interaction between EVs and the endothelium may be amplified by the release of pro-inflammatory cytokines [42] and platelet activating factor [23] from endothelial cells. In turn, endothelial-derived EVs further activate the endothelium, thus further perpetuating the inflammatory response [29]. Taken together, EVs seem to mediate increased adherence of immune cells to the endothelium with the induction of a pro-inflammatory response.

EV-mediated inflammatory responses in effector cells in tissues

EVs in body fluids or tissues can modulate the local inflammatory responses. During rheumatoid arthritis, EVs from synovial fluid trigger the production of pro-inflammatory mediators by synovial fibroblasts, via the transfer of arachidonic acid from leukocytes to fibroblasts, thereby contributing to the destructive activity of fibroblasts [43]. In adipose tissue, EVs from adipocytes increased

CD16 and CCR5 production on monocytes, thereby inducing monocyte migration into the adipose tissue, contributing to the chronic inflammatory phenotype in obesity [44]. In a model of traumatic brain injury, microglial-derived EVs are released into the circulation [45]. These EVs initiated neuroinflammation after injection into the cortex of healthy animals. In addition, EVs derived from monocytes also promote inflammation by interaction with endothelial cells, monocytes, fibroblasts, and smooth muscle cells [14]. Taken together, there is increasing evidence suggesting that EVs from different cellular sources act in concert with the endothelium in inducing and perpetuating inflammatory host responses.

EV-mediated pro-coagulant response

One of the first identified roles of EVs has been its participation in coagulation. RBC-derived EVs can expose PS, a negatively-charged phospholipid to which (activated) coagulation factors can bind, thereby promoting thrombin generation [1]. Another pathway by which RBC-EVs may promote coagulation is that these EVs may be a source of von Willebrand factor [13]. Endothelial- and monocyte-derived EVs may also support coagulation by the expression of tissue factor (TF). The amount of TF bearing monocyte- and platelet-derived EVs have been related to infarction severity in patients [46]. Additinoally, in cancer patients, the levels of tumor-derived EVs exposing TF are associated with the development of venous thromboembolism, although cause and effect are unclear [47]. Together, in patients with an increased risk, EVs are associated with thrombotic events.

Increased coagulation activation is a hallmark of a pro-inflammatory response. The endothelium undergoes pro-thrombotic changes and platelets become activated, exhibiting adhesive properties. In diffuse intravascular coagulation (DIC), there is formation of microthrombi with the consumption of platelets and coagulation factors, associated with adverse outcome [48]. EVs probably also play a role in the hyper-coagulative response in inflammation. In human endotoxemia, the levels of total EVs and platelet-derived EVs are increased, bearing TF, showing increased thrombin generating activity [25]. In patients with septic shock, the systemic levels of TF-exposing EVs are increased [49]. Additionally, in severe sepsis patients, endothelial-derived EVs are associated with the occurrence of DIC [50], suggesting that EVs play a role in clinically-relevant coagulation disorders in sepsis. EVs bearing TF derived from tissues also contribute to local inflammation. In patients with acute lung injury, the concentrations of TF-bearing EVs in the lung are higher compared to controls [51]. Furthermore, in patients with sterile inflammation due to

arthritis, TF-exposing EVs are present in joint fluid of inflamed joints [52]. Taken together, EVs can promote coagulation and are often pro-coagulant. In particular, platelet-derived EVs are associated with thrombotic events in patients at risk, whereas EVs from all sources can contribute to inflammation-induced coagulation.

From a therapeutic point of view, the role of EVs in disease states characterized by a reduced ability to clot is also interesting. Under these conditions, the amount and activity of EVs seem to correlate with the ability to clot. In coagulopathic bleeding trauma patients, the levels of platelet-derived EVs were lower compared to minimally-injured control patients, and were associated with reduced thrombin generating capacity [53]. Given that the levels of RBC- and leukocyte-derived EVs did not differ between groups, it has been suggested that in particular decreased levels of platelet-derived EVs may contribute to an anti-coagulant phenotype. Findings in a murine model of traumatic brain injury (TBI) also underline that low levels of circulating platelet-derived EVs are associated with anti-coagulant activity [54]. Mice showed impaired clot formation following head injury, which was partially restored by the addition of platelet-derived EVs from healthy donor mice to the blood of the mice who had sustained TBI. Whether platelet-derived EVs could be a potential therapeutic target to modulate coagulation responses remains to be determined.

EV-mediated host response in inflammatory disease states

In most models of sterile inflammation, EVs seem to perpetuate organ injury. In a mouse model of hepatic ischemia-reperfusion injury, endothelial cells, platelets, and T cells release EVs into the circulation, containing F2-isoprostanes, indicating oxidative damage to membrane lipids [18]. In particular, EVs from macrophages and platelets were able to trigger TNFα release from hepatocytes, thereby perpetuating hepatic injury, a process that is ROS-dependent. Similarly, in a mouse model of hemorrhage and resuscitation, injection of RBC-derived EVs increased pulmonary neutrophil accumulation and histologic evidence of lung injury compared with mice resuscitated without EVs [11]. Additionally, in an endotoxemia mouse model, injection of RBC-derived EVs aggravated leukocyte recruitment to the lungs and increased levels of proinflammatory cytokines, which was abrogated in C5aR-deficient mice, suggesting that RBC-derived EVs are pro-inflammatory via complement activation [12].

In sepsis, the contribution of EVs to inflammation is probably more complicated. EVs from different cellular origin may play a role at multiple sites, as both the activated endothelium releases EVs which may communicate with immune

cells, and EVs shed from immune cells interact with the endothelium. As discussed above, the levels of circulating EVs from platelets, leukocytes, and endothelial cells are increased in sepsis. EVs promote the adhesion of platelets and/or leukocytes to the endothelium, thus playing the role of trigger in the production of pro-inflammatory cytokines such as IL-1β, IL-8, and TNFα [41]. Of note, it is not only EVs from the host immune response that drive the inflammatory response in sepsis. In vitro, staphylococcal super antigen-like protein 5 (SSL5) from *Staphylococcus aureus* dose-dependently induced the generation of platelet-derived EVs, which then bind to monocytes, causing platelet aggregation and release of inflammatory mediators IL-1β, TNFα, and MCP-1 [38]. These EVs induced by *S. aureus* also enhanced monocyte migration. Taken together, there seems to be a role for EVs in perpetuating the inflammatory response in sepsis.

EVs play an important role in trauma, a state which is also characterized by a hyper-inflammatory host response with activated endothelium, together with immune suppression, as demonstrated by a markedly reduced ability of immune cells to generate a pro-inflammatory response. Circulating EVs may have several functions. Low levels of platelet-derived EVs correlate with disturbed coagulation potential in trauma, as described before. In addition, EVs regulate the host response in trauma. The reduced immune response towards endotoxin challenge in trauma is associated with low levels of circulating EVs in trauma compared to controls [26]. The host response to endotoxin was restored by the addition of EVs, suggesting that these EVs drive the synthesis of pro-inflammatory cytokines in response to LPS [26]. Most circulating endogenous EVs are derived from platelets, suggesting that platelets are the most important source of EVs involved in host response post-injury. Whether exogenously-added EVs could boost an immune response in trauma remains to be determined.

EVs also have anti-inflammatory effects

The anti-inflammatory effects are not the focus of this review and are, therefore, not discussed in detail. However, in the discussion that pro-inflammatory effects from EVs may be hurdle from a therapeutic viewpoint, it needs to be acknowledged that EVs can also exert anti-inflammatory effects, which seem to be primarily mediated by neutrophil-derived EVs. Neutrophil-derived EVs induce downregulation of the transcription of pro-inflammatory cytokines [55] and the release of stored TGF-β1 [56] from macrophages. Leukocyte-derived EVs isolated from sepsis patients increased the phagocytic capacity of cells [14]. Similarly, neutrophil-derived EVs from sepsis patients induced monocyte phagocytosis ex vivo [57]. In an infectious sepsis mouse model, injection of leukocyte-derived EVs had anti-inflammatory effects associated with immune dysfunction, characterized by an increased bacterial load, decreased neutrophil

recruitment, increased expression of IL-10 and a negative effect on mortality [35]. Furthermore, platelet-derived EVs have anti-inflammatory effects. T cells exposed to platelet-derived EVs had reduced production of interferon γ (IFNγ), TNFα, and IL-6 secretion [39]. Other anti-inflammatory effects of platelet-derived EVs were recently summarized [40].

CONCLUSION

EVs act by triggering signaling pathways and exchanging information, and thereby have gained interest as carriers for targeted drug delivery. When contemplating the use of EVs as carriers, it should be taken into account that EVs can augment an inflammatory response, thereby contributing to organ injury. Even though it should be noted that most of these effects of EVs have been studied in vitro and the clinical relevance of these findings is highly unclear, this pro-inflammatory potential of EVs may hamper its use as carriers in various conditions, which may particularly hold true for sterile inflammatory conditions.

Removal of EVs as a method to reduce a pro-inflammatory response during hyper-inflammatory conditions, may be an alternative potential beneficial intervention. When considering the pro-coagulant response, the association between platelet-derived EVs and DIC in sepsis seems a consistent finding throughout the studies. Whether these EVs could be removed, e.g., by filtering, to treat DIC in sepsis without increasing the risk of bleeding is, however, undecided.

The use of EVs to restore an inhibited host response is another intriguing potential application. In this concept, the timing of intervention is probably crucial. Sepsis is characterized by both hyper-inflammation, as well as by immune tolerance, i.e., the inability of immune cells to respond to endotoxins. These conditions occur simultaneously but probably not in a balanced fashion, and also with a highly variable phenotype between patients [58]. Additionally, trauma is characterized by a pro-inflammatory response coinciding with a decreased ability to respond to bacterial antigens. In trauma patients, addition of EVs has been shown to restore cellular reactivity to LPS in an ex vivo design [26]. Whether EVs can be used to "boost" the immune system during a state of reduced host immune response needs further investigation.

Taken together, EVs have clearly entered the scene. Since these vesicles are small and difficult to study [2], recent and still ongoing efforts to improve isolation, detection, and characterization will further elucidate their roles in physiology and pathology.

REFERENCES

1. van der Pol, E., et al., *Classification, functions, and clinical relevance of extracellular vesicles.* Pharmacol Rev, 2012. **64**(3): p. 676-705.
2. Coumans, F.A.W., et al., *Methodological Guidelines to Study Extracellular Vesicles.* Circ Res, 2017. **120**(10): p. 1632-1648.
3. Inamdar, S., R. Nitiyanandan, and K. Rege, *Emerging applications of exosomes in cancer therapeutics and diagnostics.* Bioeng Transl Med, 2017. **2**(1): p. 70-80.
4. Xitong, D. and Z. Xiaorong, *Targeted therapeutic delivery using engineered exosomes and its applications in cardiovascular diseases.* Gene, 2016. **575**(2 Pt 2): p. 377-384.
5. MacKenzie, A., et al., *Rapid secretion of interleukin-1beta by microvesicle shedding.* Immunity, 2001. **15**(5): p. 825-35.
6. Wang, J.G., et al., *Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner.* Blood, 2011. **118**(8): p. 2366-74.
7. Qu, Y., et al., *Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages.* J Immunol, 2007. **179**(3): p. 1913-25.
8. Watanabe, J., et al., *Endotoxins stimulate neutrophil adhesion followed by synthesis and release of platelet-activating factor in microparticles.* J Biol Chem, 2003. **278**(35): p. 33161-8.
9. Hawari, F.I., et al., *Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: a mechanism for generation of soluble cytokine receptors.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1297-302.
10. Straat, M., et al., *Extracellular Vesicles from Red Blood Cell Products Induce a Strong Pro-Inflammatory Host Response, Dependent on Both Numbers and Storage Duration.* Transfus Med Hemother, 2016. **43**(4): p. 302-305.
11. Belizaire, R.M., et al., *Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation.* J Am Coll Surg, 2012. **214**(4): p. 648-55; discussion 656-7.
12. Zecher, D., A. Cumpelik, and J.A. Schifferli, *Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement.* Arterioscler Thromb Vasc Biol, 2014. **34**(2): p. 313-20.
13. Straat, M., et al., *Monocyte-mediated activation of endothelial cells occurs only after binding to extracellular vesicles from red blood cell products, a process mediated by beta-integrin.* Transfusion, 2016. **56**(12): p. 3012-3020.
14. Halim, A.T., N.A. Ariffin, and M. Azlan, *Review: the Multiple Roles of Monocytic Microparticles.* Inflammation, 2016. **39**(4): p. 1277-84.
15. Mastronardi, M.L., et al., *Microparticles from apoptotic monocytes enhance nitrosative stress in human endothelial cells.* Fundam Clin Pharmacol, 2011. **25**(6): p. 653-60.
16. Thomas, L.M. and R.D. Salter, *Activation of macrophages by P2X7-induced microvesicles from myeloid cells is mediated by phospholipids and is partially dependent on TLR4.* J Immunol, 2010. **185**(6): p. 3740-9.

17. Esser, J., et al., *Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration.* J Allergy Clin Immunol, 2010. **126**(5): p. 1032-40, 1040 e1-4.
18. Teoh, N.C., et al., *Microparticles mediate hepatic ischemia-reperfusion injury and are the targets of Diannexin (ASP8597).* PLoS One, 2014. **9**(9): p. e104376.
19. Mesri, M. and D.C. Altieri, *Endothelial cell activation by leukocyte microparticles.* J Immunol, 1998. **161**(8): p. 4382-7.
20. Gasser, O. and J.A. Schifferli, *Microparticles released by human neutrophils adhere to erythrocytes in the presence of complement.* Exp Cell Res, 2005. **307**(2): p. 381-7.
21. Scanu, A., et al., *Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes: differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins.* J Leukoc Biol, 2008. **83**(4): p. 921-7.
22. Martin, S., et al., *Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression.* Circulation, 2004. **109**(13): p. 1653-9.
23. Wolf, P., et al., *Platelet-activating factor is crucial in psoralen and ultraviolet A-induced immune suppression, inflammation, and apoptosis.* Am J Pathol, 2006. **169**(3): p. 795-805.
24. Xie, R.F., et al., *Platelet-derived microparticles induce polymorphonuclear leukocyte-mediated damage of human pulmonary microvascular endothelial cells.* Transfusion, 2015. **55**(5): p. 1051-7.
25. Mooberry, M.J., et al., *Procoagulant microparticles promote coagulation in a factor XI-dependent manner in human endotoxemia.* J Thromb Haemost, 2016. **14**(5): p. 1031-42.
26. Balvers, K., et al., *Endogenous microparticles drive the proinflammatory host immune response in severely injured trauma patients.* Shock, 2015. **43**(4): p. 317-21.
27. Jansen, F., et al., *Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles.* Circulation, 2013. **128**(18): p. 2026-38.
28. Lee, S.K., et al., *Role of tumour necrosis factor receptor-1 and nuclear factor-kappaB in production of TNF-alpha-induced pro-inflammatory microparticles in endothelial cells.* Thromb Haemost, 2014. **112**(3): p. 580-8.
29. Liu, Y., et al., *Endothelial microparticles activate endothelial cells to facilitate the inflammatory response.* Mol Med Rep, 2017. **15**(3): p. 1291-1296.
30. Xiong, Z., et al., *Red blood cell microparticles show altered inflammatory chemokine binding and release ligand upon interaction with platelets.* Transfusion, 2011. **51**(3): p. 610-21.
31. Kent, M.W., et al., *The pro-inflammatory potential of microparticles in red blood cell units.* Transfus Med, 2014. **24**(3): p. 176-81.
32. Eyre, J., et al., *Monocyte- and endothelial-derived microparticles induce an inflammatory phenotype in human podocytes.* Nephron Exp Nephrol, 2011. **119**(3): p. e58-66.
33. Smit, K.F., et al., *Effects of helium on inflammatory and oxidative stress-induced endothelial cell damage.* Exp Cell Res, 2015. **337**(1): p. 37-43.

34. Sarkar, A., et al., *Monocyte derived microvesicles deliver a cell death message via encapsulated caspase-1.* PLoS One, 2009. **4**(9): p. e7140.
35. Johnson, B.L., 3rd, et al., *Neutrophil derived microparticles increase mortality and the counter-inflammatory response in a murine model of sepsis.* Biochim Biophys Acta Mol Basis Dis, 2017. **1863**(10 Pt B): p. 2554-2563.
36. Mazzeo, C., et al., *Exosome secretion by eosinophils: A possible role in asthma pathogenesis.* J Allergy Clin Immunol, 2015. **135**(6): p. 1603-13.
37. Boilard, E., et al., *Platelets amplify inflammation in arthritis via collagen-dependent microparticle production.* Science, 2010. **327**(5965): p. 580-3.
38. Bei, J.J., et al., *Staphylococcal SSL5-induced platelet microparticles provoke proinflammatory responses via the CD40/TRAF6/NFkappaB signalling pathway in monocytes.* Thromb Haemost, 2016. **115**(3): p. 632-45.
39. Sadallah, S., et al., *Ectosomes released by platelets induce differentiation of CD4+T cells into T regulatory cells.* Thromb Haemost, 2014. **112**(6): p. 1219-29.
40. Edelstein, L.C., *The role of platelet microvesicles in intercellular communication.* Platelets, 2017. **28**(3): p. 222-227.
41. Meziani, F., et al., *Bench-to-bedside review: circulating microparticles--a new player in sepsis?* Crit Care, 2010. **14**(5): p. 236.
42. Nomura, S., et al., *Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines.* Thromb Res, 2000. **98**(4): p. 257-68.
43. Berckmans, R.J., et al., *Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes.* Arthritis Res Ther, 2005. **7**(3): p. R536-44.
44. Renovato-Martins, M., et al., *Microparticles derived from obese adipose tissue elicit a pro-inflammatory phenotype of CD16(+), CCR5(+) and TLR8(+) monocytes.* Biochim Biophys Acta Mol Basis Dis, 2017. **1863**(1): p. 139-151.
45. Kumar, A., et al., *Microglial-derived microparticles mediate neuroinflammation after traumatic brain injury.* J Neuroinflammation, 2017. **14**(1): p. 47.
46. Chiva-Blanch, G., et al., *Platelet-, monocyte-derived and tissue factor-carrying circulating microparticles are related to acute myocardial infarction severity.* PLoS One, 2017. **12**(2): p. e0172558.
47. Gardiner, C., et al., *Extracellular vesicles, tissue factor, cancer and thrombosis - discussion themes of the ISEV 2014 Educational Day.* J Extracell Vesicles, 2015. **4**: p. 26901.
48. Ono, T., et al., *Severe secondary deficiency of von Willebrand factor-cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: its correlation with development of renal failure.* Blood, 2006. **107**(2): p. 528-34.
49. Nieuwland, R., et al., *Cellular origin and procoagulant properties of microparticles in meningococcal sepsis.* Blood, 2000. **95**(3): p. 930-5.
50. Delabranche, X., et al., *Microparticles are new biomarkers of septic shock-induced disseminated intravascular coagulopathy.* Intensive Care Med, 2013. **39**(10): p. 1695-703.

51. Bastarache, J.A., et al., *Procoagulant alveolar microparticles in the lungs of patients with acute respiratory distress syndrome.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(6): p. L1035-41.
52. Berckmans, R.J., et al., *Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism.* Arthritis Rheum, 2002. **46**(11): p. 2857-66.
53. Matijevic, N., et al., *Cellular microparticle and thrombogram phenotypes in the Prospective Observational Multicenter Major Trauma Transfusion (PROMMTT) study: correlation with coagulopathy.* Thromb Res, 2014. **134**(3): p. 652-8.
54. Midura, E.F., et al., *Microparticles impact coagulation after traumatic brain injury.* J Surg Res, 2015. **197**(1): p. 25-31.
55. Eken, C., et al., *Ectosomes released by polymorphonuclear neutrophils induce a MerTK-dependent anti-inflammatory pathway in macrophages.* J Biol Chem, 2010. **285**(51): p. 39914-21.
56. Eken, C., et al., *Ectosomes of polymorphonuclear neutrophils activate multiple signaling pathways in macrophages.* Immunobiology, 2013. **218**(3): p. 382-92.
57. Prakash, P.S., et al., *Human microparticles generated during sepsis in patients with critical illness are neutrophil-derived and modulate the immune response.* J Trauma Acute Care Surg, 2012. **73**(2): p. 401-6; discussion 406-7.
58. van Vught, L.A., et al., *The Host Response in Patients with Sepsis Developing Intensive Care Unit-acquired Secondary Infections.* Am J Respir Crit Care Med, 2017. **196**(4): p. 458-470.

CHAPTER 3

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Pathogenesis of non-antibody mediated transfusion-related acute lung injury from bench to bedside

ABSTRACT

Transfusion-related acute lung injury (TRALI) is a major cause of transfusion-related mortality. Causative factors are divided in antibody mediated TRALI and non-antibody mediated TRALI. Antibody mediated TRALI is caused by passive transfusion of cognate antibodies and non-antibody mediated TRALI is caused by transfusion of aged cellular blood products. This review focuses on mechanisms in non-antibody mediated TRALI which includes soluble mediators accumulating during storage of red blood cells (RBCs) and platelets (PLTs), as well as changes in morphology and function of aged PLTs and RBCs. These mediators cause TRALI in two-hit animal models and have been implicated in TRALI onset in clinical studies. Pre-clinical studies show a clear relation between TRALI and increased storage time of cellular blood products. Observational clinical studies however report conflicting data. Knowledge of pathophysiological mechanisms of TRALI is necessary to improve storage conditions of blood products, develop prevention strategies and develop a therapy for TRALI.

INTRODUCTION

Transfusion-related acute lung injury (TRALI), a syndrome of respiratory distress caused by transfusion of blood products, is a major cause of transfusion-related mortality [1-4]. In the absence of biomarkers, TRALI is defined according to the TRALI conference and US National Heart, Lung and Blood Institute definition as onset of acute lung injury within 6 hours of blood transfusion without an additional risk factor for acute lung injury (Table 1) [2, 3, 5]. Patients suffering TRALI develop symptoms of dyspnea, fever, hypotension and sometimes hypertension, hypoxia, passing leukopenia, thrombopenia and bilateral infiltrates on chest X-ray. Typical post-mortem findings in the lung of patients who die of TRALI are pulmonary edema, diffuse alveolar damage, hyaline membrane formation and extensive granulocyte infiltration and aggregation in the alveoli [6, 7]. TRALI incidence varies between 0.08-15.1% per patient transfused and 0.01-1.12% per product transfused. Critically ill patients bear an increased risk for developing TRALI after blood transfusion [8, 9]. The high incidence of TRALI in critically ill patients can be explained in light of the "two-hit hypothesis": a "first hit", an underlying clinical condition of the patient, causes priming of the pulmonary neutrophil. The "second hit", the transfusion of a cellular blood product, causes activation of the neutrophils in the pulmonary compartment resulting in TRALI. Hematogical malignancy, cardiovascular disease, sepsis, fluid overload, emergency cardiac surgery, massive transfusion, mechanical ventilation, high APACHE-II score, increasing age, shock, alcohol dependence kidney failure and severe liver disease have been identified as risk factors for a "first hit" [8, 10-14].

Originally, infusion of cognate donor antibodies of the human leucocyte antigens (HLA) and human neutrophil alloantigen (HNA) was recognized as causative factor in TRALI. In these cases antigen-antibody interaction led to activation of primed neutrophils, so called antibody-mediated TRALI [15, 16]. However, antibodies are not involved in all cases fulfilling the clinical definition of TRALI [17-19] and recipients of a product which contains antibodies do not always develop TRALI, even in the presence of the cognate antigen [20]. This resulted in the hypothesis of non-antibody mediated TRALI. The causative agent in non-antibody mediated TRALI is still unknown. Several mechanisms have been proposed in which accumulation of cell derived substances in donor blood and storage related changes of the donor red blood cells (RBCs) and donor platelets (PLTs) cause TRALI [18]. This review summarizes evidence on the pathogenesis of non-antibody-mediated TRALI from both preclinical and clinical studies. Given the association between TRALI and

adverse outcome in several patient populations [13, 21], identification of causative factors is paramount to develop prevention and treatment strategies for TRALI. Knowledge of pathophysiological mechanisms of TRALI is necessary to improve storage conditions of blood products and refine transfusion policies.

Table 1. Definition transfusion-related acute lung injury (TRALI).

TRALI	Acute onset within 6 hours of blood transfusion PaO2 /FI O2 <300 mm Hg, or worsening of P to F ratio Bilateral infiltrative changes on chest radiograph No sign of hydrostatic pulmonary edema (pulmonary arterial occlusion pressure ≤18 mm Hg or central venous pressure ≤15 mm Hg) No other risk factor for acute lung injury
Possible TRALI	Same as for suspected TRALI, but another risk factor present for acute lung injury
Delayed TRALI	Same as for (possible) TRALI and onset within 6–72 h of blood transfusion

Definition according to the TRALI conference and US National Heart, Lung and Blood Institute Definition [2, 3, 5].

METHODS

Embase and PubMed were accessed for relevant English literature. Search strategy for PubMed was ((((transfusion-related acute lung injury[tw] OR TRALI[tw])) OR (("Acute Lung Injury"[MeSH]) AND ("Blood Transfusion"[Mesh] OR transfusion[tw])))) AND ("Models, Animal"[Mesh] OR animal model*[tw] OR "Cohort Studies"[MeSH Terms] OR cohort[tiab] OR "Humans"[MeSH] OR "Animals"[Mesh]) and keywords in Embase "Transfusion-related lung injury", "TRALI", "animal", "cohort analysis" and "human". Articles were selected based on title and abstract. References of all relevant articles were checked and included when considered relevant. Conference abstracts were excluded.

Pathogenesis non-antibody mediated TRALI

The current understanding of the pathogenesis of non-antibody mediated TRALI suggests it is caused by transfusion of a stored cellular blood product in the presence of a "first hit". A second hit then induces lung injury [22]. This is in contrast with antibody mediated TRALI in which majority of TRALI cases are related to cognate antibodies in plasma containing transfusion products. In short, TRALI is thought to be mediated by neutrophils [23]. Pulmonary endothelium release cytokines and chemokines which facilitate neutrophil migration to the lung. There, L-selectin

mediates loose binding of the neutrophil on the epithelium after which firm adhesion is mediated by E-selectin, platelet-derived P-selectin and intracellular adhesion molecules (ICAM-1). The transfusion product activates these neutrophils and lung injury develops. The neutrophils adhere to the injured capillary endothelium and migrate into the air space where they release oxidants, proteases, platelet-activating factor (PAF) and neutrophil extracellular traps (NETs). The air space is filled with protein-rich oedema and cytokines interleukin-1, -6, and -8, (IL-1, IL-6, and IL-8, respectively). These stimulate chemotaxis and stimulate neutrophils to form elastase-α1-antitrypsin (EA) complex. The clinical symptoms of acute respiratory distress is caused by influx of protein-rich oedema into the alveolus which leads to the inactivation of surfactant (Figure 1) [9, 24, 25]. Possibly symptoms of acute lung injury are not only caused by release of proteases by activated neutrophils, but also by ischemic lung damage as an effect of platelet aggregation in the pulmonary capillaries. Of interest neutrophil deficient patients also have been described to develop TRALI [26] and histochemical coloring of lung sections of patients who died of TRALI do not always show neutrophil influx in the alveolar space [27]. The past years research has focused on pro-inflammatory mediators which accumulate in stored cell containing blood that serve as a "second hit". More recent the transfused aged red blood cell (RBC) and platelet (PLT) themselves have been implicated as well in the onset of TRALI [13, 28].

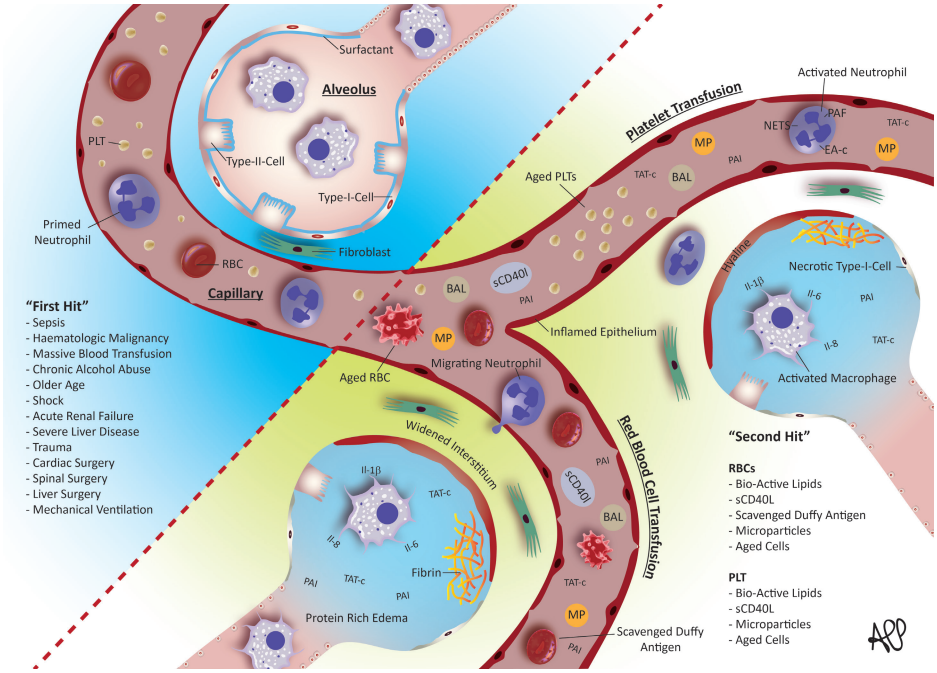

Figure 1. Pathophysiology of transfusion-related acute lung injury (TRALI).
A "first hit", an underlying clinical condition of the patient, results in priming of neutrophils and attracting them to the lung capillary by release of cytokines and chemokines by lung endothelium. L-selectin loosely binds the neutrophils after which E-selectin, platelet-derived P-selectin and intracellular adhesion molecules (ICAM-1) facilitate firm adhesion. The "second hit", the transfusion of a blood product, causes activation of the neutrophils resulting in TRALI. Neutrophil activation results in neutrophils margination through the interstitium into the alveoli which are filled with protein-rich edema. Here, cytokines, interleukine-1β, -6, -8 (Il-1β, Il-6, Il-8) are secreted which further stimulate neutrophil chemotaxis and neutrophil formation of elastase-α1-antitrypsin (EA) complex. Increase in thrombin-antithrombin complexes (TATc) and reduction of plasminogen activator activity (PAA) indicate activation of coagulation. The "second hit", the transfusion product may contain accumulated bioactive lipids (BAL), soluble CD40 ligand (sCD40L), aged RBCs with reduced levels of Duffy antigen or UV-B illuminated platelets. Hypothesized mediators are microparticles (MP), non-transferrin bound iron and aged RBCs or platelets. RBCs: red blood cells; PLTs: platelets. PAI: plasminogen activator inhibitor.

Recent insights in TRALI pathogenesis

The role of hemin and neutrophil extracellular traps (NETs) has recently been related to TRALI pathogenesis. Hemin is iron-containing protoporphyrin IX. It is essential for the formation of heme-containing proteins including hemoglobin, myoglobin, nitric oxide synthases and cytochromes. Hemin can be released under various pathological conditions as β-thalassemia, glucose-6-phosphate dehydrogenase deficiency, hemorrhage, hemolysis and muscle injury. An excess of free

circulating hemin can results in formation of reactive oxygen species and cellular injury [29]. NETs can be released by activated neutrophils to trap pathogens and thus prevent pathogen spreading [30]. They are composed of DNA fibers decorated with histones and antimicrobial proteins. Their formation follows a specific pattern of histone hypercitrullination, chromatin decondensation, dissolution of granular and nuclear membranes and cytolysis. Although NETs have been associated with beneficial antimicrobial function by trapping gram- negative and gram+ positive bacteria [30], they also have been related to amongst others colitis ulcerosa [31], small-vessel vasculitis [32] and preeclampsia [33]. Recently NETs have been detected in the circulation of patients with TRALI [34]. To determine whether these were causative or consequence in TRALI the effect of NETS have been studied *in vitro*. *In vitro* NETs induce enhanced permeability in primed human umbilical vein endothelial cells (HUVECs) [35] and NETs were found in two TRALI mouse models. In these *in vivo* models mice were primed with LPS after which infusion of MHC-I antibody functioned as "second hit". Mice developed TRALI with extensive NETs release [34, 35]. TRALI symptoms could be prevented by DNase1 which prevents NETs formation by neutrophils. No *in vivo* TRALI model has to this date focused on the role of NETs in non-antibody mediated TRALI. Of interest, *in vitro* models support the hypothesis that NETs also have a function in non-antibody mediated TRALI as hemin has been shown to activate neutrophils *in vitro* [36] and induce NETs formation [37].

Aging blood products

During storage, blood products undergo changes referred to as the "storage lesion". The RBC changes and suffers vesiculation, membrane loss and lysis, reduced glutathione, cellular levels of 2,3-diphosphoglycerate, adenosine triphosphate and nitric oxide, decreased membrane expression of CD47, and increased oxidation of cellular lipids and proteins (Figure 2) [38]. These processes induce storage dependent decrease in pH, increase of potassium and loss of hemoglobin. The stored platelet changes shape from discoid to spheroid, glucose consumption and lactate production increases and enhanced expression of GPIIIa, GPIIb and P-selectin expression can be found. The platelet apoptosis machinery activates amongst many other changes that affect *in vivo* recovery, survival and hemostatic activity of the transfused platelet [39]. The "storage lesion" effects post-transfusion survival and function. Therefore in most countries storage of RBCs has been maximized to 35 - 42 days and platelets storage to maximal 5 - 7 days to ensure sufficient post-transfusion function [40].

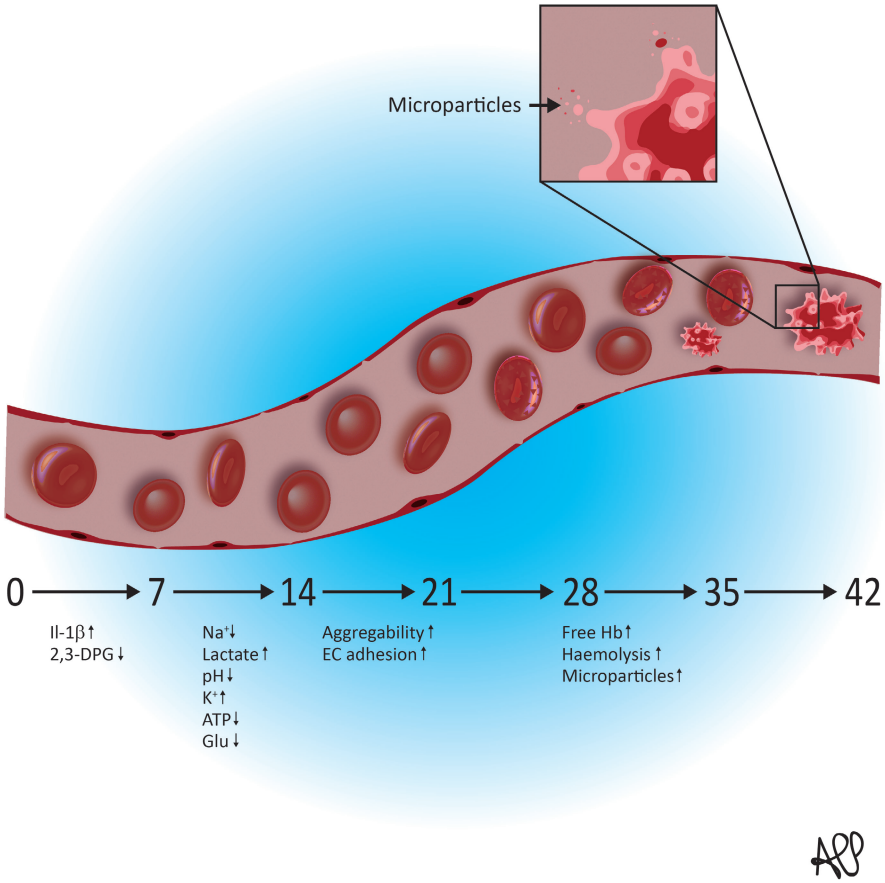

Figure 2. RBC storage lesion.
During storage, the RBC changes and suffers vesiculation, membrane loss and lysis, reduced glutathione, reduced cellular levels of 2,3-diphosphoglycerate, adenosine triphosphate and nitric oxide, decreased membrane expression of CD47, and increased oxidation of cellular lipids and proteins The changes that occur in the stored RBC lead to reduced microvascular flow, depletion of 2,3 diphosphoglycerate (2,3-DPG) which shifts the oxyhemoglobin dissociation curve to the left and reduces oxygen delivery, reductions in concentrations of nitric oxide and many other changes [38]. Il-1β: interleukin-1β; Na^+: sodium; K^+: potassium; ATP: adenosine triphosphate; Glu: glucose; EC: endothelial cell ; Hb: hemoglobin.

In several patient populations transfusion of stored cellular transfusion products has been related to adverse outcomes and increased mortality, although most studies are observational and highly heterogenous [41, 42]. The role of aged blood products in TRALI is still controversial. In animal models, aged PLTs and RBCs are clearly related to TRALI (Table 2 and 3), in clinical studies data is conflicting for

both RBCs and PLTs which could be caused by design of these studies. Most studies are retrospective and patients included received both fresh and stored products (Table 4). Although storage time is generally not considered to be of importance in onset of non-antibody mediated TRALI, one retrospective study in cardio surgery patients has identified storage time of plasma as risk factor for all-cause mortality [43]. Several factors in this study could explain the difference in all-cause mortality in patients receiving "young" plasma (plasma stored ⩽323 days) and "old" plasma (plasma stored >323 days). Groups differed in the number of patients who underwent re-exploration (young FFP 36%vs old FFP 25%) and platelet transfusion (young FFP 0.29 units versus old FFP 0.42 units). Also techniques to produce and store FFP, such as leucodepletion since 2001 and the male-only plasma measure since 2007 could have affected the storage times of FFP during the 10-year period of the study [44]. The effect of aged plasma has up to this date not been studied in animal models. We will describe which factors have been implicated in TRALI in both preclinical and clinical studies (Figure 3). We will separately discuss the role of soluble mediators, which accumulate during storage, and the role of aging of the red blood cell and platelet in the pathogenesis of TRALI.

Table 2. Models for non-antibody mediated TRALI involving red blood cell products or sCD40L. Various models for non-antibody mediated TRALI use a "two hit" model. The product used for production of the "second hit" is mentioned under "Implicated Product". Any additional conclusions derived from the model are mentioned in the column "Addition".

Study	Model	Animal	Donor	"First Hit"	Implicated Product (Storage time in days) / Cell	"Second Hit"	Age Dependent	Addition
Bennett 1972 [124]	In Situ: lung perfusion	Dog Baboon	Autologous	-	RBCs (1D, 21D)	Aged RBCs	YES	Aged RBCs
Bennett 1973 [125]	In Situ: lung perfusion	Dog	Autologous	-	RBCs (1D, 21D)	Aged RBCs	YES	Aged RBCs
Silliman 1998 [46]	Ex vivo: lung perfusion	Rat	Human	LPS	RBCs (0D, 42D)	Supernatant LysoPCs	YES	PAF-receptor antagonist attenuates TRALI.
Khan 2006 [59]	In vitro	N/A	HMVEC	LPS	RBCs (1D, 28D, 42D) PMN	sCD40L	YES	sCD40L accumulates in stored blood products
Mangalmurti 2009 [68]	In vivo	Mouse	Mouse	LPS	RBCs (1D, 2D, 10D)	RBCs with scavenged Duffy antigen	YES	Washing RBCs does not attenuate TRALI
Kelher 2009 [23]	In vivo	Rat	Human	LPS	RBCs (1D, 28D, 42D)	Supernatant LysoPCs Cognate antigen	YES	PMN depletion prevents TRALI
Silliman 2010 [72]	In vivo	Rat	Human	LPS	RBCs (1D, 42D)	Supernatant	YES	Riboflavin and UV-B do not induce enhanced TRALI
Vlaar [48]	In vivo	Rat	Rat	LPS	RBCs (1D, 14D)	Supernatant RBCs	YES	Washing RBCs prevents TRALI LysoPCs normal

Harr 2011[75]	In vivo	Rat	Rat	Trauma	RBCs (0D)	Autologous whole blood in low-dose heparin but not high-dose, citrate, or clopidogrel	N/A	ALI possibly micro thrombi mediated
Nicholson 2011 [126]	In vivo	Rat	Rat	Trauma	RBCs (0D, 7D, 14D, 21D, 28D, 35D)	RBCs		No control group
Silliman 2011[53]	In vivo	Rat	Human	LPS	RBCs (1D, 42D), leukoreduced and non-leukoreduced	Nonpolar Lipids	N/A	Inhibiting expression of CD40L by ciglitazone does not attenuate TRALI
Tung 2012 [64]	In vivo	Sheep	Human	LPS	RBCs (1D, 42D)	Supernatant	YES	
Fung 2013 [127]	In vivo	Sheep	Sheep	Trauma	RBCs (<5D, 35-42D)	RBCs	YES	Modest increase sCD40L
Silliman 2014 [45]	In vivo	Rat	Human	LPS	RBCs (1D, 42D)	Supernatant		No TRALI due to too mild "first hit"

LPS = lipopolysaccharide, RBCs = red blood cells, PLTs = platelets, PAF = platelet activating factor, TRALI = transfusion related acute lung injury, PMN = polymorphnucleur cells, HMVEC = human microvascular endothelial cells

Table 3. Models for non-antibody mediated TRALI involving platelet products or isolated LysoPCs. Various models for non-antibody mediated TRALI use a "two hit" model. The product used for production of the "second hit" is mentioned under "Implicated Product". Any additional conclusions derived from the model are mentioned in the column "Addition".

Study	Model	Animal	Donor	"First Hit"	Implicated Product (Storage time in days) / Cell	"Second Hit"	Age Dependent	Addition
Wyman 2002 [49]	In vitro	N/A	HMVEC	LPS	N/A	LysoPCs	N/A	
Silliman 2003 [7]	Ex vivo	Rat	Human	LPS	PLTs (0D, 5D)	Supernatant LysoPCs	YES	PAF-receptor antagonist attenuates TRALI
Khan 2006 [59]	In vitro	N/A	HMVEC	LPS	PLT (1D, 3D, 5D, 7D) PMN	sCD40L	YES	sCD40L accumulates in stored platelets

Silliman 2010 [72]	In vivo	Rat	Human	LPS	PLTs (1D, 7D)	Supernatant illuminated platelets	YES	Riboflavin and UV-B do not induce enhanced TRALI
Vlaar 2010 [47]	In vivo	Rat	Rat	LPS	PLTs (1D, 5D)	Supernatant Platelets	YES	Washing platelets prevents TRALI LysoPCs slightly elevated CD40L normal
Tung 2011 [128]	In vivo	Sheep	Human	LPS	PLTs (1D, 5D)	Supernatant	YES	
Gelderman 2011 [71]	In vivo	Mouse	Human	LPS	Model 1: PLTs (1D) Model 2: anti-MHC-1 antibody	Platelets UVB illuminated MHC-1 antibody	N/A	
Chi 2012 [74]	In vivo	Mouse	Human	LPS	PLTs (1D)	Platelets UVB illuminated	N/A	
Zhi 2013 [73]	In vivo	Mouse	Human	LPS	PLTs (1D)	Platelets UVB illuminated	N/A	PKC signaling inhibitor attenuates TRALI P2Y12 inhibitor attenuates TRALI MIP-2 inhibitor attenuates TRALI
Zhi 2013 [129]	In vivo	Mouse	Human	LPS	PLT (1D)	Platelets UVC illuminated	N/A	Platelet UVC illumination gives platelet aggregation in lungs but no lung injury
Chi 2014 [76]	In vivo	Mouse	Human	LPS	PLT (1D)	Platelets UVB illuminated		Platelet UVB illumination and riboflavin do not induce TRALI

LPS = lipopolysaccharide, RBCs = red blood cells, PLTs = platelets, PAF = platelet activating factor, TRALI = transfusion related acute lung injury, PMN = polymorphnucleur cells, HMVEC = human microvascular endothelial cells

Soluble mediators

Bioactive lipids

In stored transfusion products bioactive lipids accumulate that are thought to cause TRALI *in vivo* and *in vitro* [7, 23, 45-49]. The polar lipids that accumulate in both RBCs and PLTs have been identified as lysophosphatidylcholines (LysoPCs), lipids structurally similar to platelet activating factor (PAF) and to the ligand for the G2A receptor [7, 50]. *In vitro*, the G2A receptor on neutrophils can be activated by LysoPCs and thus cause chemotaxis and release of components of the microbicidal arsenal via activation of G-protein subunits that have cell signaling roles [51, 52]. The non-polar lipids have been identified as arachidonic acid, 5-hydroxeicosatetranoic acid (HETE), 12-HETE and 15-HETE [53]. Polar lipids accumulate in both whole blood RBCs and PLTs but to a lesser extent in leukoreduced RBCs where platelet contamination decreases by 4 to 5 logs [7, 50]. Non-polar lipids can be isolated in RBCs but not in PLTs. Therefore, LysoPCs, the polar lipids, are thought to be platelet derived and arachidonic acid, 5-HETE, 12-HETE and 15-HETE, the non-polar lipids, to be RBC derived [53, 54]. The role of both LysoPCs and non-polar lipids in TRALI is still debated. The LysoPCs have been implicated to cause acute lung injury in a series of 10 patients suffering TRALI [18]. The same research group developed two *ex vivo* rat models to study the role of LysoPCs [7, 46]. These rats were stimulated with lipopolysaccharide (LPS), a gram negative endotoxin as "first hit". The lungs were extracted and perfused *ex vivo* with plasma from 42 days old human red blood cells (RBCs) as "second hit". To prevent activation of human complement and fibrinogen, plasma was preheated to 56°C before transfusion. In this study, supernatant from outdated but not fresh RBCs caused lung injury. LysoPCs isolated from aged blood products also caused evident lung injury when used as "second hit" [7, 46]. A third murine model developed by the same group confirmed the potential of LysoPCs to cause acute lung injury *in vivo*. Rats primed with LPS developed acute lung injury when injected with either supernatant of aged RBCs or with LysoPCs. In this study, LysoPCs were confirmed to accumulate in stored blood [23]. Our group could not confirm that lipids play a role in two *in vivo* rat transfusion models. In these models LPS was also used as "first hit" followed by either aged RBCs or aged PLTs. LysoPCs did not accumulate in both murine and human aged RBCs and were only slightly elevated in aged PLTs [47, 48] which was also confirmed by two other groups [54, 55]. Clinical studies also report conflicting results on the role of lysoPCs [56, 57]. In a cohort of cardiac surgery patients, the total amount of LysoPCs transfused in the group of patients who developed TRALI was higher than in the control group. However, when corrected for concentration of LysoPCs per product no difference was found between the TRALI group and control group [14]. Similar results were

found in a study where total transfused LysoPCs was higher in the TRALI group. In this study group however, increased total LysoPCs most likely reflected the higher volume of plasma transfused as plasma contains more LysoPCs [8]. Moreover, *in vitro* priming studies showed that accumulation of LysoPCs is not cell derived, but plasma derived and storage temperature dependent as opposed to storage time related. Neutrophil priming in these reports was LysoPCs independent [54, 57]. These different results are possibly explained by different storage procedures. Studies in which LysoPCs were implicated in TRALI originate from the United States whereas the studies where no role was found for LysoPCs come from either Europe or Asia [8, 11, 12, 14, 47, 48, 54, 57]. The role of nonpolar lipids arachidonic acid, 5-HETE, 12-HETE and 15-HETE has been investigated in an *in vivo* rat model. Rats were primed with LPS after which they received an identical volume of neutral lipids at concentrations equal to 1 day or 42 days stored whole blood or leukoreduced RBCs. Nonpolar lipids from stored RBCs caused acute lung injury and accumulated in both stored leukoreduced and stored non-leukoreduced RBCs [53]. Accumulation of 5-HETE but not of arachidonic acid could be prevented by filtration of donor blood before storage, possibly due to filtration of enzymes required for converting arachidonic acid into 5-HETE. *In vivo* heat-treated supernatant of these filtered RBCs did not induce TRALI in rats primed with LPS, *in vitro* neutrophil priming activity was attenuated after filtration. This is somewhat unexpected as TRALI is thought to be mediated by storage time of blood products, but apparently can be prevented by filtration before storing the products. The authors of this study hypothesize that enzymes required for conversion of arichdonic acid 5-HETE are filtered. Maybe also enzymes required for conversion of molecules to TRALI-implicated soluble mediators may be filtered, thus preventing storage related lung injury [45]. The role of pre-storage filtration and non-polar lipids in human TRALI has not been explored yet and needs further investigation.

Soluble CD40L

CD40 is a member of the tumor necrosis factor (TNF) receptor family expressed on endothelial and epithelial cells, monocytes, and macrophages. Its ligand CD40L is a pro-inflammatory mediator produced by platelets that accumulates in stored blood in either soluble (sCD40L) or cell associated forms [58-60]. Soluble CD40L activates macrophages and elicits the production and release of multiple pro-inflammatory cytokines. Increased sCD40L in stored platelets has been associated with transfusion reactions [61] and increased respiratory burst in polymorphnuclear leukocytes (PMNs) [62]. In animal models, acute lung injury caused by LPS or reactive oxygen species (ROS) is attenuated by inhibition of the CD40-CD40L system

[56, 63]. The effect of transfusion of sCD40L has been studied in several models. Human microvascular endothelial cells (HMVEC) exposed to PMN stimulated by LPS as "first hit" and sCD40L as "second hit" were shown to be less viable compared to control HMVEC incubated with medium [59]. In an *in vivo* model, sheep primed with LPS intravenously developed acute lung injury after transfusion with human heat treated supernatant of 42 days old RBCs. In this study, only a modest increase in sCD40L levels was found in stored RBCs compared to earlier studies. Still the sheep developed overt lung injury. Additional tests showed that this relatively low level of sCD40L was due to the heat treatment used to eliminate human complement and fibrinogen. This implicated that sCD40L only plays a minor role in TRALI [64]. In a "two-hit" TRALI-model, rats developed lung injury after transfusion of 5 days stored PLTs after priming with LPS. In this study levels of sCD40L in stored PLTs were not increased compared to fresh PLTs [47]. Clinical studies also produce conflicting results. Samples of 62 whole blood platelet transfusions implicated in TRALI reactions in patients were compared with platelet transfusions in control hospitalized patients without TRALI. In this study platelets associated with TRALI were found to have higher concentrations of sCD40L compared to control platelets [59]. On the other hand, in a cohort of prospectively included cardiac surgery patients no elevated sCD40L was found in the group that developed TRALI after transfusion compared to control [65]. Even though sCD40L is a potent mediator in inflammation, a majority of studies suggest only a minor role for sCD40L in TRALI.

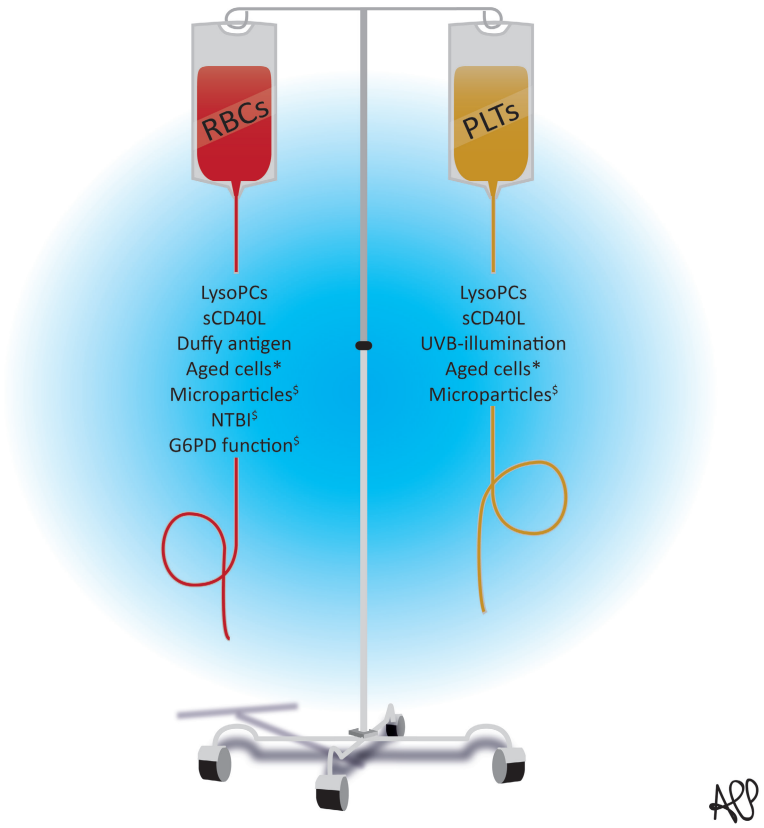

Figure 3. Storage lesion and transfusion-related acute lung injury.
Several soluble and cellular mediators accumulate during storage of cellular blood products and have been associated with non-antibody mediated transfusion-related acute lung injury (TRALI). Fresh frozen plasma has been related to antibody mediated TRALI, but not with non-antibody mediated TRALI. * : associated in animal models; $: hypothesized mediators. RBCs: red blood cells; PLTs: platelets; sCD40l: soluble CD40 ligand; NTBI: non-transferrin bound iron.

Cellular mediators

The aging of the erythrocyte

The changes that occur in the stored erythrocyte such as decreased deformability which leads to reduced microvascular flow, depletion of 2,3 diphosphoglycerate (2,3-DPG) which shifts the oxyhemoglobin dissociation curve to the left and reduces oxygen delivery, reductions in concentrations of nitric oxide and many other changes (Figure 1) have been implicated in reduced 24-hour post transfusion RBCs survival. These effects have been implicated to poorer outcome after transfusion

but have not been directly associated with the onset of TRALI [66]. There is however pre-clinical evidence which links the aging of RBCs with the onset of TRALI. During storage human erythrocyte also lose Duffy antigen expression and chemokine scavenging function. The Duffy antigen is a minor blood group antigen that binds multiple inflammatory chemokines with high affinity thus making erythrocyte bound chemokines inaccessible to circulating neutrophils [67]. In a study investigating the role of Duffy antigen in TRALI, scavenging of Duffy antigen was found to be related to onset of acute lung injury. In the studied *in vivo* mouse model, mice were primed with intravenous LPS after which they were transfused with either fresh or aged RBCs. Transfusion of aged RBCs caused increased neutrophil counts, cytokine and chemokine concentrations in the lung and increased pulmonary microvascular permeability. This effect was even more pronounced in transfusion of blood derived from Duffy-antigen knock-out mice compared to wild-type mice implicating a key role for the Duffy-antigen [68]. Transfusion of aged RBCs thus causes a significant reduction of chemokine scavenging function by RBCs and results in acute lung injury.

The aging platelet

As in erythrocytes, platelets undergo time-dependent structural and functional changes when stored. Stored platelets shrink, show cytoplasm condensation, plasma membrane blebbing and extension of filopodia [69]. Ultraviolet-B (UV-B) illumination has the potential to damage nucleic acids. Therefore it is used to eliminate leukocytes and pathogens in a-nucleate cellular transfusion products as for example PLTs [70]. Experiments with platelets treated with either UV-B illumination or riboflavin and UV-B illumination showed that illuminated aged platelets but not supernatant of illuminated aged platelets caused acute lung injury in two-hit murine models indicating an role for the aging platelet in TRALI [71-74]. Peripheral blood smears and histochemistry of lung sections showed increased platelet aggregation in the rats that developed acute lung injury [73, 75]. Inhibition of protein kinase C (PKC) signal pathway and the thrombocyte P2Y12-receptor by clopidogrel attenuated this lung injury [73]. However, a recent study with riboflavin treated, UV-B illuminated platelets did not replicate these findings [76]. This difference might be attributed to different storage conditions. In the most recent study, PLTs were illuminated in closed bags [76] where previous studies PLTs were illuminated in an open system [71, 73, 74]. This could cause enhanced production of ROS. Also, riboflavin could have a protective effect against TRALI. Treatment strategies targeting the aging platelet have been studied in both animal models and clinical studies. In an *in vivo* animal study clopdigrel, a P2Y12 ADP receptor inhibitor which prevents

platelets aggregation, attenuates TRALI [76]. This is not supported however by a retrospective cohort study. In this study no difference was found in development of TRALI between a cohort of ICU patients who used aspirin and a matched control group [77].

Research fields in TRALI

Storage time

Even though pre-clinical studies show a clear relation between TRALI and aged cellular blood products, observational clinical studies report conflicting data. As of yet, no prospective studies have addressed the role of storage time but the ABLE [78], RECESS [79], Red Cell Storage Duration and Outcomes in Cardiac Surgery [80], and the TRANSFUSE study [81] are underway. These studies will not shed light on the direct relation between transfusion of aged blood products and TRALI, but they will substantiate extrapolation of the relation between storage time and acute lung injury that has been found in animal models to the human population. Preclinical studies on the other hand can focus on the pathogenesis of TRALI.

Erythrocytes deformability and endothelial adhesion

Normal erythrocytes have unique flow properties that ensure optimal circulation. These flow conditions are mainly influenced by erythrocyte deformability, self-aggregability and adherence to the blood vessel wall endothelium [82]. When erythrocytes age, they lose their deformability, suffer increased self-aggregability and increased adherence to endothelium which causes occlusion of the (pulmonary) microcirculation [82]. Endothelial adherence is even further enhanced in inflammatory conditions which activate vascular endothelium [83, 84]. Increased erythrocyte adhesion to endothelial cells may also play a role in TRALI. The role of adhesion has not yet been investigated in TRALI, but it can be hypothesized that the mechanism of occlusion of the microcirculation by adhering erythrocytes causes pulmonary ischemia, and thus contributes to the clinical picture of TRALI.

Storage conditions

Acid citrate dextrose was the first modern whole blood storage solution when it was developed in 1943. Since then many changes in the storage process and solution have taken place to ensure maximal survival of stored cells and prevent, for as far as possible, the "storage lesion" [85]. The effect that storage and additive solutions can have on the transfusion product is illustrated by a study in which the shape transition of erythrocytes from discoid to spherocytic was investigated. This shape change is time related but could be reversed by addition of glucose, pyruvate,

inosine, adenine and phosphate [86]. Storage procedures could also play a role in TRALI. The development of LysoPCs for example is, at least in Europe compared to US based studies, temperature dependent and plasma dependent. In platelets stored in 100% plasma significantly more LysoPCs developed than in platelets stored in SSP+ additive solution. Moreover, storage of plasma at 22°C induced significantly more LysoPCs development than storage at 4°C [57].

Iron Metabolism

The relation between iron overload and cytokine release that occurs after transfusion of aged RBCs has recently been investigated in healthy volunteers. Most RBCs clearance occurs within the first hour after transfusion [87]. One human RBCs unit contains 220 to 250 mg of iron, therefore, rapid RBCs clearance of up to 25% of even a single unit, acutely delivers a massive load of hemoglobin iron to the monocyte/macrophage system [88]. This load overwhelms the iron handling capacity of the monocyte/macrophage system which results in freely circulating non-transferrin bound iron (NTBI). NTBI is thought to cause activation of the inflammasome by production of ROS after which Il-1β, a pro-inflammatory cytokine, is released. In both an animal [89] and a healthy volunteer model, transfusion of aged RBCs but not fresh RBCs induced a pro-inflammatory response, associated with increased levels of NTBI [90]. This effect could be mitigated by administering an iron chelator although these are known to have anti-oxidant capacities next to iron-binding properties [89]. Of note is that in the animal study, transfusion of washed and unwashed aged RBCs caused a rise in cytokines that could not be replicated by transfusion of fresh RBCs or RBCs ghosts, stroma-free lysate derived from aged RBCs or supernatant of aged RBCs [89]. The role of iron metabolism has not yet been investigated in TRALI but deserves attention because of the pro-inflammatory response that has been associated with increased levels of NTBI in stored RBCs.

Microparticles

Microparticles (MPs) are small vesicles with a lipid bilayer which may contain membrane proteins, cytoplasmic components or RNA from the cell of which they were derived [91]. MP formation follows after the neutral cell membrane charge changes into negative which results in loss of phospholipid asymmetry. This induces phosphatidylserine (PS) externalization, causing budding of the plasma membrane [92]. MP formation is triggered by different stimuli, such as shear stress, complement attack, oxidative stress and pro- apoptotic stimuli [93]. MPs themselves again are pro-inflammatory and pro-coagulant which they achieve either via direct stimulation of target cells by receptor interaction or by transfer of their contents [94].

Erythrocytes and platelets undergo MP formation during storage which is considered part of the "storage lesion" [95]. Several studies show that the level of MPs increases during storage time [95-97]. Although MPs from RBCs (R-MPs) are the predominant species, significant amounts of MPs from PLTs (P-MPs) and leukocytes (L-MPs) are also generated during blood storage [86]. The role of MPs in TRALI has not yet been investigated although R-MPs have been found to contribute to neutrophil priming and activation in a murine model of hemorrhagic shock and resuscitation [98]. As activation of neutrophils also plays a central role in the pathogenesis of TRALI, R-MPs and P-MPs are hypothesized to promote onset of TRALI [91, 92]. MPs can activate neutrophils in several ways. The surface of MPs contain lipids with platelet activating factor (PAF)-like activity [99] and proteomic analysis showed an enrichment in complement and immunoglobulins of R-MPs during storage which may also play a role in activation of neutrophils via Fc receptors [100].

P-MPs are able to activate neutrophils directly. Furthermore they have the potential to stimulate endothelial cells and monocytes which results in a release of a range of inflammatory cytokines (IL-1, IL-6, IL-8, TNFα) [101] and subsequently may result in priming and activation of neutrophils.

TRALI is also characterized by onset of systemic and pulmonary coagulopathy in both pre-clinical and clinical studies [47]. MPs are able to induce activation of coagulation in different ways. Tissue factor (TF) is expressed on many MPs under inflammatory conditions [102] and is an initiator of coagulopathy in the lung [103]. The large amounts of PS on the surface of MPs are able to activate TF or interact with coagulation factors VIIa, IX and X leading to thrombin generation and amplification of the coagulation cascade [104]. The pro- coagulant activity of MPs from platelets is 50- to 100- fold higher than their parent platelet [105] which may render them important contributors to the onset of systemic and pulmonary coagulopathy in TRALI. The level of MP formation in stored PLTs and RBCs depends on the process and storage conditions of the products as MPs generation is higher in stored non-leukoreduced compared to leukoreduced RBCs [86].

If MPs are involved in TRALI onset several strategies can be employed to prevent acute lung injury. In a two event animal model of TRALI pre-storage filtration of RBCs decreased lipid priming activity and mitigated TRALI [45]. Pre-storage leukofiltration also significantly decreases post-storage P-MP levels and might contribute to prevention of TRALI [106]. It is unclear whether filtration or washing of stored RBCs pre-transfusion might further mitigate TRALI as MPs may still be formed

during storage of pre-storage filtered RBCs. Therefore, another intervention may be the use of additive solution during storage as this is associated with reduced level of MP formation [86]. RBCs stored in additive solutions consisting of glucose, pyruvate, inosine, adenine, and phosphate showed a reduction of MPs formation possibly due to reduction of oxidative stress [107]. Recognition of the potential harmful effects of P-MP en R-MP in the context of onset of TRALI is important, as this could be a target for future prevention and treatment strategies.

Table 4. Biological factors in stored blood implicated in clinical studies on risk of transfusion related acute lung injury.

	Type	Population	Country	Year	Role for aging blood product?		Role for accumulation of LysoPCs?
					RBCs	PLTs	
Silliman 2003 [7]	Prospective, active	Hospital	Canada	1991-1995	No	Yes	Yes
Vlaar 2010 [13]	Retrospective, active	ICU	The Netherlands	2004-2007	No	No	--
Gajic 2007 [8]	Prospective, active	ICU - medical	USA	2005-2007	No	No	Yes
Vlaar 2010 [14]	Prospective, active	ICU	The Netherlands	2006-2009	Yes	No	No
Middelburg 2012 [28]	Retrospective, passive	National	The Netherlands	2005-2007	No	Yes	--
Toy 2012 [12]	Prospective, active	Regional	USA	2006-2009	No	No	No

Table adapted from Vlaar et al [9]. RBCs = red blood cells, PLTs = platelets.

Prevention

Despite the progress that has been made in TRALI research, still no treatment is available for TRALI. Reduction of TRALI cases is predominantly achieved by prevention. The potential role of washing of transfusion products, implementation of a fresh blood product strategy, and pre-storage filtration are currently under investigation as potential measures to prevent TRALI.

Washing of transfusion products

Washing of transfusion products potentially has an effect on both the cellular and the plasma component of the stored product. Stored RBCs are more fragile and prone to hemolysis or sequestration. Washing of stored RBCs may selectively

eliminate these more fragile cells and thus prevent intravascular hemolysis [108]. On the other hand, washing may also damage cells increasing plasma free hemoglobin instead of preventing intravascular hemolysis [109]. Washing effects the plasma component by removal of mediators that accumulate during storage, for example potassium, cell-free hemoglobin, bio-active lipids and microparticles, and it eliminates residual antigens [108, 110, 111]. Washing of stored platelets effectively removes the plasma without effecting the *in vitro* function of platelets [112] although they might affect the *in vivo* recovery [113]. Several studies indicate that washing of cells prevents TRALI, thus supporting the role of soluble mediators in acute lung injury. In two studies TRALI could be prevented by washing RBCs and platelets before transfusion in rats primed with LPS [47, 48], a third study on the other hand did not prevent acute lung injury in a two-hit *in vivo* mouse model by washing cells before transfusion [68]. In a retrospective study comparing TRALI incidence before and after introduction of standard leukoreducing of transfusion products, no TRALI was observed in a cohort of patients who received washed transfusions [114]. Washed stored cell containing blood products may be an important alternative for fresh blood products and is clinical feasible [115]. However well performed clinical studies are first needed to confirm that fresh cell containing blood products or washed stored cell containing blood products are indeed able to reduce or prevent onset of TRALI.

Filtration

Transfusion products can be leucoreduced by filtration or removal of the buffy coat. Standard leukoreduction by filtration removes 3 logs of leukocytes and 2 logs of platelets. This reduces HLA antigen exposure, non-hemolytic transfusion reactions, levels of cytokine accumulation during storage, sCD40L accumulation and cytomegalovirus exposure [53, 59, 116]. Universal leucoreduction has been related to a decreased incidence of TRALI [114] and recently pre-storage filtration that removes antibodies, leukocytes, platelets and lipids was found to have a positive effect on *in vitro* RBCs storage lesion [117]. This filter has also been investigated in an *in vivo* animal model. As described above, rats were primed in this model with LPS after which they were treated with heat-treated supernatant of RBCs. RBCs were either prepared with standard leucoreduction or with more extensive pre-storage filtration. In this model TRALI was prevented by pre-storage filtration [45]. The *in vivo* effects of pre-storage filtration on cellular function has not yet been investigated, nor is there any data on the role of pre-storage filtration in human TRALI.

Fresh blood products

Although the evidence from animal models that aged transfusion products play a major role in TRALI is compelling, observational clinical studies report conflicting data. Aged blood products have been associated with multiple complications as increasing rates of sepsis, infection, multi-organ failure, myocardial infarction, acute renal failure, thrombosis resulting in poorer outcome and increased mortality [118]. A systematic review on the effect of stored blood on morbidity and mortality identified fifty-five studies that had reported the effects of age of transfused RBCs on mortality or morbidity in adult patients. In about half of these studies a negative effect of stored blood products was reported. Several described studies specifically focused on the association between respiratory failure and storage of blood products but found no clear relation [119].

No effects of stored blood products have directly been associated with the onset of TRALI [66]. In light of these highly conflicting data, prospective clinical studies are needed to support a "fresh only" policy to prevent non-antibody mediated TRALI. Although it might be tempting to change transfusion policy to prevent TRALI, the impact on blood services would be unquestionable. At the moment RBCs can be stored 35 to 42 days depending on local policy and PLTs 5 to 7 days. If storage time would be reduced demands on donors would increase substantially and risk of transfusion blood shortage would rise. As the incidence of TRALI is relatively low such a trial would need large numbers of patients and take many years to complete. Even when such trials become available, conclusions should be drawn with caution. Differences in blood processing, storage condition and even transfusion policy will prevent extrapolation of conclusions to clinical practices which were not involved in these trials. Still, with the thought of impact of TRALI on patient health and the evidence from preclinical data, such a trial will be needed to further investigate measures for TRALI prevention and treatment.

Treatment

As of yet, no treatment exist other than supportive measures. Most patients need supplemental oxygen therapy, 70-90% of patients even need mechanical ventilation [120]. The prognosis of TRALI was thought to be relatively good with a mortality of 5-10% [120-122]. Recent studies however found increased morbidity in high risk patients with mortality as high as 17%-47% in TRALI patients [8, 13, 14, 123]. A successful reduction in antibody-mediated TRALI-cases has been caused by excluding female donors for production of plasma derived products [9]. For non-antibody mediated TRALI no strategy yet has been developed to prevent acute lung injury.

For now, the accepted strategy to prevent TRALI is to apply a restrictive transfusion policy, especially in the critically ill. Transfusion cannot be avoided altogether but a patient tailored approach could be effective in preventing TRALI. Amongst others, fluid overload, shock, sepsis and organ failure have been recognized as risk factors for TRALI. If the circumstances permit delaying the transfusion until the patient is in a better clinical condition, could prevent some TRALI cases [13].

CONCLUSION

Transfusion-related acute lung injury (TRALI) is a severe complication of transfusion. Research has shifted the past years from antibody-mediated TRALI to factors in the aging cellular blood product that cause acute lung injury. The causative factor is still not known, but there are many candidate pathways which need to be investigated. Prospective clinical studies are underway and will hopefully be able to further elucidate the clinical relevance of the aging blood product. Preclinical studies will be needed to inform us about biological transfusion factors implicated in the pathogenesis of non-antibody mediated TRALI.

REFERENCES

1. Stainsby, D., et al., *Serious hazards of transfusion: a decade of hemovigilance in the UK.* Transfus Med Rev, 2006. **20**(4): p. 273-82.
2. Kleinman, S., *A perspective on transfusion-related acute lung injury two years after the Canadian Consensus Conference.* Transfusion, 2006. **46**(9): p. 1465-8.
3. Goldman, M., et al., *Proceedings of a consensus conference: towards an understanding of TRALI.* Transfus Med Rev, 2005. **19**(1): p. 2-31.
4. Holness, L., et al., *Fatalities caused by TRALI.* Transfus Med Rev, 2004. **18**(3): p. 184-8.
5. Toy, P., et al., *Transfusion-related acute lung injury: definition and review.* Crit Care Med, 2005. **33**(4): p. 721-6.
6. Ausley, M.B., Jr., *Fatal transfusion reactions caused by donor antibodies to recipient leukocytes.* Am J Forensic Med Pathol, 1987. **8**(4): p. 287-90.
7. Silliman, C.C., et al., *Plasma and lipids from stored platelets cause acute lung injury in an animal model.* Transfusion, 2003. **43**(5): p. 633-40.
8. Gajic, O., et al., *Transfusion-related acute lung injury in the critically ill: prospective nested case-control study.* Am J Respir Crit Care Med, 2007. **176**(9): p. 886-91.
9. Vlaar, A.P. and N.P. Juffermans, *Transfusion-related acute lung injury: a clinical review.* Lancet, 2013. **382**(9896): p. 984-94.
10. Rana, R., et al., *Transfusion-related acute lung injury and pulmonary edema in critically ill patients: a retrospective study.* Transfusion, 2006. **46**(9): p. 1478-83.
11. Silliman, C.C., et al., *Transfusion-related acute lung injury: epidemiology and a prospective analysis of etiologic factors.* Blood, 2003. **101**(2): p. 454-62.
12. Toy, P., et al., *Transfusion-related acute lung injury: incidence and risk factors.* Blood, 2012. **119**(7): p. 1757-67.
13. Vlaar, A.P., et al., *Risk factors and outcome of transfusion-related acute lung injury in the critically ill: a nested case-control study.* Crit Care Med, 2010. **38**(3): p. 771-8.
14. Vlaar, A.P., et al., *The incidence, risk factors, and outcome of transfusion-related acute lung injury in a cohort of cardiac surgery patients: a prospective nested case-control study.* Blood, 2011. **117**(16): p. 4218-25.
15. Bux, J., et al., *Transfusion-related acute lung injury due to HLA-A2-specific antibodies in recipient and NB1-specific antibodies in donor blood.* Br J Haematol, 1996. **93**(3): p. 707-13.
16. Kakaiya, R.M., et al., *Prevalence of HLA antibodies in remotely transfused or alloexposed volunteer blood donors.* Transfusion, 2010. **50**(6): p. 1328-34.
17. Gans, R.O., et al., *Transfusion-related acute lung injury.* Intensive Care Med, 1988. **14**(6): p. 654-7.
18. Silliman, C.C., et al., *The association of biologically active lipids with the development of transfusion-related acute lung injury: a retrospective study.* Transfusion, 1997. **37**(7): p. 719-26.
19. Toy, P., et al., *Recipients of blood from a donor with multiple HLA antibodies: a look-back study of transfusion-related acute lung injury.* Transfusion, 2004. **44**(12): p. 1683-8.

20. Kopko, P.M., et al., *Transfusion-related acute lung injury: report of a clinical look-back investigation.* JAMA, 2002. **287**(15): p. 1968-71.
21. Li, G., et al., *Long-term survival and quality of life after transfusion-associated pulmonary edema in critically ill medical patients.* Chest, 2010. **137**(4): p. 783-9.
22. Vlaar, A.P., *Transfusion-related acute lung injury: Current understanding and preventive strategies.* Transfus Clin Biol, 2012. **19**(3): p. 117-24.
23. Kelher, M.R., et al., *Plasma from stored packed red blood cells and MHC class I antibodies causes acute lung injury in a 2-event in vivo rat model.* Blood, 2009. **113**(9): p. 2079-87.
24. Silliman, C.C. and M. Kelher, *The role of endothelial activation in the pathogenesis of transfusion-related acute lung injury.* Transfusion, 2005. **45**(2 Suppl): p. 109S-116S.
25. Silliman, C.C., *The two-event model of transfusion-related acute lung injury.* Crit Care Med, 2006. **34**(5 Suppl): p. S124-31.
26. Finlayson, J., et al., *Transfusion-related acute lung injury in a neutropenic patient.* Intern Med J, 2011. **41**(8): p. 638-41.
27. Danielson, C., et al., *Pulmonary pathology of rapidly fatal transfusion-related acute lung injury reveals minimal evidence of diffuse alveolar damage or alveolar granulocyte infiltration.* Transfusion, 2008. **48**(11): p. 2401-8.
28. Middelburg, R.A., et al., *Storage time of blood products and transfusion-related acute lung injury.* Transfusion, 2012. **52**(3): p. 658-67.
29. Li, S.D., et al., *Hemin-mediated hemolysis in erythrocytes: effects of ascorbic acid and glutathione.* Acta Biochim Biophys Sin (Shanghai), 2006. **38**(1): p. 63-9.
30. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.
31. Savchenko, A.S., et al., *Long pentraxin 3 (PTX3) expression and release by neutrophils in vitro and in ulcerative colitis.* Pathol Int, 2011. **61**(5): p. 290-7.
32. Kessenbrock, K., et al., *Netting neutrophils in autoimmune small-vessel vasculitis.* Nat Med, 2009. **15**(6): p. 623-5.
33. Gupta, A.K., et al., *Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia.* Hum Immunol, 2005. **66**(11): p. 1146-54.
34. Thomas, G.M., et al., *Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice.* Blood, 2012. **119**(26): p. 6335-43.
35. Caudrillier, A., et al., *Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury.* J Clin Invest, 2012. **122**(7): p. 2661-71.
36. Kono, M., et al., *Morphological and flow-cytometric analysis of haemin-induced human neutrophil activation: implications for transfusion-related acute lung injury.* Blood Transfus, 2013. **11**(1): p. 53-60.
37. Kono, M., et al., *Heme-related molecules induce rapid production of neutrophil extracellular traps.* Transfusion, 2014. **54**(11): p. 2811-9.
38. Tinmouth, A., et al., *Clinical consequences of red cell storage in the critically ill.* Transfusion, 2006. **46**(11): p. 2014-27.
39. Ohto, H. and K.E. Nollet, *Overview on platelet preservation: better controls over storage lesion.* Transfus Apher Sci, 2011. **44**(3): p. 321-5.
40. Hess, J.R., *Measures of stored red blood cell quality.* Vox Sang, 2014. **107**(1): p. 1-9.

41. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
42. Patel, S.V., et al., *Risks associated with red blood cell transfusion in the trauma population, a meta-analysis.* Injury, 2014. **45**(10): p. 1522-33.
43. van Straten, A.H., et al., *Effect of storage time of transfused plasma on early and late mortality after coronary artery bypass grafting.* J Thorac Cardiovasc Surg, 2011. **141**(1): p. 238-43 e1-2.
44. van der Bom, J.G., et al., *Comment on "Effect of storage time of transfused plasma on early and late mortality after coronary artery bypass grafting".* J Thorac Cardiovasc Surg, 2011. **142**(2): p. 473-4.
45. Silliman, C.C., et al., *Experimental prestorage filtration removes antibodies and decreases lipids in RBC supernatants mitigating TRALI in vivo.* Blood, 2014. **123**(22): p. 3488-95.
46. Silliman, C.C., et al., *Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model.* J Clin Invest, 1998. **101**(7): p. 1458-67.
47. Vlaar, A.P., et al., *Supernatant of stored platelets causes lung inflammation and coagulopathy in a novel in vivo transfusion model.* Blood, 2010. **116**(8): p. 1360-8.
48. Vlaar, A.P., et al., *Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model.* Anesthesiology, 2010. **113**(1): p. 92-103.
49. Wyman, T.H., et al., *A two-insult in vitro model of PMN-mediated pulmonary endothelial damage: requirements for adherence and chemokine release.* Am J Physiol Cell Physiol, 2002. **283**(6): p. C1592-603.
50. Silliman, C.C., et al., *Analysis of the priming activity of lipids generated during routine storage of platelet concentrates.* Transfusion, 1996. **36**(2): p. 133-9.
51. Khan, S.Y., et al., *Lysophosphatidylcholines activate G2A inducing G(alphai)(-)(1)-/G(alphaq/)(1)(1)- Ca(2)(+) flux, G(betagamma)-Hck activation and clathrin/beta-arrestin-1/GRK6 recruitment in PMNs.* Biochem J, 2010. **432**(1): p. 35-45.
52. Carlos, T.M. and J.M. Harlan, *Leukocyte-endothelial adhesion molecules.* Blood, 1994. **84**(7): p. 2068-101.
53. Silliman, C.C., et al., *Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury.* Transfusion, 2011. **51**(12): p. 2549-54.
54. Maslanka, K., et al., *Lysophosphatidylcholines: bioactive lipids generated during storage of blood components.* Arch Immunol Ther Exp (Warsz), 2012. **60**(1): p. 55-60.
55. U.J. Sachs, N.W., W. Wasel, G. Bein, H. Berghöfer, W. Schilow, *Supernatants from stored leukodepleted packed red blood cells do not regularly exhibit changes in their (lyso-) phosphatidylcholine composition and do not cause TRALI in an ex vivo rat lung model.* Vox Sang, 2010. **99**(Suppl. s1): p. p. 456.
56. Adawi, A., et al., *Disruption of the CD40-CD40 ligand system prevents an oxygen-induced respiratory distress syndrome.* Am J Pathol, 1998. **152**(3): p. 651-7.
57. Vlaar, A.P., et al., *Accumulation of bioactive lipids during storage of blood products is not cell but plasma derived and temperature dependent.* Transfusion, 2011. **51**(11): p. 2358-66.

58. Inwald, D.P., et al., *CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation.* Circ Res, 2003. **92**(9): p. 1041-8.
59. Khan, S.Y., et al., *Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury.* Blood, 2006. **108**(7): p. 2455-62.
60. Wenzel, F., et al., *Comparison of soluble CD40L concentrations and release capacities in apheresis and prestorage pooled platelet concentrates.* Clin Hemorheol Microcirc, 2011. **47**(4): p. 269-78.
61. Blumberg, N., et al., *An association of soluble CD40 ligand (CD154) with adverse reactions to platelet transfusions.* Transfusion, 2006. **46**(10): p. 1813-21.
62. Xie, R.F., et al., *The effect of platelet-derived microparticles in stored apheresis platelet concentrates on polymorphonuclear leucocyte respiratory burst.* Vox Sang, 2014. **106**(3): p. 234-41.
63. Hashimoto, N., et al., *CD40 plays a crucial role in lipopolysaccharide-induced acute lung injury.* Am J Respir Cell Mol Biol, 2004. **30**(6): p. 808-15.
64. Tung, J.P., et al., *Age of blood and recipient factors determine the severity of transfusion-related acute lung injury (TRALI).* Crit Care, 2012. **16**(1): p. R19.
65. Tuinman, P.R., et al., *Lack of evidence of CD40 ligand involvement in transfusion-related acute lung injury.* Clin Exp Immunol, 2011. **165**(2): p. 278-84.
66. Vlaar, A.P., M. Straat, and N.P. Juffermans, *The relation between aged blood products and onset of transfusion-related acute lung injury. A review of pre-clinical data.* Clin Lab, 2011. **57**(3-4): p. 267-72.
67. Darbonne, W.C., et al., *Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin.* J Clin Invest, 1991. **88**(4): p. 1362-9.
68. Mangalmurti, N.S., et al., *Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation.* Blood, 2009. **113**(5): p. 1158-66.
69. Leytin, V. and J. Freedman, *Platelet apoptosis in stored platelet concentrates and other models.* Transfus Apher Sci, 2003. **28**(3): p. 285-95.
70. Seghatchian, J. and G. de Sousa, *Pathogen-reduction systems for blood components: the current position and future trends.* Transfus Apher Sci, 2006. **35**(3): p. 189-96.
71. Gelderman, M.P., et al., *Ultraviolet B light-exposed human platelets mediate acute lung injury in a two-event mouse model of transfusion.* Transfusion, 2011. **51**(11): p. 2343-57.
72. Silliman, C.C., et al., *Mirasol Pathogen Reduction Technology treatment does not affect acute lung injury in a two-event in vivo model caused by stored blood components.* Vox Sang, 2010. **98**(4): p. 525-30.
73. Zhi, L., et al., *Activation of platelet protein kinase C by ultraviolet light B mediates platelet transfusion-related acute lung injury in a two-event animal model.* Transfusion, 2013. **53**(4): p. 722-31.
74. Chi, X., et al., *Host platelets and, in part, neutrophils mediate lung accumulation of transfused UVB-irradiated human platelets in a mouse model of acute lung injury.* PLoS One, 2012. **7**(9): p. e44829.
75. Harr, J.N., et al., *Activated platelets in heparinized shed blood: the "second hit" of acute lung injury in trauma/hemorrhagic shock models.* Shock, 2011. **36**(6): p. 595-603.

76. Chi, X., L. Zhi, and J.G. Vostal, *Human platelets pathogen reduced with riboflavin and ultraviolet light do not cause acute lung injury in a two-event SCID mouse model.* Transfusion, 2014. **54**(1): p. 74-85.
77. Tuinman, P.R., et al., *The effect of aspirin in transfusion-related acute lung injury in critically ill patients.* Anaesthesia, 2012. **67**(6): p. 594-9.
78. Lacroix, J., et al., *The Age of Blood Evaluation (ABLE) randomized controlled trial: study design.* Transfus Med Rev, 2011. **25**(3): p. 197-205.
79. Steiner, M.E., et al., *Addressing the question of the effect of RBC storage on clinical outcomes: the Red Cell Storage Duration Study (RECESS) (Section 7).* Transfus Apher Sci, 2010. **43**(1): p. 107-16.
80. CG., K., *Red cell storage duration and outcomes in cardiac surgery.* Clinical Trials Identifier Gov: NCT00458783.
81. DJ, C., *STandaRd Issue TrANsfusion versuS Fresher red blood cell Use in intenSive carE — a randomised controlled trial.* ClinicalTrials.gov NCT01638416, 2013.
82. Barshtein, G., N. Manny, and S. Yedgar, *Circulatory risk in the transfusion of red blood cells with impaired flow properties induced by storage.* Transfus Med Rev, 2011. **25**(1): p. 24-35.
83. Anniss, A.M. and R.L. Sparrow, *Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions.* Am J Hematol, 2007. **82**(6): p. 439-45.
84. Eichelbronner, O., et al., *Endotoxin promotes adhesion of human erythrocytes to human vascular endothelial cells under conditions of flow.* Crit Care Med, 2000. **28**(6): p. 1865-70.
85. Hess, J.R., *Red cell storage.* J Proteomics, 2010. **73**(3): p. 368-73.
86. Jy, W., et al., *Microparticles in stored red blood cells as potential mediators of transfusion complications.* Transfusion, 2011. **51**(4): p. 886-93.
87. Luten, M., et al., *Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods.* Transfusion, 2008. **48**(7): p. 1478-85.
88. Ozment, C.P. and J.L. Turi, *Iron overload following red blood cell transfusion and its impact on disease severity.* Biochim Biophys Acta, 2009. **1790**(7): p. 694-701.
89. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
90. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
91. Mause, S.F. and C. Weber, *Microparticles: protagonists of a novel communication network for intercellular information exchange.* Circ Res, 2010. **107**(9): p. 1047-57.
92. Rubin, O., et al., *Microparticles in stored red blood cells: submicron clotting bombs?* Blood Transfus, 2010. **8 Suppl 3**: p. s31-8.
93. Simak, J. and M.P. Gelderman, *Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers.* Transfus Med Rev, 2006. **20**(1): p. 1-26.

94. McVey, M., A. Tabuchi, and W.M. Kuebler, *Microparticles and acute lung injury.* Am J Physiol Lung Cell Mol Physiol, 2012. **303**(5): p. L364-81.
95. Kriebardis, A.G., et al., *RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components.* Transfusion, 2008. **48**(9): p. 1943-53.
96. Xiong, Z., et al., *Red blood cell microparticles show altered inflammatory chemokine binding and release ligand upon interaction with platelets.* Transfusion, 2011. **51**(3): p. 610-21.
97. Rubin, O., et al., *Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools.* Vox Sang, 2008. **95**(4): p. 288-97.
98. Belizaire, R.M., et al., *Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation.* J Am Coll Surg, 2012. **214**(4): p. 648-55; discussion 656-7.
99. Cardo, L.J., D. Wilder, and J. Salata, *Neutrophil priming, caused by cell membranes and microvesicles in packed red blood cell units, is abrogated by leukocyte depletion at collection.* Transfus Apher Sci, 2008. **38**(2): p. 117-25.
100. Bosman, G.J., et al., *The proteome of red cell membranes and vesicles during storage in blood bank conditions.* Transfusion, 2008. **48**(5): p. 827-35.
101. Morel, O., et al., *Microparticles: a critical component in the nexus between inflammation, immunity, and thrombosis.* Semin Immunopathol, 2011. **33**(5): p. 469-86.
102. Lechner, D. and A. Weltermann, *Circulating tissue factor-exposing microparticles.* Thromb Res, 2008. **122 Suppl 1**: p. S47-54.
103. van der Poll, T., *Tissue factor as an initiator of coagulation and inflammation in the lung.* Crit Care, 2008. **12 Suppl 6**: p. S3.
104. Mackman, N., *Role of tissue factor in hemostasis and thrombosis.* Blood Cells Mol Dis, 2006. **36**(2): p. 104-7.
105. Sinauridze, E.I., et al., *Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets.* Thromb Haemost, 2007. **97**(3): p. 425-34.
106. Sugawara, A., et al., *Preventing platelet-derived microparticle formation--and possible side effects-with prestorage leukofiltration of whole blood.* Arch Pathol Lab Med, 2010. **134**(5): p. 771-5.
107. Antonelou, M.H., et al., *Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol.* Transfusion, 2010. **50**(2): p. 376-89.
108. Bennett-Guerrero, E., et al., *Randomized study of washing 40- to 42-day-stored red blood cells.* Transfusion, 2014. **54**(10): p. 2544-52.
109. Masalunga, C., et al., *Increased hemolysis from saline pre-washing RBCs or centrifugal pumps in neonatal ECMO.* J Perinatol, 2007. **27**(6): p. 380-4.
110. Gruber, M., et al., *Washing of banked blood by three different blood salvage devices.* Transfusion, 2013. **53**(5): p. 1001-9.
111. Smith, T., W. Riley, and D. Fitzgerald, *In vitro comparison of two different methods of cell washing.* Perfusion, 2013. **28**(1): p. 34-7.
112. Jilma-Stohlawetz, P., et al., *The effect of plasma removal from apheresis platelet concentrates on platelet function.* Vox Sang, 2012. **102**(3): p. 258-60.

113. Karafin, M., et al., *The impact of apheresis platelet manipulation on corrected count increment.* Transfusion, 2012. **52**(6): p. 1221-7.
114. Blumberg, N., et al., *An association between decreased cardiopulmonary complications (transfusion-related acute lung injury and transfusion-associated circulatory overload) and implementation of universal leukoreduction of blood transfusions.* Transfusion, 2010. **50**(12): p. 2738-44.
115. de Vroege, R., et al., *Washing of stored red blood cells by an autotransfusion device before transfusion.* Vox Sang, 2007. **92**(2): p. 130-5.
116. Bilgin, Y.M., L.M. van de Watering, and A. Brand, *Clinical effects of leucoreduction of blood transfusions.* Neth J Med, 2011. **69**(10): p. 441-50.
117. Sowemimo-Coker, S.O., *Evaluation of an experimental filter designed for improving the quality of red blood cells (RBCs) during storage by simultaneously removing white blood cells and immunomodulators and improving RBC viscoelasticity and Band 3 proteins.* Transfusion, 2014. **54**(3): p. 592-601.
118. Wang, D., et al., *Transfusion of older stored blood and risk of death: a meta-analysis.* Transfusion, 2012. **52**(6): p. 1184-95.
119. Lelubre, C. and J.L. Vincent, *Relationship between red cell storage duration and outcomes in adults receiving red cell transfusions: a systematic review.* Crit Care, 2013. **17**(2): p. R66.
120. Wallis, J.P., et al., *Single hospital experience of TRALI.* Transfusion, 2003. **43**(8): p. 1053-9.
121. Moore, S.B., *Transfusion-related acute lung injury (TRALI): clinical presentation, treatment, and prognosis.* Crit Care Med, 2006. **34**(5 Suppl): p. S114-7.
122. Popovsky, M.A., *Transfusion-Related Acute Lung Injury: Incidence, Pathogenesis and the Role of Multicomponent Apheresis in Its Prevention.* Transfus Med Hemother, 2008. **35**(2): p. 76-79.
123. Looney, M.R., et al., *Prospective study on the clinical course and outcomes in transfusion-related acute lung injury*.* Crit Care Med, 2014. **42**(7): p. 1676-87.
124. Bennett, S.H., et al., *Pulmonary injury resulting from perfusion with stored bank blood in the baboon and dog.* J Surg Res, 1972. **13**(6): p. 295-306.
125. Bennett, S.H., et al., *Pulmonary effects of autotransfused blood. A comparison of fresh autologous and stored blood with blood retrieved from the pleural cavity in an in situ lung perfusion model.* Am J Surg, 1973. **125**(6): p. 696-702.
126. Nicholson, S.E., et al., *Transfusion-related acute lung injury in a rat model of trauma-hemorrhage.* J Trauma, 2011. **70**(2): p. 466-71.
127. Fung, Y.L., et al., *Stored blood transfusion induces transient pulmonary arterial hypertension without impairing coagulation in an ovine model of nontraumatic haemorrhage.* Vox Sang, 2013. **105**(2): p. 150-8.
128. Tung, J.P., et al., *A novel in vivo ovine model of transfusion-related acute lung injury (TRALI).* Vox Sang, 2011. **100**(2): p. 219-30.
129. Zhi, L., X. Chi, and J.G. Vostal, *In vitro and in vivo characterization of ultraviolet light C-irradiated human platelets in a 2 event mouse model of transfusion.* PLoS One, 2013. **8**(11): p. e79869.

CHAPTER 4

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Monocyte mediated activation of endothelial cells occurs only after binding to extracellular vesicles from red blood cell products, a process mediated by beta integrin

ABSTRACT

BACKGROUND: Red blood cell (RBC) transfusion is associated with organ failure. The mechanism remains unknown, but may include adherence of blood cells to the microvasculature. We hypothesized that RBC-derived extracellular vesicles (EVs) interact with monocytes to activate endothelial cells.

STUDY DESIGN AND METHODS: Human umbilical vein endothelial cells were incubated with supernatant from fresh and stored RBC units either containing EVs or depleted from EVs, with or without the addition of immune cells. We measured expression of adhesion markers by flow cytometry and markers of coagulation and inflammation in the culture medium. We studied phagocytosis of EVs by monocytes by using confocal microscopy and flow cytometry.

RESULTS: Incubation of endothelial cells with monocytes alone did not induce up regulation of adhesion markers. The addition of both monocytes and supernatant from RBCs containing EVs resulted in up regulation of endothelial expression of intercellular adhesion molecule 1 and E-selectin when compared to baseline. Up regulation was absent when stimulated with RBC supernatant depleted from EVs. EVs are phagocytosed by monocytes, which was partly abrogated after coincubation with two different complement receptor 3 (CR3)-blocking antibodies. Addition of RBC-derived EVs also increased levels of von Willebrand factor (VWF). There were no differences between groups related to storage time.

CONCLUSION: EVs from RBC transfusion bags activate monocytes with subsequent up regulation of endothelial cell adhesion markers. EVs are phagocytosed by monocytes through CR3. Furthermore, these EVs proved to be a source of VWF. These effects are unrelated to storage time. Thereby, EVs from RBC transfusion bags induce a pro-inflammatory and pro-coagulant endothelial cell response.

INTRODUCTION

A red blood cell (RBC) transfusion is frequently administered to patients on the Intensive Care Unit (ICU), with up to 45% of patients receiving a blood transfusion [1]. However, the risk-benefit of RBC transfusion is unclear, as observational studies in several patient populations show that RBC transfusion is associated with organ failure, including acute lung injury and acute kidney injury [2-4]. The causative factor in the blood products associated with RBC-related organ failure remains unknown, but includes effects on the vasculature [5]. RBCs regulate blood flow by influencing vasomotor tone of blood vessels. This regulation is impaired after prolonged storage by reducing nitric oxide (NO) bio availability, both in experimental [6] and clinical settings [7]. Furthermore, in an animal model, transfused RBCs adhere to the endothelium in the microcirculation, which is further enhanced after prolonged RBC storage [8]. The mechanism of interaction between RBCs and endothelium is unknown. We previously found that washing of RBCs attenuates organ injury in a rat transfusion model [9], suggesting a soluble factor in the blood product. This factor is thus far not identified, but there may be a role for extracellular vesicles (EVs).

EVs are small phospholipid vesicles released from most cell types. EVs facilitate intercellular exchange of information without direct cell-to-cell contact. High concentrations of RBC-derived EVs are present in the supernatant of RBC transfusion bags [10], which were shown to have pro-inflammatory and pro-coagulant effects [11-13]. We hypothesize that RBC-derived EVs can activate endothelial cells through activation of host immune cells and that this effect is a function of storage time.

MATERIAL & METHODS

Isolation of human umbilical vein endothelial cells (HUVECs)

We collected HUVECs from human umbilical veins as described previously [14]. Our Ethical Committee waived for informed consent under number W12-167#12.17.096. The HUVECs were cultured in gelatin (0.75%) coated 25 cm^2 flasks (passage 0) and 75 cm^2 flasks (passages 1 and 2). We performed the experiments with cells from the third passage and these cells were grown in a 12-well plate coated with gelatin, containing endothelial cell growth medium with supplement (Promocell) to which we added 10% Fetal Bovine Serum (Tico Europe), 100 U/mL Penicillin and 0.1 mg/mL Streptomycin (Sigma-Aldrich), 2.5 µg/mL Amphotericin B (Sigma-Aldrich) and

2 mM L-glutamine (Gibco). After reaching confluence, the cells were maintained for 1 day until steady state was achieved before the experiments were started.

Preparation of blood samples

We obtained RBC units from Sanquin blood bank (Amsterdam, The Netherlands) after written informed consent from the donors. These RBC units were prepared and stored according to Sanquin blood bank standards. In short, donations are stored overnight at ambient temperature. Blood is then centrifuged at 20°C, 1600g for 8 min, deceleration rate 3 (Sorvall RC12BP, ThermoFisher Scientific). The centrifuged blood is separated automatically with a blood component extractor (Compomat G5, Fresenius) in plasma, buffy coat and RBC suspensions. After separation, SAGM, 150 mmol/L NaCl, 1.25 mmol/L adenine, 50 mmol/L glucose, 29 mmol/L mannitol) [SAGM]; Fresenius Kabi) is added to the RBCs via the filter on the Compomat. After being carefully mixed, the RBCs will be filtered by gravity over the integrated leukoreduction filter into the empty additive solution bag. The RBC product characteristics meet the following targets: volume 250 to 280 mL, hematocrit 0.5 to 0.65, hemoglobin (Hb) level of more than 40 g/unit, platelet (PLT) count of less than 10×10^9/L, residual white blood cell (WBC) count of less than 1×10^6/unit, hemolysis at end of shelf life of less than 0.8%, ATP at end of shelf life more than 2.7 µmol/g Hb.

RBC units were sampled at day 4 and day 35 and diluted 1:1 with filtered phosphate-buffered saline (PBS). The diluted sample was centrifuged again at 1550g at 20°C. Finally, the EV-containing supernatant obtained after the second centrifugation step was snap frozen and stored at -80°C until further use. On the day of each experiment, we thawed samples from both fresh (4 days old) and stored (35 days old) RBC bags and centrifuged these for 60 minutes at 150.000g, 4°C. Hereafter, we isolated the EV-free supernatant. The EV-containing sample was prepared by resuspending the EV-pellet in a fixed volume of supernatant.

Characterization of EVs

We quantified EVs using tunable resistive pulse sensing (TRPS; qNano; Izon), which determines the particle size distribution and concentration of particles in suspension [15]. To determine the origin of the EVs present in the RBC supernatant, we performed flow cytometry with a dedicated and sensitive flow cytometer (A50 Micro; Apogee). To avoid swarm detection, samples were measured at a maximum event rate of 2000 events/sec. This event rate was achieved by dilution (1-300 fold) of the cell-free supernatants of fresh and stored RBC units before labeling. Thereafter, diluted sample (20 µL) was incubated for 60 minutes with antibodies (2.5 µL) against

CD235 (PE; Dako; 50 µg/mL) to determine RBC-derived EV, CD61 (PE; Becton Dickinson [BD]; 6.25 µg/mL) to determine PLT-derived EV, and CD45 (PE; BD, 5 µg/mL) to determine WBC-derived EV. After incubation, PBS containing 0.32% citrate (200 µL) was added and samples were measured on a flow cytometry (A50 Micro) for 1 minute at a flow rate of 4,51 µL/min, with the trigger on LALS and SALS. Data were analyzed with computer software (FlowJo, FlowJO LLC).

Cell culture protocol

In some experiments, we added immune cells to the culture assay. We isolated these immune cells by drawing blood from healthy volunteers (after written informed consent was obtained) in 10 mL Sodium Heparin tubes, diluting this blood 1:1 with PBS and isolating granulocytes and monocytes by using Ficoll-Paque (GE Healthcare Life Sciences). After isolation, white blood cells were counted on a hematocytometer (Z2 Coulter Particle Counter; Beckman Coulter Corporation). At the start of the experiment, the culture medium of the HUVECs was replaced with 925 µL fresh culture medium containing either 150.000 monocytes, 3.000.000 neutrophils or no cells. We used tumor necrosis factor α (TNF-α; 10 ng/mL) as a positive control and PBS as a negative control. After 1 hour, we added 100 µL of RBC supernatant sampled at day 4 (fresh) either containing EVs or depleted from EVs or RBC supernatant sampled at day 35 (stored) either containing EVs or depleted from EVs. A pilot trial was done to determine optimal stimulation duration, showing that 5 hours of stimulation resulted in upregulation of adhesion markers. After 5 hours of stimulation with RBC supernatant, medium was collected, snap frozen, and stored at -80°C until further use. Subsequently, cells were detached with 300 µL trypsin and washed in PBS- fetal calf serum 1%, after which the supernatant was removed and centrifuged for 10 minutes at 600g at 4°C and stored until analysis at -80°C.

We repeated each set of experimental conditions five or ten times in HUVECs derived from umbilical cords from five different donors. Every set of conditions was completely performed in HUVECs from the same donor.

Monocyte experiments

We isolated monocytes from blood from healthy donors using positive selection with CD14 beads on a MACS system (Miltenyi Biotec). RBC supernatant samples containing EVs (1200 µL) were incubated with 1 µL of PKH26 membrane dye (Sigma-Aldrich) in the dark at room temperature during 10 minutes. Subsequently, EVs were centrifuged for 60 min at 150.000g and the EV- pellet was resuspended in a fixed volume of supernatant.

PKH stained RBC-derived EVs were quantified with qnano and n=1,000,000 were added to 250.000 monocytes in a polysterene tube in a total volume of 200 µL in Hepes buffer (132 mmol/L NaCl, 20 mmol/L HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 6 mmol/L KCL, 1 mmol/L MgSO, 1.2 mmol/L K_2HPO_4 (all from Sigma-Aldrich) supplemented with 0.5% (vol/vol) human serum albumin, 1 mmol/L $CaCl_2$ (Sigma-Aldrich) and 10 mmol/L glucose (Sigma-Aldrich) and were placed in a 37°C water bath for 1.5 hours. After incubation, samples were washed with Hepes twice.

We analyzed the monocytes without and containing PKH stained EVs by confocal microscopy (Zeiss). Monocytes were also incubated with antibodies against CD11b and CD18. CytoB (2.5 mg/mL, 1:500) was used as a negative control. Monocytes were then analyzed with flow cytometry until 5000 events were counted, and we recorded the forward scatter (FSC), sideward scatter (SSC) and the fluorescence.

Assays

Collected culture medium from the cells was thawed and the following measurements were performed. Levels of Interleukin-6 (IL-6), Interleukin-8 (IL-8; R&D Systems) and syndecan-1 (Diaclone) were determined by enzyme- linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Levels of syndecan-1 and von Willebrand factor (vWF) antigen (vWF:Ag) levels were determined with an in-house ELISA using commercially available polyclonal antibodies against vWF (DAKO). Levels of vWF were also measured in both the EV-free and EV-containing supernatant of the RBC bags.

Statistical analysis

Results are depicted as median and interquartile range (IQR). Results of EV-characterization were depicted as mean and standard deviation. To study the effect of EVs, comparisons between groups were performed using the percentage of change compared to their negative control (PBS), that is, the change compared to baseline of a certain parameter when EVs were added versus the change compared to baseline of a certain parameter when EVs were omitted. We initially performed a Kruskal Wallis and when a post-hoc analysis was warranted, we performed a Mann-Whitney U test. Results of EV characterization were analyzed using independent samples t-test. Statistics were performed using software (SPSS 22, SPSS inc.; and Graphpad Prism 5, Graphpad software).

RESULTS

Baseline effect of co-incubation of endothelial cells with immune cells

In the HUVEC model, ICAM-1 is expressed on 40% of cells, VCAM-1 is expressed on 10% of cells and E-selectin is expressed on 2.3% of cells (Table 1). The addition of granulocytes increased expression of all cell adhesion markers significantly, whereas co-incubation with monocytes had no effect on expression of cell adhesion markers (Table 1), nor on secretion of markers of endothelial activation or cytokines into the culture medium (Table 1). TNFα was used as a positive control and upregulated all adhesion markers tested. Thereby, monocytes alone did not provoke an endothelial cell response in this model.

Table 1. Baseline measurement of the endothelial cell culture condition with and without the addition of immune cells, showing ICAM-1, VCAM-1, E-selectin, TF, vWF, TNFα, IL-6, and IL-8.

	No cells	Monocytes	Granulocytes
Adhesion markers			
ICAM-1-1 (% positive cells)	39.9 [26.6-44.2]	44.6 [29.5-50.8]	65.8 [38.7-74.4]*
VCAM-1-1 (% positive cells)	12.2 [6.2-14.9]	14.4 [9.0-17.8]	33.5 [12.7-46.7]*
E-selectin (% positive cells)	2.8 [2.0-6.1]	3.0 [2.2-4.9]	25.1 [3.9-31.6]
TF (% positive cells)	3.9 [3.5-7.1]	5.0 [3.1-7.4]	14.7 [5.1-16.6]
Shed endothelial markers			
vWF (%)	0.7 [0.5-0.8]	0.6 [0.5-0.8]	0.9 [0.6-1.1]
Syndecan-1 (ng/mL)	3.2 [2.0-7.1]	2.5 [2.0-6.6]	3.4 [2.0-7.6]
Shed cytokines (pg/mL)			
TNF α (pg/mL)	4.7 [3.0-35.5]	8.1 [3.0-29.5]	3.0 [3.0-27.3]
IL-6 (pg/mL)	117.4 [25.1-176.5]	119.5 [28.1-198.7]	336.1 [311.1-1327.3]*
IL-8 (pg/mL)	1523.2 [1248.4-1648.0]	1426.7 [1111.1-1901.2]	2090.3 [1298.0-3934.2]*

Data are expressed as median [IQR]. *p <0.05 versus 'No cells'.

Characterization of EVs in the RBC supernatants

EVs from fresh RBC units were 217 (±57) nm in size, which did not differ from the size of EVs from stored RBC units, which were 191 (±46) nm (p=0.3). The amount of EVs increased during storage, from 27 × 10^8 (±15 × 10^8) to 848 × 10^8 (±599 × 10^8;

p = 0.037). The EVs were primarily RBC-derived EVs (Table 2). Storage tended to decrease the amount of EVs derived from WBCs and PLTs.

Table 2. Characterization of EVs in supernatant of RBCs units.

Origin	Fresh EVs	Stored EVs	p-value
WBCs (% of total)	0.93 (± 0.74)	0.17 (± 0.09)	0.052
PLTs (% of total)	4.03 (± 3.34)	0.26 (± 0.19)	0.065
RBCs (% of total)	95.04 (± 4.06)	99.57 (± 0.27)	0.067

Data are expressed as mean [SD].

EVs upregulate expression of endothelial ICAM and E-selectin when co-cultured with monocytes.

In the absence of WBCs, expression of endothelial adhesion markers did not differ between HUVECs incubated with EV-depleted or EV-containing RBC supernatant (Figure 1). However, when HUVECs were incubated with EV-containing RBC supernatant in the presence of monocytes, expression of ICAM-1 and E-selectin significantly increased compared to HUVECs incubated with EV-depleted RBC supernatant. Expression of VCAM-1 also increased in response to EV-containing RBC supernatant in the presence of monocytes, but this result was not statistically significant. In contrast to monocytes, the addition of granulocytes to EV-containing supernatant did not influence expression of adhesion markers.

EVs did not influence markers of endothelial integrity

Tissue factor (TF) was expressed on a small percentage of HUVECs (Table 1). Incubation with EV-containing RBC supernatant did not upregulate TF expression compared to EV-depleted supernatant, neither in the presence nor the absence of WBCs (Figure 2). Also, EVs did not result in shedding of syndecan-1. Thereby, EVs did not seem to activate the endothelium. Incubation of endothelial cells with RBC supernatant either with or without EVs, did not induce the secretion of TNFα, IL-6 or IL-8 into the culture medium (Figure 3).

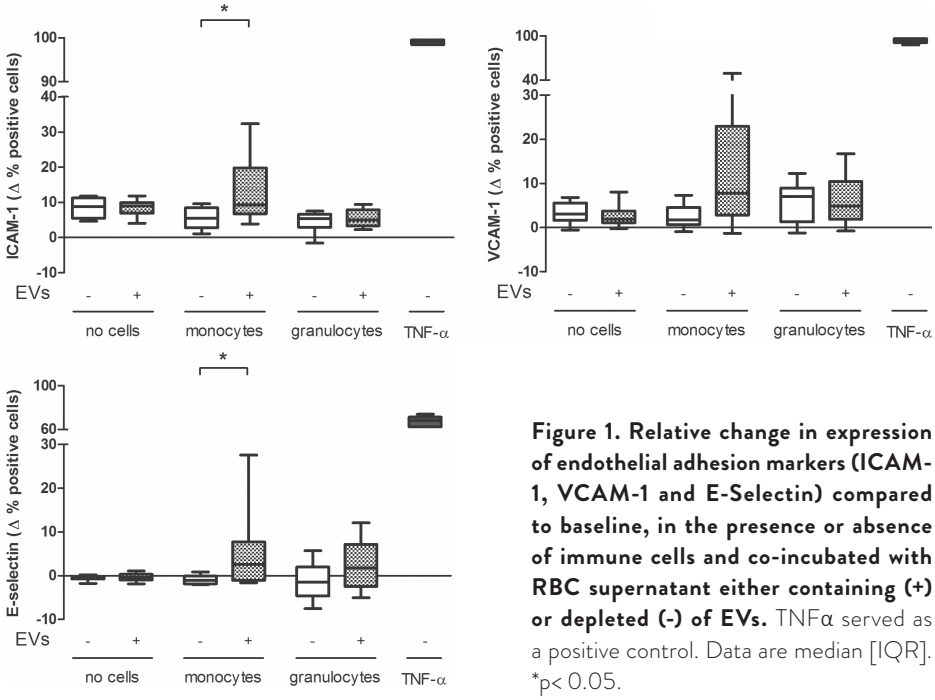

Figure 1. Relative change in expression of endothelial adhesion markers (ICAM-1, VCAM-1 and E-Selectin) compared to baseline, in the presence or absence of immune cells and co-incubated with RBC supernatant either containing (+) or depleted (-) of EVs. TNFα served as a positive control. Data are median [IQR]. *p< 0.05.

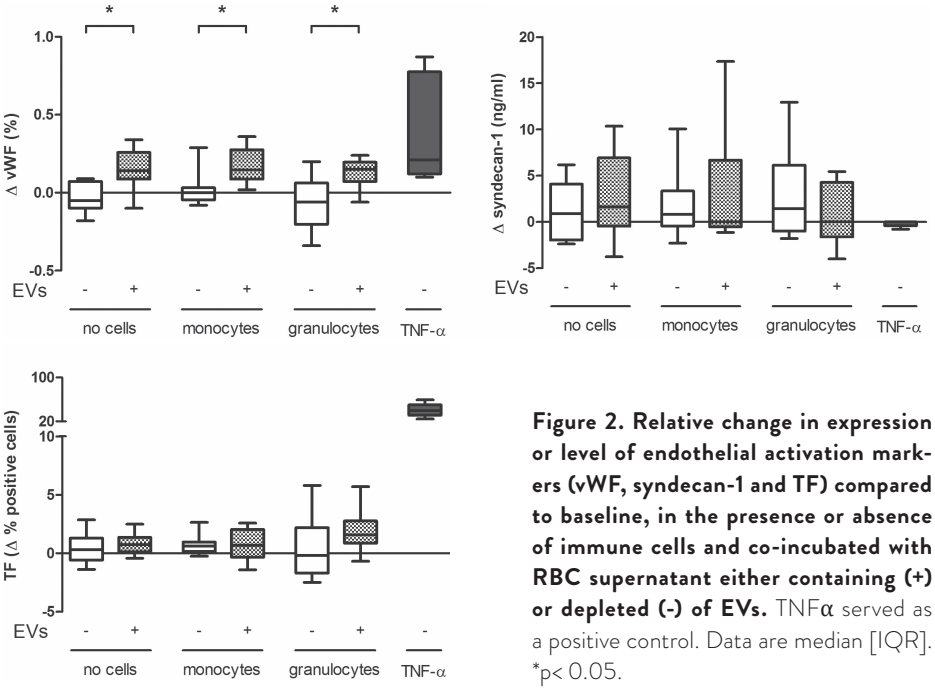

Figure 2. Relative change in expression or level of endothelial activation markers (vWF, syndecan-1 and TF) compared to baseline, in the presence or absence of immune cells and co-incubated with RBC supernatant either containing (+) or depleted (-) of EVs. TNFα served as a positive control. Data are median [IQR]. *p< 0.05.

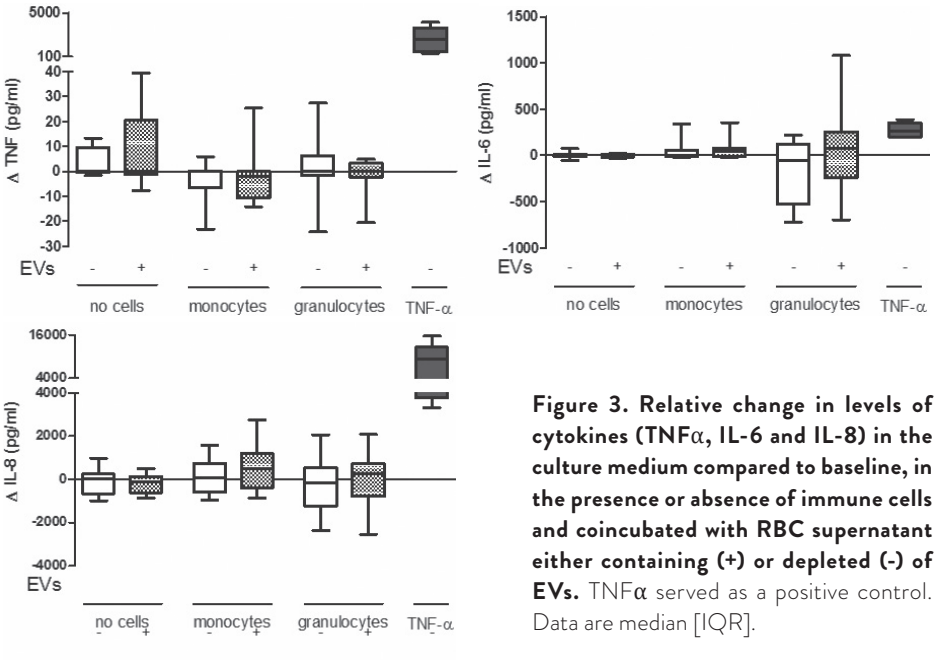

Figure 3. Relative change in levels of cytokines (TNFα, IL-6 and IL-8) in the culture medium compared to baseline, in the presence or absence of immune cells and coincubated with RBC supernatant either containing (+) or depleted (-) of EVs. TNFα served as a positive control. Data are median [IQR].

EVs increase vWF levels

Incubation of HUVECS with EVs resulted in increased levels of vWF antigen into the culture medium, which occurred also without the addition of immune cells. To determine the source of vWF, samples of RBC units containing EVs were compared to RBC samples depleted from EVs. The level of vWF antigen in RBC samples with EVs was higher compared to samples of RBC units depleted from EVs (2.3 ± 0.9 vs 0.1 ± 0.04 %, $p<0.001$).

EVs are phagocytosed by monocytes via complement receptor 3

Having observed that EVs increase endothelial adhesion markers only in the presence of monocytes, we further delineated the interaction of monocytes with EVs. After incubation of monocytes with stained EVs, confocal microscopy showed that the EVs were taken up by monocytes (Figure 4). In line with this, monocytes incubated with PKH-stained EVs were positive when analyzed by flow cytometry, demonstrating that EVs bind to monocytes. This process was partially inhibited by incubation with antibodies directed against the α chain of complement receptor 3 (CR3; α_m, CD11b) as well as to the β chain (β_2, CD18). Both anti-CD11b or antiCD18 resulted in a decrease in EV-positive monocytes (Figure 5).

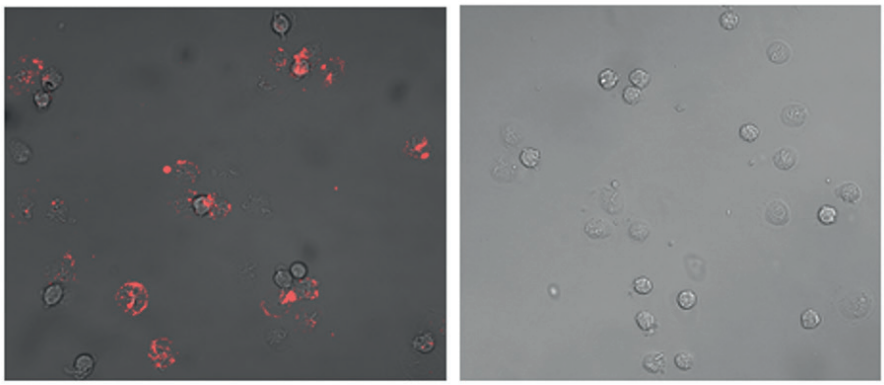

Figure 4. Confocal images of monocytes incubated with (A) and without (B) PKH-stained extracellular vesicles (indicated by the red color).

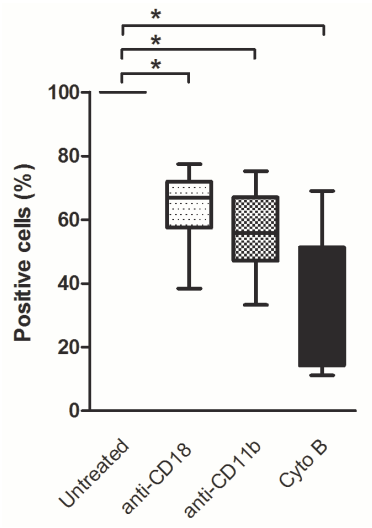

Figure 5. Monocytes positive for PKH after incubation with PKH-stained EVs, untreated or in the presence of the β integrin antibodies anti-CD18 or anti-CD11b or cytochalasin B (Cyto B) as a negative control. Data are median [IQR]. *p<0.05.

Effects of EVs on the endothelium are independent of storage time

To investigate whether storage time influenced the effects of EV-containing RBC supernatant on endothelial cell activation mediated by monocytes, HUVECS were stimulated in the presence of monocytes to which either fresh or stored RBC supernatant was added containing EVs (Figure 6). There was no difference in inflammatory response of endothelial cells between HUVECs incubated with EVs from fresh RBC supernatant and EVs from stored RBC supernatant, nor was there any difference in upregulation of TF or the shedding of vWF and syndecan-1.

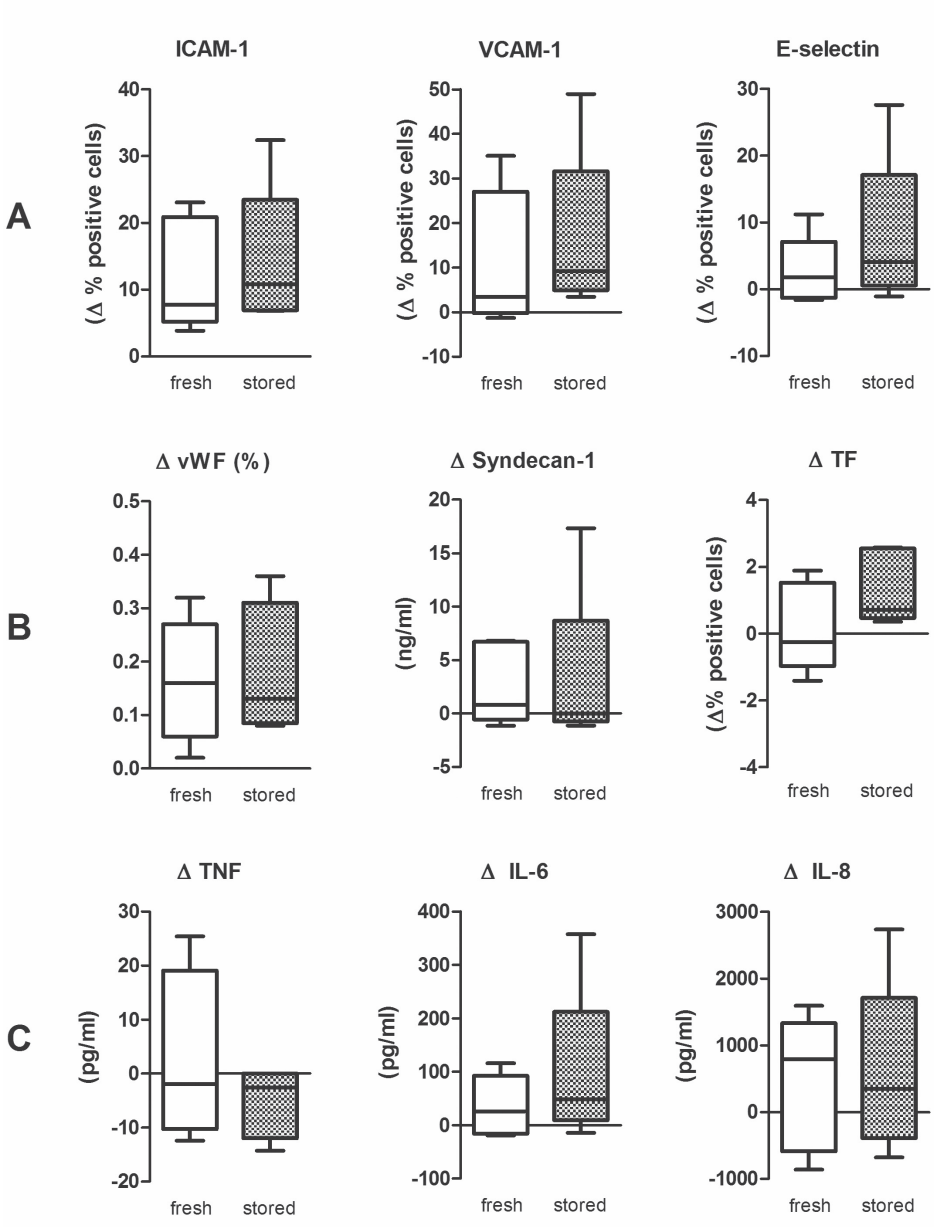

Figure 6. Relative change in expression of endothelial adhesion (A) and activation markers (B) and cytokine levels (C) compared to baseline, in the presence of monocytes and co-incubated with supernatant containing EVs from either fresh or stored RBC products. Data are median [IQR].

DISCUSSION

EVs from RBC products interact with monocytes via a mechanism involving adherence of EVs to monocytes which is at least in part mediated by CR3 on the monocytes, whereafter these activated monocytes induce upregulation of endothelial cell adhesion molecules ICAM and E-selectin. Also, these EVs seem to be a source of vWF, which is involved both as a plasma ligand for cell adhesion as well in the formation of micro-thrombi. Thereby, EVs from RBC can activate a pro-inflammatory and pro-coagulant response from endothelial cells. These processes are unrelated to storage time.

RBC transfusion is associated with immune modulation [16-20], demonstrating both pro- and anti-inflammatory effects. In a clinical study in pediatric patients, RBC transfusion resulted in an increase in soluble ICAM-1, which is a marker of activated endothelium. Our data extend these findings by showing that the compound in the blood product resulting in activation of endothelial adhesion markers are RBC-derived EVs, via a process that requires monocytes. These EVs need monocytes in order to upregulate adhesion markers, whereas monocytes alone do not increase expression. We observed that EVs bind to monocytes, a process that is blocked by antibodies to CR3. Thereby, EVs seem to activate monocytes via integrins, which in concert with EVs may then increase (secondary) adhesion of cells to the endothelium. It was shown *in vitro,* that both lipopolysaccharide and oxidative stress promote adherence of donor RBCs to the endothelium [21, 22], which may suggest that RBC transfusion may impede blood flow in inflammatory recipients. This observation may be an explanation for the consistent clinical observation of the association of RBC transfusion with organ injury in the critically ill [2-4]. Alternatively, immune cells may adhere to the endothelium. Whether RBCs or other cells adhere to the endothelium following up-regulation of adhesion markers by donor RBC-derived EVs requires further study.

In this study, we also investigated whether RBC-derived EVs have a pro-coagulant effect on endothelium. RBC-derived EVs have an abundant expression of TF and are also referred to as a 'blood-borne' TF source. In addition, RBC-derived EVs express phosphatidylserine (PS), which was shown to induce thrombin generation *in vitro* [23]. In this study, EVs did not stimulate endothelial TF expression. Of note however, RBC-derived EVs are a source of vWF. Thereby, another mechanism of activation of coagulation by EVs unrelated to TF is increasing circulating levels of vWF.

The amount of EVs as well as the number of PS-expressing EVs accumulate in RBC products increases during storage [11, 12], suggesting that EVs from stored blood could induce a hypercoagulable state. We found no effect of storage duration on EV-induced endothelial cell activation. However, some studies show that stored RBCs but not fresh RBCs adhere to endothelial cells *in vitro* and to the microcirculation *in vivo* [8, 24]. Apparently, this increased adhesion of stored RBCs does not depend on an increased endothelial cell activation. In line with our findings, another study found that inflammation and not storage time determined the effect of donor RBCs on endothelium [22]. In accordance, large trials in ICU populations showed that age of blood does not seem to influence clinically relevant outcome parameters [25, 26].

Thereby, other factors influence the efficacy of RBC transfusion, which may include the condition of the recipient. In critically ill patients, RBC transfusion may have more pro-coagulant, pro-inflammatory and pro-adhesive effects than in chronic anemia patients who do not have a hyperinflammatory status. Other mechanisms of the association between RBC transfusion and outcome may include manufacturing processes [27, 28], or issues related to donors [29]. Our data suggest that washing or filtering of RBC units to deplete them from EVs prior to transfusion may be a beneficial intervention.

CONCLUSION

EVs from RBC products activate endothelial cells via a mechanism involving adherence of EVs to monocytes, which is mediated by β integrin and results in up regulation of endothelial adhesion markers. Whether depleting of RBC products of EVs prior to transfusion is beneficial on a relevant outcome level, should be further explored.

REFERENCES

1. Corwin, H.L., et al., *The CRIT Study: Anemia and blood transfusion in the critically ill--current clinical practice in the United States.* Crit Care Med, 2004. **32**(1): p. 39-52.
2. Gauvin, F., et al., *Association between length of storage of transfused red blood cells and multiple organ dysfunction syndrome in pediatric intensive care patients.* Transfusion, 2010. **50**(9): p. 1902-13.
3. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
4. Moore, F.A., E.E. Moore, and A. Sauaia, *Blood transfusion. An independent risk factor for postinjury multiple organ failure.* Arch Surg, 1997. **132**(6): p. 620-4; discussion 624-5.
5. Peters, A.L., et al., *Pathogenesis of non-antibody mediated transfusion-related acute lung injury from bench to bedside.* Blood Rev, 2015. **29**(1): p. 51-61.
6. Alexander, J.T., et al., *Red blood cells stored for increasing periods produce progressive impairments in nitric oxide-mediated vasodilation.* Transfusion, 2013. **53**(11): p. 2619-28.
7. Neuman, R., et al., *Effects of storage-aged red blood cell transfusions on endothelial function in hospitalized patients.* Transfusion, 2014.
8. Chin-Yee, I.H., et al., *Transfusion of stored red blood cells adhere in the rat microvasculature.* Transfusion, 2009. **49**(11): p. 2304-10.
9. Vlaar, A.P., et al., *Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model.* Anesthesiology, 2010. **113**(1): p. 92-103.
10. Rubin, O., et al., *Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation.* Transfusion, 2013. **53**(8): p. 1744-54.
11. Gao, Y., et al., *Elevated levels of thrombin-generating microparticles in stored red blood cells.* Vox Sang, 2013. **105**(1): p. 11-7.
12. Jy, W., et al., *Microparticles in stored red blood cells as potential mediators of transfusion complications.* Transfusion, 2011. **51**(4): p. 886-93.
13. Straat, M., et al., *Extracellular Vesicles from Red Blood Cell Products Induce a Strong Pro-Inflammatory Host Response, Dependent on Both Numbers and Storage Duration.* Transfus Med Hemother 2015. **Epub**.
14. Weber, N.C., et al., *Intermitted pharmacologic pretreatment by xenon, isoflurane, nitrous oxide, and the opioid morphine prevents tumor necrosis factor alpha-induced adhesion molecule expression in human umbilical vein endothelial cells.* Anesthesiology, 2008. **108**(2): p. 199-207.
15. van der Pol, E., et al., *Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing.* J Thromb Haemost, 2014. **12**(7): p. 1182-92.
16. Claridge, J.A., et al., *Blood transfusions correlate with infections in trauma patients in a dose-dependent manner.* Am Surg, 2002. **68**(7): p. 566-72.
17. Taylor, R.W., et al., *Impact of allogenic packed red blood cell transfusion on nosocomial infection rates in the critically ill patient.* Crit Care Med, 2002. **30**(10): p. 2249-54.

18. Rohde, J.M., et al., *Health care-associated infection after red blood cell transfusion: a systematic review and meta-analysis*. JAMA, 2014. **311**(13): p. 1317-26.
19. Blajchman, M.A., *Immunomodulation and blood transfusion*. Am J Ther, 2002. **9**(5): p. 389-95.
20. Vamvakas, E.C. and M.A. Blajchman, *Transfusion-related immunomodulation (TRIM): an update*. Blood Rev, 2007. **21**(6): p. 327-48.
21. Mittag, D., et al., *Stored red blood cell susceptibility to in vitro transfusion-associated stress conditions is higher after longer storage and increased by storage in saline-adenine-glucose-mannitol compared to AS-1*. Transfusion, 2015. **55**(9): p. 2197-206.
22. Anniss, A.M. and R.L. Sparrow, *Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions*. Am J Hematol, 2007. **82**(6): p. 439-45.
23. Zecher, D., A. Cumpelik, and J.A. Schifferli, *Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement*. Arterioscler Thromb Vasc Biol, 2014. **34**(2): p. 313-20.
24. Anniss, A.M. and R.L. Sparrow, *Storage duration and white blood cell content of red blood cell (RBC) products increases adhesion of stored RBCs to endothelium under flow conditions*. Transfusion, 2006. **46**(9): p. 1561-7.
25. Lacroix, J., et al., *Age of transfused blood in critically ill adults*. N Engl J Med, 2015. **372**(15): p. 1410-8.
26. Steiner, M.E., et al., *Effects of red-cell storage duration on patients undergoing cardiac surgery*. N Engl J Med, 2015. **372**(15): p. 1419-29.
27. Bakkour, S., et al., *Manufacturing method affects mitochondrial DNA release and extracellular vesicle composition in stored red blood cells*. Vox Sang, 2016.
28. Heddle, N.M., et al., *Red blood cell processing methods and in-hospital mortality: a transfusion registry cohort study*. Lancet Haematol, 2016. **3**(5): p. e246-54.
29. Chasse, M., et al., *Clinical effects of blood donor characteristics in transfusion recipients: protocol of a framework to study the blood donor-recipient continuum*. BMJ Open, 2015. **5**(1): p. e007412.

CHAPTER 5

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The effect of red blood cell transfusion on iron metabolism in critically ill patients

ABSTRACT

BACKGROUND: Anemia of Inflammation (AI) has a high prevalence in critically ill patients. In AI, iron metabolism is altered, as high levels of inflammation-induced hepcidin reduce the amount of iron available for erythropoiesis. AI is treated with red blood cell (RBC) transfusions. The effect of RBC transfusion on iron metabolism during inflammatory processes in adults is unknown. We investigated the effect of RBC transfusion on iron metabolism in critically ill patients.

STUDY DESIGN AND METHODS: In a prospective cohort study in 61 critically ill patients who received one RBC unit, levels of iron parameters were determined before, directly after and 24 hours after transfusion in septic and non-septic patients.

RESULTS: Serum iron levels were low, and increased following transfusion (p=0.02). However, RBC transfusion had no effect on transferrin saturation (p=0.14) and ferritin levels (p=0.74). Hepcidin levels increased after RBC transfusion (p=0.01), while IL-6 levels decreased (p=0.03). In septic patients, RBC transfusion induced a decrease in haptoglobin levels compared to baseline, which did not occur in non-septic patients (p=0.01). The effect of RBC transfusion on other iron parameters did not differ between septic and non-septic patients.

CONCLUSION: Transfusion of a RBC unit transiently increases serum iron levels in ICU patients. The increase in hepcidin levels after transfusion can further decrease iron release from intracellular storage making it available for erythropoiesis. RBC transfusion is associated with a decrease in haptoglobin levels in septic compared to non-septic patients, but did not affect other markers of hemolysis.

INTRODUCTION

Anemia has a high prevalence in the critically ill, occurring in up to 95% of all patients after day 3 of intensive care unit (ICU) admission [1]. Although its cause is often multifactorial, inflammation is thought to contribute to anemia in the majority of critically ill patients [2]. In anemia of Inflammation (AI), iron homeostasis is altered, due to high levels of hepcidin [3]. Production of hepcidin is increased in response to inflammatory cytokines such as interleukin 6 (IL-6). Hepcidin inhibits cellular iron export through degradation of the iron exporter ferroportin, causing the sequestration of iron in cells (like macrophages and enterocytes), resulting in low levels of iron available for erythropoiesis [3]. Currently, anemia is treated with red blood cell (RBC) transfusions. More than one fourth of critically ill patients receive at least one RBC transfusion during their ICU stay [4]. A RBC unit contains 220 to 250 mg of iron bound to hemoglobin [5]. Given that up to 25% of the RBCs are cleared shortly after transfusion [6-8], a RBC transfusion may result in the delivery of an iron load approximating 50-60 mg to the monocyte/macrophage system, from where it is released into the plasma [9, 10]. In comparison, the average intestinal iron intake is only 12-18 mg per day [11]. Thereby, RBC transfusion could increase both iron levels as well as iron availability for erythropoiesis. On the other hand, RBC transfusion may cause iron overload [7, 12], leading to the presence of non-transferrin bound iron (NTBI) [9]. NTBI catalyzes the Haber-Weiss reaction in which toxic reactive oxygen species are formed, causing oxidative stress and tissue damage [13]. Also, increased iron availability was found to correlate with increased mortality in ICU patients [14]. Thereby, RBC transfusions may be detrimental for the iron metabolism. In previous studies, differential effects of RBC transfusion were found, resulting in both an increase in NTBI levels in healthy volunteers [10], as well as no effect on NTBI levels in volunteers infused with lipopolysaccharide [13]. Besides the infusion of lipopolysaccharide, these studies also differed in RBC storage time – 42 days [10] and 35 days [13] – which may explain the different outcome. The aim of this study was to investigate the effect of RBC transfusion on iron metabolism in critically ill patients. Given that iron metabolism is more severely disturbed in septic than in non-septic patients [14, 15], the effect of RBC transfusion on the iron metabolism of septic patients was compared to non-septic patients.

MATERIAL & METHODS

Study design

A prospective, observational cohort study was conducted in the ICU of 2 tertiary hospitals in The Netherlands and included patients for 4 years (trialregister.nl NTR 6596). This study was approved by The Institutional Review Board of the Academic Medical center, Amsterdam (NL61833.018.17). Written informed consent was obtained from all participants or their legal representatives.

Patient selection

Eligible patients were patients who received one RBC unit. Exclusion criteria were 1) actively bleeding patients; 2) patients who received multiple RBC units 3) patients who received RBC, plasma or thrombocyte transfusion in the last 24 hours prior to inclusion. Patients were defined as having sepsis according to the Sepsis-3 criteria [16]; Sequential Organ Failure Assessment (SOFA) score ⩾2 in combination with a suspected or proven infection which was treated with antibiotics.

Sampling and analysis

Blood was drawn into heparin tubes on three time points: just prior to transfusion (baseline); within 1 hour post-transfusion; and 24 hours post-transfusion. Samples were centrifuged at 1500g for 20 minutes at room temperature. The plasma was stored at -80 °C. Iron, transferrin, ferritin and haptoglobin were measured by immunoturbidimetric methods. Transferrin saturation was calculated by plasma iron (µmol/L) / (25.2 x transferrin (g/L)). Hepcidin (R&D Systems, MN, USA) and IL-6 (R&D Systems, MN, USA) were measured by enzyme-linked immunosorbent assay kits. Cell free hemoglobin (Hb) and free heme were measured with QuantiChrom assay kits (BioAssay Systems, Hayward, CA). Iron, transferrin, transferrin saturation, cell free Hb and free heme were measured in blood samples of all three time points. Ferritin, haptoglobin, hepcidin and IL-6 were measured in blood samples at baseline and 24 hours post-transfusion, since a rapid effect following transfusion was not expected.

Statistical analysis

In endotoxemic volunteers, a sample size of 25 patients has 95% power with a two-sided significance level of 0.05 and an effect size of 0.76 to show difference in iron levels comparing pre and post-transfusion [13]. We included more patients in our study, because this sample size calculation was based on healthy volunteers and not on critically ill patients, who are highly heterogeneous. For continuous variables,

the t-test, or in case of not normally distributed data, the Mann-Whitney-U test was used. For categorical variables, the Chi-square test was used. Data was expressed as median [IQR]. To determine the effect of RBC transfusion on ICU patients, a paired samples T-Test was performed, or a Wilcoxon Signed Ranks test if the data was skewed. To assess the differential effects of RBC transfusion on septic compared to non-septic patients, the post-transfusion change (delta) of each iron parameter was determined compared to baseline for each patient. For hepcidin, ferritin, haptoglobin and IL-6 the delta between baseline and post 24h was used. Given that a rapid effect following transfusion was expected for iron, transferrin (saturation), free heme and free Hb, the delta between baseline and post 1h was used. Differences between deltas were analyzed with the unpaired t-test for normally distributed data; or a Mann-Whitney-U test if the data was skewed. Spearman correlation was used to assess the correlation between RBC storage time and changes in iron parameters. Statistical significance was considered to be at p 0.05. All statistical analyses were performed using *R Statistics*.

RESULTS

Subjects

61 patients were included, of which 34 patients were septic and 27 patients were non-septic at study inclusion (figure 1). Baseline characteristics are given in table 1. Non-septic patients were more often male. Age, SOFA score and Hb levels of patients were not statistically different between the groups. The storage time of the RBC units did not differ between groups either.

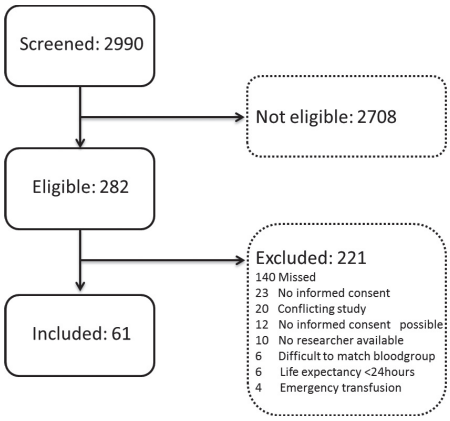

Figure 1. Inclusion chart.
A patient was eligible for the study when the patient received 1 unit of RBCs, when there was no bleeding and when the patient had not received RBC, plasma or thrombocyte transfusion in the last 24 hours prior to inclusion.

Table 1. Baseline characteristics

	Total n = 61	**No sepsis n = 27**	**Sepsis n = 34**	**p-value**
Male, ***n (%)***	33 (59)	20 (74)	13 (38)	0.01
Age, years ***median [IQR]***	63 [57–72]	61 [57–71]	64.5 [59–74]	0.30
SOFA score, ***median [IQR]***	7 [5–9]	7 [6–9]	8 [5–9]	0.23
Specialty, ***n (%)***				0.06
Cardiology	12 (20)	7 (26)	5 (15)	
Cardiothoracic surgery	14 (23)	9 (33)	5 (15)	
Internal medicine	13 (21)	4 (15)	9 (26)	
Neurology	1 (2)	1 (4)	0 (0)	
Surgery	18 (30)	3 (11)	14 (41)	
Traumatology	4 (7)	3 (11)	1 (3)	
RBC storage time, ***days***	13 [6–22]	14 [6–21]	13 [6–22]	0.92
Hb level, ***mmol/L median [IQR]***	4.2 [3.9–4.6]	4.3 [4.1–4.6]	4.1 [3.9–4.6]	0.56
Hospital mortality, ***n (%)***	19 (31)	5 (19)	14 (41)	0.11

* Data are reported as number (%) or median [IQR].

Effect of RBC transfusion on iron parameters in ICU patients

Table 2 shows the median of Hb levels and each iron parameter per time point. Hb level was low and show a significant increase after transfusion. Iron levels were below the reference level and show a significant increase directly after transfusion. After 24 hours, the iron levels had decreased to baseline values again. Pre-transfusion levels of transferrin and transferrin saturation were below reference levels and these parameters showed no significant change after transfusion. Ferritin levels were higher than the reference level prior to transfusion and showed no significant change after transfusion. RBC transfusion resulted in an increase in hepcidin levels and IL-6 decreased statistically significant after transfusion. Prior to RBC transfusion, haptoglobin levels were higher than the reference level, but showed no significant change after transfusion. Also, cell free Hb and cell free heme levels did not change after RBC transfusion (table 2).

Table 2. Effect of RBC transfusion on Hb level and parameters of iron metabolism.

Variable	Timepoint			p-value
	Baseline	Post 1h	Post 24h	
Hb (mmol/L)	4.2 [3.9–4.6]	4.9 [4.6–5.4]	5 [4.6–5.2]	<0.01*
Iron (µmol/L)	4.3 [2.6–7.7]	4.7 [2.7 – 7.9]	4.3 [2.8–7.6]	0.02
Transferrin (g/L)	1.5 [1.3–1.7]	1.5 [1.3–1.7]	1.5 [1.3–1.8]	0.77
Transferrin saturation (%)	10 [8–20]	12 [8–21]	14 [9–19]	0.14
Ferritin (µg/L)	708 [264–1150]	-	615 [264–1253]	0.74
Haptoglobin (g/L)	2.1 [1.0–3.5]	-	2.0 [1.2–3.5]	0.45
Hepcidin (ng/mL)	204 [88–408]	-	214 [93–543]	0.01
IL-6 (pg/mL)	36.6 [11.2–75.8]	-	26.6 [12.2–59.7]	0.03
Free Hb (µM)	6.7 [5.0–10.5]	6.9 [4.8–9.9]	6.4 [5.1–10.6]	0.61
Free heme (µM)	24.3 [19.4–38.9]	25.1 [18.9–36.7]	24.7 [19.5–32.4]	0.74

Data are expressed as median [IQR]. Hb, iron, transferrin (saturation), free Hb and free heme were measured at three time points. Ferritin, haptoglobin, hepcidin and IL-6 were measured at two time points. P values were calculated between baseline and 24h post transfusion for ferritin, haptoglobin, hepcidin and IL-6. For Hb, iron, transferrin (saturation), free Hb and free heme p values were calculated between baseline and 1h post transfusion. * p=0.69 for Hb level 1h post transfusion compared to Hb level 24h post transfusion.

Effect of RBC transfusion on iron parameters in septic compared to non-septic ICU patients

Table 3 shows the post-transfusion change (delta) of iron parameters after RBC transfusion compared to baseline in septic compared to non-septic patients. RBC transfusion induced a decrease in haptoglobin levels compared to baseline in septic patients, but not in non-septic patients. Hepcidin levels tended to increase more compared to baseline in septic compared to non-septic patients. Other iron parameters did not show a different effect of RBC transfusion in septic compared to non-septic patients (table 3).

Table 3. Post transfusion change of parameters of iron metabolism in non-septic and septic patients.

Iron parameter	% change		p-value
	No Sepsis	Sepsis	
Iron	3.9 [-3.6–9.5]	3.1 [-4–13.5]	0.92
Transferrin	0.0 [-1.5–3.9]	1.0 [-2.7–3.8]	0.89
Transferrin saturation	0.0 [0.0–12.6]	0.0 [-6.1–16.7]	0.83

Table 3. Continued.

Iron parameter	% change		p-value
Ferritin	5.4 [-6.0–15.2]	-0.7 [-7.8–8.6]	0.14
Haptoglobin	9.4 [-4.8–23.3]	-2.7 [-7.1–8.1]	0.01
Hepcidin	4.1 [-21.6–36.3]	24.6 [-3.1–85.1]	0.07
IL-6	-16.8 [-48.4–17.5]	-11.7 [-35.7–30.8]	0.56
Free Hb	1.2 [-12.2–16.7]	1.7 [-5.9–14.4]	0.65
Free heme	-0.4 [-10.1–17.8]	3.3 [-8.2–7.8]	0.49

Data is expressed as median [IQR]. Deltas (post-transfusion change) were calculated between baseline and 24h post transfusion for ferritin, haptoglobin, hepcidin and IL-6. For iron, transferrin (saturation), free Hb and free heme the deltas were calculated between baseline and 1h post transfusion.

Correlation between iron parameters and RBC storage time

In this cohort of patients, RBC storage time did not correlate with post-transfusion change in haptoglobin (ρ=0.009, p=0.95), iron (ρ=0.19, p=0.15) and ferritin (ρ=-0.03, p=0.83). RBC storage did correlate with hepcidin, although the correlation was poor (ρ=0.31, p=0.02) (figure 2).

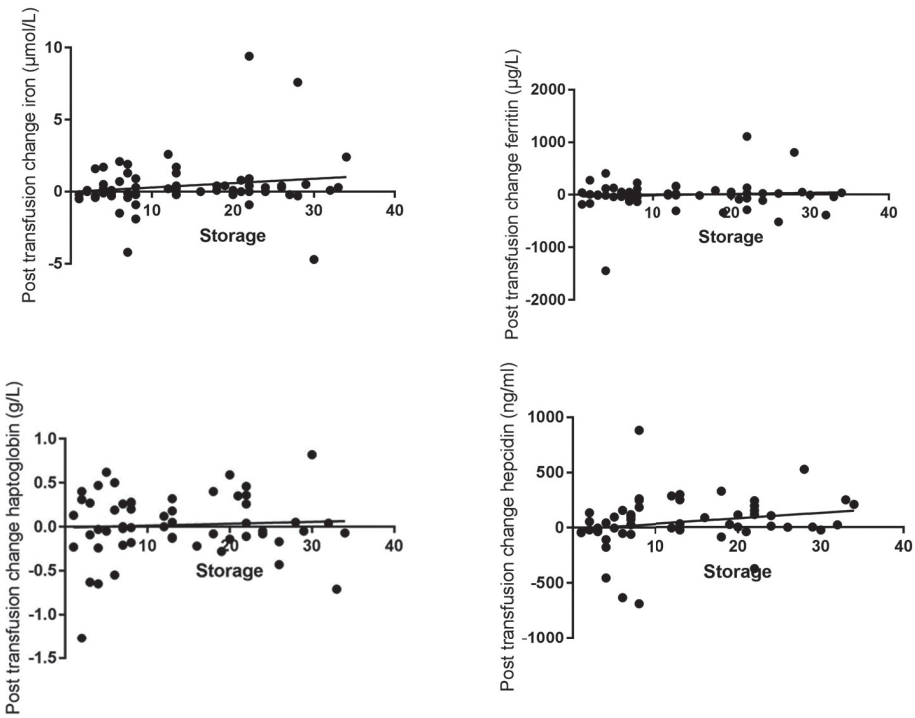

Figure 2. Correlation plots between post transfusion change and storage time.

DISCUSSION

This study investigated the effect of a single RBC transfusion on the iron metabolism of critically ill patients. The main findings in this study are that 1) RBC transfusion increases plasma iron levels, but not transferrin saturation, 2) RBC transfusion results in increased hepcidin levels, 3) RBC transfusion results in a decrease in haptoglobin levels in septic patients compared to non-septic patients.

Plasma iron levels showed a significant increase directly after transfusion in this study. However, transferrin saturation was low and did not increase after transfusion, suggesting that iron availability for erythropoiesis did not increase. Twenty-four hours after transfusion, the iron levels decreased to baseline. This decline in iron may be due to consumption of iron for erythropoiesis. Alternatively, iron may be sequestrated by mononuclear phagocytes [17]. In line with this thought, hepcidin levels were increased after RBC transfusion in our study population. Hepcidin is a hormone that prevents iron release from macrophages [3] and is increased in

response to both high iron levels and inflammatory stimuli [3]. It has been shown before that RBC transfusion induces IL-6 production [18]. However, in our study, IL-6 levels were decreased 24 hours after transfusion. Thereby, although we may have missed a transient IL-6 increase prior to the measurement at time point 24 hour post transfusion [18], we think it is unlikely that RBC-induced inflammation is the reason for the hepcidin increase. A more likely explanation for our findings is that iron loading due to clearance of the RBCs after transfusion may have resulted in hepcidin upregulation. Taken together, RBC transfusion may result in a lower iron release from intracellular stores for erythropoiesis by upregulation of hepcidin. Hereby, besides raising the hemoglobin level, RBC transfusion may hamper resolution of anemia of inflammation. Our study is in line with previous reports that show that RBC transfusion increases iron levels directly after transfusion in healthy volunteers [10, 13] and return to baseline 24 hours later [10], indicating a transient effect. In these studies specifically older RBC units were transfused [10, 13], whereas in our study RBCs with a median storage time of 13 days were transfused. This suggests that the transient increase of iron after RBC transfusion was not due to RBC storage time. In line with that, RBC storage time did not correlate with post-transfusion change in iron levels.

Haptoglobin levels decreased after transfusion, but only in the septic patients. This could suggest that RBC transfusion induces intravascular hemolysis in septic patients. This RBC-induced intravascular hemolysis was shown to occur with stored RBCs but not with fresh RBCs in a previous study in critically ill children [19], but not in healthy volunteers [10, 20]. In our study, RBC storage time did not correlate with post-transfusion changes in haptoglobin levels. Also, the decrease in haptoglobin levels was small, and the median absolute level of haptoglobin levels was 2.0 g/L after transfusion, suggesting that hemolysis was not severe. Of note, we did not include a non-transfused group of sepsis patients, so we cannot rule out that the decrease in haptoglobin is due to the transfusion. In addition, other markers of hemolysis, like free heme and free Hb levels were similar in septic and non-septic patients. NTBI was not measured, as transferrrin saturation was very low in this study. Taken together, the effect of one unit of RBCs on hemolysis in septic patients in this study is very limited. However, whether transfusion of multiple or repeated RBC units induces more profound hemolysis in septic patients, which may explain the association between transfusion and adverse clinical outcome, should be investigated in a future study.

This study has several limitations. First, we could have missed transient changes in iron parameters occurring between the 1 and 24 hours sampling time points in this study [10, 20]. Second, due to logistical problems, this study had a long inclusion period with several breaks during the study period, which caused the high number of missed patients. Third, this study could be underpowered for some of the iron parameters. However, clinical practice is to transfuse single units to correct for anemia. Thereby, the study design is clinically relevant. Lastly, as most RBC units in this study had a storage time between 6 and 22 days, the results of this study may not be generalizable to older stored transfusions. However, whether storage time is clinically relevant may be questioned, given the large clinical trials showing no effect of storage time on outcome [21-23].

CONCLUSION

In conclusion, our findings suggest that a RBC transfusion exerts a transient increase in iron levels in critically ill patients and an increase in hepcidin levels, which may further hamper iron release from intracellular stores. Furthermore, RBC transfusion may exert a different effect in patients with sepsis compared to non-septic patients.

REFERENCES

1. Silva Junior, J.M., et al., *Red blood cell transfusions worsen the outcomes even in critically ill patients undergoing a restrictive transfusion strategy.* Sao Paulo Med J, 2012. **130**(2): p. 77-83.
2. Prakash, D., *Anemia in the ICU: anemia of chronic disease versus anemia of acute illness.* Crit Care Clin, 2012. **28**(3): p. 333-43, v.
3. Ganz, T., *Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation.* Blood, 2003. **102**(3): p. 783-8.
4. Vincent, J.L., et al., *Worldwide audit of blood transfusion practice in critically ill patients.* Crit Care, 2018. **22**(1): p. 102.
5. Ozment, C.P. and J.L. Turi, *Iron overload following red blood cell transfusion and its impact on disease severity.* Biochim Biophys Acta, 2009. **1790**(7): p. 694-701.
6. Zeiler T, M.J., Kretschmer V. , *Flow-cytometric determination of survival time and 24-hour recovery of transfused red blood cells.* . Transfus Med Hemother, 2003. **30**(14): p. 9.
7. Luten, M., et al., *Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods.* Transfusion, 2008. **48**(7): p. 1478-85.
8. Dumont, L.J. and J.P. AuBuchon, *Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials.* Transfusion, 2008. **48**(6): p. 1053-60.
9. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
10. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
11. *U.S. Department of Agriculture ARS. What we eat in America, NHANES, 2009–2010.* US Dep Agric Agric reserach Serv. , 2012:2009.
12. Harmatz, P., et al., *Severity of iron overload in patients with sickle cell disease receiving chronic red blood cell transfusion therapy.* Blood, 2000. **96**(1): p. 76-9.
13. Peters, A.L., et al., *Transfusion of 35-day stored red blood cells does not result in increase of plasma non-transferrin bound iron in human endotoxemia.* Transfusion, 2017. **57**(1): p. 53-59.
14. Tacke, F., et al., *Iron Parameters Determine the Prognosis of Critically Ill Patients.* Crit Care Med, 2016. **44**(6): p. 1049-58.
15. Boshuizen, M., et al., *Iron metabolism in critically ill patients developing anemia of inflammation: a case control study.* Ann Intensive Care, 2018. **8**(1): p. 56.
16. Singer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 801-10.
17. Ganz, T., *Hepcidin--a regulator of intestinal iron absorption and iron recycling by macrophages.* Best Pract Res Clin Haematol, 2005. **18**(2): p. 171-82.

18. Cholette, J.M., et al., *Washing red blood cells and platelets transfused in cardiac surgery reduces postoperative inflammation and number of transfusions: results of a prospective, randomized, controlled clinical trial.* Pediatr Crit Care Med, 2012. **13**(3): p. 290-9.
19. L'Acqua, C., et al., *Red blood cell transfusion is associated with increased hemolysis and an acute phase response in a subset of critically ill children.* Am J Hematol, 2015. **90**(10): p. 915-20.
20. Rapido, F., et al., *Prolonged red cell storage before transfusion increases extravascular hemolysis.* J Clin Invest, 2017. **127**(1): p. 375-382.
21. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
22. Rygard, S.L., et al., *Effects of shorter versus longer storage time of transfused red blood cells in adult ICU patients: a systematic review with meta-analysis and Trial Sequential Analysis.* Intensive Care Med, 2018. **44**(2): p. 204-217.
23. Steiner, M.E., et al., *Effects of red-cell storage duration on patients undergoing cardiac surgery.* N Engl J Med, 2015. **372**(15): p. 1419-29.

CHAPTER 6

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Red blood cell transfusion results in adhesion of neutrophils in human endotoxemia and in critically ill patients with sepsis

ABSTRACT

BACKROUND: Red blood cell (RBC) transfusion is associated with adverse effects, which may involve activation of the host immune response. The effect of RBC transfusion on neutrophil reactive oxygen species (ROS) production and adhesion *ex vivo* was investigated in endotoxemic volunteers and in critically ill patients that received a RBC transfusion. We hypothesized that RBC transfusion would cause neutrophil activation, the extent of which depends on the storage time and the inflammatory status of the recipient.

STUDY DESIGN AND METHODS: Volunteers were injected with lipopolysaccharide (LPS) and transfused with either saline, fresh or stored autologous RBCs. In addition, 47 critically ill patients with and without sepsis receiving either fresh (<8days) or standard stored RBCs (2-35days) were included. Neutrophils from healthy volunteers were incubated with the plasma samples from the endotoxemic volunteers and from the critically ill patients, after which priming of neutrophil ROS production and adhesion were assessed.

RESULTS: In the endotoxemia model, *ex vivo* neutrophil adhesion, but not ROS production, was increased after transfusion, which was not affected by RBC storage duration. In the critically ill, *ex vivo* neutrophil ROS production was already increased prior to transfusion and was not increased following transfusion. Neutrophil adhesion was increased following transfusion, which was more notable in the septic patients than in non-septic patients. Transfusion of fresh RBCs, but not standard issued RBCs, resulted in enhanced ROS production in neutrophils.

CONCLUSION: RBC transfusion was associated with increased neutrophil adhesion in a model of human endotoxemia as well as in critically ill patients with sepsis.

INTRODUCTION

Red blood cell (RBC) transfusions in the critically ill are associated with nosocomial infections [1-4], organ dysfunction and mortality in observational studies [5, 6]. In a meta- analysis, a reduced risk of infection after transfusion was found in a restrictive transfusion strategy compared with a liberal transfusion strategy [7]. This may support the notion that transfusion modulates the recipient immune response, also referred to as transfusion related immunomodulation (TRIM). Multiple TRIM effects may exist. RBC transfusion is associated with nosocomial infections, implying an immunosuppressive effect of transfusion. On the other hand, RBC transfusion is also associated with the induction of a pro-inflammatory cytokine response, with aggravation of the underlying inflammation of the recipient and the occurrence of organ failure, in particular lung injury [8-11]. This process involves the interaction of immune cells such as neutrophils with endothelial cells.

Neutrophils are essential in orchestrating an inflammatory response [12]. Neutrophils migrate to the site of infection by adhesion to activated endothelium, followed by extravasation and chemotaxis into the tissues, where they phagocytose and kill the disease-causing pathogen by the release of antibacterial proteases or by the production of reactive oxygen species (ROS) by the NADPH oxidase [13]. Neutrophil activation can result in increased production of ROS production to certain stimuli, such as the bacterial peptide N-Formylmethionyl-leucyl-phenylaline (fMLP) [14], a process termed priming. In addition, adhesion of neutrophils to the endothelium is increased [15, 16]. Inflammatory conditions such as sepsis, are characterized by an increased ability to induce priming of neutrophil ROS production as well as upregulation of neutrophil activation markers [17-19]. Enhanced neutrophil activation was found to be associated with the development of organ failure as well as with an increased risk of mortality [20-22].

Experimental data suggest that RBC transfusion can result in activation of neutrophils. Supernatant from stored RBC products has been found to activate neutrophils *in vitro* [23-25] and in a rat model [26]. Possibly, this may be due to soluble bioactive substances accumulating during storage, such as CD40 ligand [27], lipids [28-32], cytokines [33-35], microparticles [36, 37] or free heme [38-41], which are all able to prime neutrophil ROS production in experimental conditions. Besides accumulation of activating agents in the RBC product related to storage duration, also the inflammatory status of the recipient may modulate effects of RBC transfusion on neutrophil function. In an *in vitro* flow model, increased adhesion of RBCs

to endothelial cells was found when cells were stimulated with endotoxin [42, 43]. Besides the upregulation of adhesion molecules on the endothelium, also activation of the vascular endothelium by endotoxin can result in the release of chemokines, which can rapidly induce neutrophil activation [28, 44]. Also, critically ill patients seem particularly susceptible for the adverse effects of RBC transfusion [6]. This indicates that the underlying immune response of the host plays a role.

This study investigated whether a RBC transfusion induces priming of neutrophil ROS production and adhesion. To this end, plasma samples of endotoxemic human volunteers receiving an autologous RBC transfusion as well as plasma samples of critically ill patients that received a RBC transfusion were tested for the capacity to induce *ex vivo* priming of ROS production and adhesion of human neutrophils of healthy controls. We hypothesized that RBC transfusion results in changes in the plasma of the recipient that increase priming of ROS production and adhesion of neutrophils. Moreover, we hypothesized that the effects on neutrophil activation are modulated both by storage duration as well as by the inflammatory status of the recipient.

MATERIAL & METHODS

Both study protocols adhere to the declaration of Helsinki and have been approved by the Medical Ethical Committee of the Academic Medical Center. Written informed consent was obtained from all subjects before entry in the study.

Endotoxemia model [45]

Healthy male volunteers (18- 35 years of age) donated a unit of whole blood, which was collected in citrate- phosphate- dextrose bag and stored for 16-22h. The RBC transfusion products were produced according to the national standards of Sanquin Blood Supply Foundation, Amsterdam, The Netherlands. After centrifugation, the units were processed into a RBC unit, a unit of plasma and a buffy coat. The RBC unit was leukoreduced by filtration (Fresenius Kabi, the Netherlands) and Saline-adenine- glucose- mannitol (150 mM NaCl, 1.25 mM adenine, 50 mM glucose, 29mM mannitol) (SAGM, Fresenius Kabi, the Netherlands) was added as storage solution. On the day of the experiment, volunteers (n=18) were injected intravenously with *Escherichia coli* lipopolysaccharide (2ng/kg, (National Institutes of Health Clinical Center, Bethesda, USA)). Two hours after LPS infusion, the volunteers were transfused with an autologous RBC unit. The subjects received either 2 day stored (2D) autologous RBCs, 35 day stored (35D) autologous RBCs or an equal volume of NaCl

0.9% (saline) (all groups n=6). Heparin anticoagulated blood samples were taken before LPS (baseline), 2 hours after LPS but prior to transfusion and 4 hours after transfusion. Samples were centrifuged at 1800g for 10min at 4°C and plasma was stored at -80˚C until further use.

Clinical study in critically ill patients

Between November, 2011 and October, 2015 critically ill patients in need of a single RBC unit to correct for anemia, were consecutively included. Patients were subdivided into septic and non-septic at the time of transfusion. Sepsis was diagnosed according to the SEPSIS-3 criteria [46]; Sequential Organ Failure Assessment (SOFA) score ⩾2 in combination with a suspected or proven infection which was treated with antibiotics. Exclusion criteria were 1) actively bleeding patients 2) patients who received multiple RBC units 3) patients who received RBC, plasma or thrombocyte transfusion in the last 24hr prior to inclusion. As per protocol, critically ill patients were transfused with a single RBC unit when their hemoglobin level was 4.3 mmol/L. In the Netherlands the maximum storage time of RBCs is 35 days, which is shorter than in the Unites States where bank polices allow storage for up to 42 days, and to reduce wastage RBCs which have been stored the longest are dispensed first. The patients were divided into groups receiving either fresh RBC (< 8 days) or standard stored RBC (2-35 days). Heparin anticoagulated blood samples were drawn from an indwelling arterial catheter prior to, straight after and 24hr after transfusion, centrifuged at 1500g for 20 minutes at room temperature and plasma was stored at -80˚C until further use.

Neutrophil isolation

Venous blood was collected from healthy volunteers in heparin anticoagulant blood tubes. Neutrophil isolation was performed using a 1.076 g/mL Percoll (Pharmacia, Uppsala, Sweden)- based density gradient. Blood samples were mixed gently with an equal volume of 10% PBS/ TNC (tri sodiumcitrate, Merck), layered over Percoll and centrifuged (1125g, 20 minutes). After removing the peripheral blood mononuclear cell (PBMC), lysis of RBCs was performed twice by adding ice- cold lysis buffer (NH_4Cl [0.155M], KCO_3 [0.01M] and EDTA (triplex III) [0.1 mM] (Merck-Millipore, Burlington, MA, USA). The isolated PMNs were washed and resuspended at 5 x 10^6/mL in Hepes buffered saline solution (containing 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 20 mM Hepes, 5.5 mM glucose (Merck-Milipore) and 0.5% (w/v) human serum albumin, pH 7.4 (Brocacef, Maarsen, The Netherlands)) [47].

Priming of neutrophil ROS production

Isolated neutrophils (1 × 10^6/mL stimulation) were incubated with 25% plasma from both study cohorts and healthy controls in buffer at 37°C for 30 minutes in a shaking bath. After the incubation, neutrophils were spun down and resuspended in 1 mL hepes buffer. Neutrophils (0.25 × 10^6/mL) in the presence of Amplex Red (25 µM) and horseradish peroxidase (0.5 U/mL) were incubated for 5 min at 37°C (Amplex Red kit, Molecular Probes, Eugene, Ore) and activated by formyl- methionyl- leucyl-phenylalanine (fMLP) (1 µM, Sigma Aldrich, St Louis, USA). The hydrogen peroxide production was measured with the HTS7000plus plate reader (Tecan, Mannendorf, Switserland). Fluorescence was measured at 30-second intervals for 30 minutes. Hepes buffer and 20 ng/mL *E. Coli* lipopolysaccharide (LPS)(Sigma- Aldrich) / LPS-binding protein (R&D Systems, Mineapolis, Minn) served as a negative and positive control. The priming properties were assessed by the amount of hydrogen production (H_2O_2) 5 min after fMLP addition in nmol/ 10^6 cells. The concentration of H_20_2 was calculated from a calibration curve. The medium control was deducted from the fMLP measurement and the value corrected by the neutrophil count was used for analysis.

Neutrophil adhesion assay

Neutrophils were fluorescently labeled with Calcein-AM (1 µM, 30min) (Molecular Probes, Leiden, Netherlands) [48]. Calcein-labeled neutrophils (1 × 10^6/mL) were incubated in an uncoated Maxisorp plate (Nunc, Wiesbaden, Germany) and 25% plasma was added. After incubation of 30 min at 37°C in an incubator, the plate was washed twice with PBS. Cells were lysed with Triton (0,5% X-100, Sigma Aldrich) for 5-10 min at room temperature. Adhesion was assessed by the fluorescence of calcein-labeled neutrophils in the infinite F200-pro plate reader (Tecan, Männedorf, Switserland) and was determined as a percentage of the total input of calcein-labeled cells (100%).

To reduce bias due to the donor effect in the priming of ROS production and adhesion assays, neutrophils from two different donors were used for each patient plasma sample and all assays were performed in duplicate.

Statistical analysis

Data were tested for normality using histograms and density plots. For categorical data, the Chi- square test was used and for continuous data, the t-test or when data were skewed the Mann- Whitney U test was used. For comparisons between treatment groups (e.g. RBC vs saline, septic vs non-septic patients, fresh vs standard

RBCs) we a priori determined to use the measurements at a single timepoint or the change between baseline and the last time point as our primary (independent) outcome in the statistical analysis. These were analysed using an unpaired t-test for normally distributed data and a Mann-Whitney U test when the data were skewed. Comparisons within treatment groups were carried out using a paired samples t-test or when data were skewed the Wilcoxon Signed Ranks test. Double sided p-values of <0.05 were considered to be statistically significant. Since we consider our study to be explorative (given the sample sizes), we chose not to correct for multiple testing or repeated measurements. All statistical analyses were performed in IBM SPSS Statistics version 24.

Power statement

Sample sizes were based on previous experimental studies [23, 25]. For the present outcomes, the sample sizes used (6 vs 12 & 22 vs 25) will have 80% power to detect an effect size of 1.5 and 0.84, respectively using a t-test with a two-sided significance level of 0.05.

RESULTS

The effect of RBC transfusion on neutrophil function in a human endotoxemia model

To investigate the effect of an autologous RBC transfusion on neutrophil activation we tested the activating properties of plasma from transfused endotoxemic individuals on freshly isolated neutrophils of healthy volunteers. As previously reported [45], LPS infusion induced a systemic inflammation reaction syndrome (SIRS), including fever, tachycardia and leucocytosis. These symptoms subsided several hours following LPS administration. In this model, the *ex vivo* capacity of plasma taken after LPS to prime neutrophil ROS production was not significantly different compared to plasma taken before LPS infusion. Following RBC transfusion, *ex vivo* priming of the neutrophil ROS production was significant higher versus baseline levels (0.77 nmol/10^6 cells [IQR 0.51-1.85] vs 0.63 nmol/10^6 cells [IQR 0.38-0.81], p=0.002 figure 1a). When compared to the control volunteers who received LPS and saline, the priming of the ROS production was not statistically different at the last time point compared to the volunteers who received a RBC transfusion (0.64 nmol/10^6 cells [IQR 0.50-0.70] vs 0.77 nmol/10^6 cells [IQR 0.51-1.85], p=0.213, figure 1a).

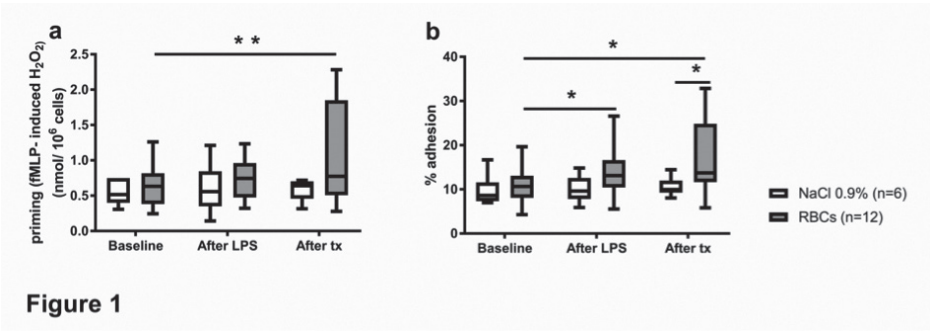

Figure 1. Neutrophil ROS production (a) and adhesion (b) activity in the endotoxemia model. 'Baseline' is before the LPS is given, 'After LPS' is 2 hours after LPS was infused and 'After tx' is 4 hours after the volunteers received either saline or an autologous RBC transfusion. LPS: lipopolysacheride, RBC: red blood cell. Data are shown as median [IQR]. *p<0.05, **p<0.01.

In the volunteers who received a RBC transfusion, the capacity of plasma to induce *ex vivo* neutrophil adhesion was increased following LPS infusion in healthy volunteers (13,1% [IQR 10,4-16,6]) and further increased by RBC transfusion when compared to baseline levels (13,7% [IQR 11,6-24,9] vs 10,6% [IQR 8,0-13,1], p=0.015 figure 1b). When compared to the saline control recipients at the last time point, the adhesion was significant higher in the volunteers who received a RBC transfusion (13,7% [IQR 11,6-24,8] vs 9,95% [IQR 9,3-12,0] in controls, p=0.024, figure 1b). Transfusion of fresh (2 days of storage) as well as of stored (35 days of storage) RBC products induced the priming of neutrophil ROS production and adhesion *ex vivo*, without a difference between these groups (Figure 2). Thus, in a human endotoxemia model, RBC transfusion is associated with an increase in *ex vivo* neutrophil adhesion, which was not affected by RBC storage duration. In the priming of neutrophil ROS production there seems to be a trend after RBC transfusion, albeit with a large variation.

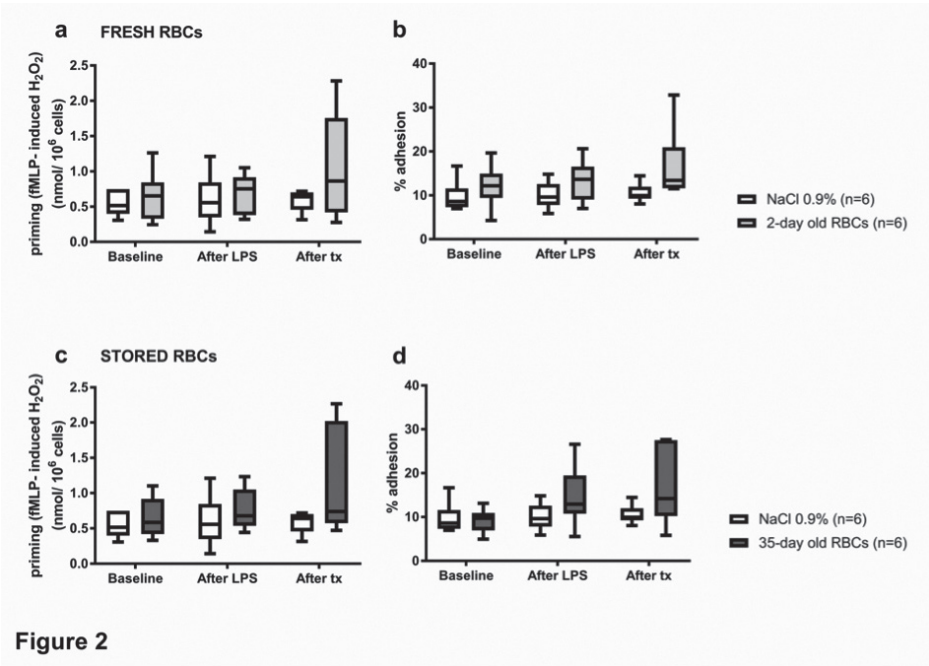

Figure 2. Effect of storage time on priming of neutrophil ROS production and adhesion activity in the endotoxemia model. 'Baseline' is before the LPS is given, 'After LPS' is 2 hours after LPS was infused and 'After tx' is 4 hours after the volunteers received either saline or an autologous RBC transfusion stored for 2 days (2D RBCs, figure 2a and 2b) or 35 days (35D RBCs, figure 2c and 2d. LPS: lipopolysaccharide, RBCs: red blood cells. Data are shown as median [IQR].

The effect of RBC transfusion on neutrophil function in critically ill patients

Given that critically ill patients receive RBC transfusion very regularly, this patient population was selected to investigate the effects of allogeneic RBC transfusion in a clinical setting. As the underlying immune status of the recipient may affect the neutrophil activation we made a distinction between septic and non- septic patients. From all sampling time points, 5 samples were missing due to logistical problems (9,6 %) and those patients were excluded. Eventually, 47 patients receiving an RBC transfusion were included in this study, of which 26 patients complied with the diagnosis of sepsis at the time of transfusion. The median duration from intensive care unit (ICU) admission until RBC transfusion was 11 days [IQR 4-16]. The baseline characteristics of each group are presented in table 1. Of note, at the time of RBC transfusion the disease severity (as assessed by the SOFA score) were similar, probably because sepsis patients had reached the convalescent phase of sepsis. In the group with non- septic patients significantly more men were present compared

to the septic group. Age, hemoglobin levels before transfusion and storage time of the RBC units were not different between the groups.

Table 1. Critically ill patient characteristics.

	All patients (n = 47)	Septic patients (n = 26)	Non- septic patients (n = 21)	P value
Age (years) *median [IQR]*	63 [57-73]	64 [56-70]	61 [58-70]	0.676
Sex Male, n (%)	25 (53)	10 (38)	15 (71)	0.024
Speciality, n (%)				0.121
Cardiology	9 (19)	3 (12)	6 (29)	
Cardiothoracic surgery	10 (21)	5 (19)	5 (24)	
Internal medicine	11 (23)	7 (27)	4 (19)	
Neurology	1 (2)	0 (0)	1 (5)	
Surgery	12 (26)	10 (38)	2 (10)	
Traumatology	4 (9)	1 (4)	3 (14)	
SOFA on transfusion day *median [IQR]*	8 [5-9]	8 [6-9]	7 [5-9]	0.425
RBC storage time (days) *median [IQR]*	8 [6-22]	8 [5-22]	12 [7-20]	0.434
Pre-transfusion Hemoglobin (mmol/L) *median [IQR]*	4.3 [3.9-4.6]	4.1 [3.8-4.6]	4.3 [4.1-4.6]	0.103
Pre-transfusion Leukocytes (1 $\times 10^9$/L) *median [IQR]*	16.3 [10.7-22.8]	18.9 [13.9-27.1]	14.8 [10.7-17.9]	0.064
Hospital mortality, n (%)	15 (33)	11 (42)	4 (19)	0.068

SOFA: Sequential Organ Failure Assessment; RBC: Red Blood Cell.

Prior to transfusion, the capacity of plasma from critically ill patients to induce *ex vivo* neutrophil ROS production was already increased when compared to plasma from healthy controls (0.82 nmol/10^6 cells [IQR 0.64-1.19] vs 0.57 nmol/10^6 cells [IQR 0.45-0.86], p=0.003, figure 3a) and was not further augmented following RBC transfusion. In septic patients, the ability of plasma to prime neutrophil ROS production at baseline was not significantly different compared to the plasma from the non-septic patients (0.81 nmol/10^6 cells [IQR 0.63-1.25] vs 0.87 nmol/10^6 cells [IQR 0.66-1.13], figure 4a). Following RBC transfusion, the post transfusion change to prime neutrophil ROS production did not differ in septic patients 24 hour after transfusion when compared to the non-septic patients (+0.05 nmol/ 10^6 cells [IQR 0-0.25] vs 0 nmol/10^6 cells [IQR -0.19-0.17], p=0.141, figure 4a). When testing the effects on adhesion, the capacity of plasma from critically ill patients to induce neutrophil

adhesion was not increased prior to transfusion when compared to plasma from healthy controls. After RBC transfusion the capacity of plasma to induce neutrophil adhesion is significantly higher compared to pre- transfusion levels (10.1% [IQR 4,8-29,0] and 16,5% [IQR 6,2-29,0] vs 9,9% [IQR 3,8-27,6], p<0.01, figure 3b).

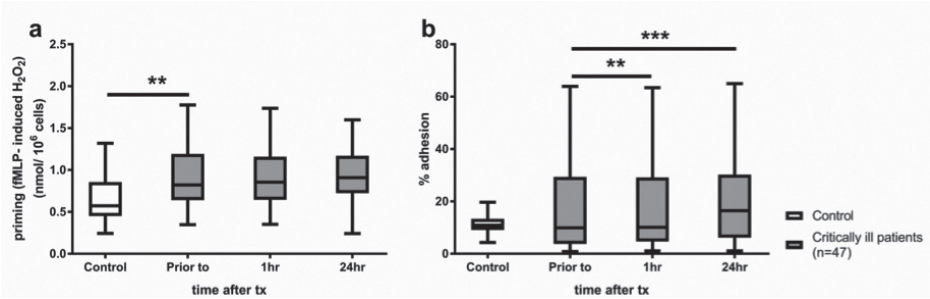

Figure 3. Priming of neutrophil ROS production (a) and adhesion (b) activity in the critically ill patients. 'Prior to' is before the RBC transfusion is given, '1hr' is 1 hour after and '24hr' is 24 hours after critically ill patients received an allogeneic RBC transfusion. RBC: red blood cell. Data are shown as median [IQR]. *p<0.05, **p<0.005, ***p<0.001.

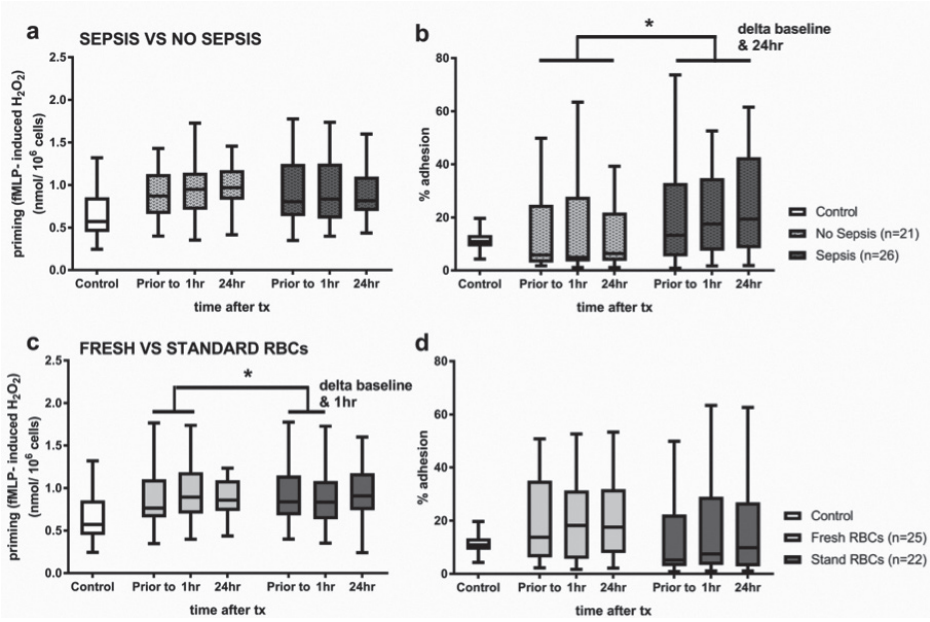

Figure 4. Priming of neutrophil ROS production (a) and adhesion (b) activity in septic and non- septic patients, and effect of storage time on neutrophil ROS production (c) and adhesion (d) activity in the critically ill patients. 'Prior to' is before the RBC transfusion is given, '1hr' is 1 hour after and '24hr' is 24 hours after critically ill patients received an allogeneic RBC transfusion. RBC: red blood cell. Data are shown as median [IQR]. *p<0.05.

When patients were divided into septic and non-septic patients, the post transfusion change to induce neutrophil adhesion was significantly higher in septic patients 24 hour after RBC transfusion when compared to non-septic patients (+3,2% [IQR 0,9-5,7] vs +0,8% [IQR -0,7-2,6], p=0.039, figure 4b), while at baseline septic and non-septic patients did not differ in the adhesion assay. In critically ill patients receiving fresh blood, storage duration was 6 days [IQR 3-8], which was significantly shorter than from the patients who received standard issued RBCs (21 days [IQR 15-31]). In patients who received fresh RBC transfusion, the post transfusion change in priming of the ROS production *ex vivo* was larger after transfusion compared to the patients who received a standard issued RBC (+0.11 nmol/ 10^6 cells [IQR -0.06-0.42] vs -0.06 nmol/ 10^6 cells [IQR -0.2-0.36], p<0.05, figure 4c). This difference is not found for adhesion, where there are no differences in the post-transfusion change between patients who received fresh RBCs compared to patients who received standard RBCs (figure 4d).

DISCUSSION

In this study, the effect of RBC transfusion on priming of neutrophil ROS production and - adhesion was investigated in human endotoxemia as well as in critically ill patients. The main findings are that (1) in human endotoxemia, RBC transfusion is associated with an increase in *ex vivo* neutrophil adhesion, which was not affected by RBC storage duration, (2) in critically ill patients, RBC transfusion is associated with an increase in the *ex vivo* neutrophil adhesion capacity in septic recipients compared to non-septic recipients and (3) in critically ill patients, fresh RBC transfusion, but not standard issued RBC transfusion, is associated with augmentation of *ex vivo* priming of neutrophil ROS production.

RBC transfusion resulted in a higher neutrophil adhesion-inducing capacity in septic recipients then in non-septic recipients; the plasmas had an equal ROS priming capacity. This may suggest that the inflammatory status of the recipient plays an important role in the association between RBC transfusion and *ex vivo* neutrophil adhesion. This finding is in line with results from experimental studies, in which endotoxin increased the ability of endothelial cells to adhere to neutrophils *in vitro* [49]. We hypothesize that during inflammation, elevated levels of pro-inflammatory substances mediate RBC-induced neutrophil adhesion [50]. Of note, neutrophils play a key role in the pathogenesis of transfusion related acute lung injury (TRALI). In this syndrome, it is thought that the presence of an inflammatory status primes pulmonary neutrophils and activates the endothelium, which increases the risk of

a TRALI reaction following transfusion. Our results are in line with this hypothesis. Taken together, our results suggest that the immunomodulatory effects of an RBC transfusion are more increased when an inflammatory status is present in the recipient.

At the start of this study, our initial hypothesis was that increased storage duration of RBCs was associated with increased effects on neutrophil function. However, we found the contrary; transfusion of fresh RBCs, but not standard issued RBCs, resulted in priming of neutrophil ROS production in critically ill patients. Large clinical trials have provided strong evidence that a prolonged storage time is not related to complications of RBC transfusions [53, 54]. On the contrary, a meta- analysis suggest that fresh RBC may even be more harmful [55], which is suggested to be related to the manufacturing method [56]. However, the manufacturing method is not likely to be the explanation for the findings in this study, as all patients received red cell filtered RBC products that were manufactured the same way. Given that storage duration did not play a role in the endotoxemia model which used autologous blood, we suggest that inflammatory mediators accumulating during storage do not play a role. We hypothesize that allogeneic properties of the transfusion have an interaction with the recipient. Of note, we previously showed that an autologous RBC transfusion does not induce immune tolerance in endotoxemic recipients [57]. Again, this points towards a role for allogenic factors in the RBC product. An alternative explanation may be donor related factors like sex mismatch [58], which has also been associated with adverse outcome. Sex mismatch was not corrected for in this study in critically ill patients and cannot be dissected from our findings. However, it is unknown whether sex mismatch may occur only with fresh RBC products.

This study has several limitations. The effect of RBC transfusion was investigated by comparison to pre-transfusion baseline levels, and not to a negative control. Thereby, effects may have occurred in time or by something other than the RBC product itself. Also, samples were not spun down to lose any residual platelets. The ability to prime neutrophil ROS production and adhesion was tested using neutrophils from healthy subjects. The endogenous neutrophils in critically ill patients receiving a RBC transfusion may react differently. In addition, due to the incubation time of 30 minutes, we may have missed transient effects of other agents on the neutrophil ROS production and adhesion. Also, although a power calculation was done, the study in critically ill patients could have been underpowered to find a significant difference in priming of the neutrophil ROS production after transfusion

due to confounders unaccounted for, such as shock. However, SOFA scores between groups were similar and large variation between individuals was also found in the endotoxemia model. In addition, we tested effects of a single RBC unit. Possibly, multiple RBC units may induce more immunomodulatory effects. However, the use of one single RBC unit to correct anaemia is comparable to the clinical practice at the ICU [53] and makes the study clinically relevant. Of note, the results in this may not apply to other countries where other manufacturing methods to produce RBC units are used.

CONCLUSION

In conclusion, RBC transfusion results in an increased capacity of plasma from the recipients to induce *ex vivo* neutrophil adhesion in a model of human endotoxemia as well as in a cohort of critically ill patients with sepsis. Fresh RBC transfusion, but not standard issued RBC transfusion, is associated with *ex vivo* priming of neutrophil ROS production. Therefore, neutrophil activation may be a mechanism of transfusion-induced adverse outcome in individuals with severe systemic inflammation. Future research should focus on which factors in the RBC product are responsible for priming of neutrophil ROS production and adhesion, as well as on the influence of short storage duration.

REFERENCES

1. Muszynski, J.A., et al., *Transfusion-related immunomodulation: review of the literature and implications for pediatric critical illness.* Transfusion, 2017. **57**(1): p. 195-206.
2. Juffermans, N.P., et al., *Transfusion-related risk of secondary bacterial infections in sepsis patients: a retrospective cohort study.* Shock, 2011. **35**(4): p. 355-9.
3. Shorr, A.F., et al., *Transfusion practice and blood stream infections in critically ill patients.* Chest, 2005. **127**(5): p. 1722-8.
4. Vamvakas, E.C., *Deleterious clinical effects of transfusion immunomodulation: proven beyond a reasonable doubt.* Transfusion, 2006. **46**(3): p. 492-4; author reply 494-5.
5. Tinmouth, A., et al., *Clinical consequences of red cell storage in the critically ill.* Transfusion, 2006. **46**(11): p. 2014-27.
6. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
7. Rohde, J.M., et al., *Health care-associated infection after red blood cell transfusion: a systematic review and meta-analysis.* JAMA, 2014. **311**(13): p. 1317-26.
8. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
9. Callan, M.B., et al., *Transfusion of 28-day-old leucoreduced or non-leucoreduced stored red blood cells induces an inflammatory response in healthy dogs.* Vox Sang, 2013. **105**(4): p. 319-27.
10. Cortes-Puch, I., et al., *Washing older blood units before transfusion reduces plasma iron and improves outcomes in experimental canine pneumonia.* Blood, 2014. **123**(9): p. 1403-11.
11. Vamvakas, E.C., *Possible mechanisms of allogeneic blood transfusion-associated postoperative infection.* Transfus Med Rev, 2002. **16**(2): p. 144-60.
12. Segal, A.W., *How neutrophils kill microbes.* Annu Rev Immunol, 2005. **23**: p. 197-223.
13. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
14. van Bruggen, R., et al., *Toll-like receptor responses in IRAK-4-deficient neutrophils.* J Innate Immun, 2010. **2**(3): p. 280-7.
15. Condliffe, A.M., E. Kitchen, and E.R. Chilvers, *Neutrophil priming: pathophysiological consequences and underlying mechanisms.* Clin Sci (Lond), 1998. **94**(5): p. 461-71.
16. Sheppard, F.R., et al., *Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation.* J Leukoc Biol, 2005. **78**(5): p. 1025-42.
17. Martins, P.S., et al., *Upregulation of reactive oxygen species generation and phagocytosis, and increased apoptosis in human neutrophils during severe sepsis and septic shock.* Shock, 2003. **20**(3): p. 208-12.

18. Nakamori, Y., et al., *Enhanced expression of intranuclear NF-kappa B in primed polymorphonuclear leukocytes in systemic inflammatory response syndrome patients.* J Trauma, 2003. **54**(2): p. 253-60.
19. Kaufmann, I., et al., *Polymorphonuclear leukocyte dysfunction syndrome in patients with increasing sepsis severity.* Shock, 2006. **26**(3): p. 254-61.
20. Hotchkiss, R.S. and I.E. Karl, *The pathophysiology and treatment of sepsis.* N Engl J Med, 2003. **348**(2): p. 138-50.
21. Yang, K.Y., J.J. Arcaroli, and E. Abraham, *Early alterations in neutrophil activation are associated with outcome in acute lung injury.* Am J Respir Crit Care Med, 2003. **167**(11): p. 1567-74.
22. Biffl, W.L., et al., *Neutrophils are primed for cytotoxicity and resist apoptosis in injured patients at risk for multiple organ failure.* Surgery, 1999. **126**(2): p. 198-202.
23. Silliman, C.C., G.W. Thurman, and D.R. Ambruso, *Stored blood components contain agents that prime the neutrophil NADPH oxidase through the platelet-activating-factor receptor.* Vox Sang, 1992. **63**(2): p. 133-6.
24. Zallen, G., et al., *Stored red blood cells selectively activate human neutrophils to release IL-8 and secretory PLA2.* Shock, 2000. **13**(1): p. 29-33.
25. Chin-Yee, I., et al., *Supernatant from stored red cells activates neutrophils.* Transfus Med, 1998. **8**(1): p. 49-56.
26. Kelher, M.R., et al., *Plasma from stored packed red blood cells and MHC class I antibodies causes acute lung injury in a 2-event in vivo rat model.* Blood, 2009. **113**(9): p. 2079-87.
27. Khan, S.Y., et al., *Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury.* Blood, 2006. **108**(7): p. 2455-62.
28. Wyman, T.H., et al., *A two-insult in vitro model of PMN-mediated pulmonary endothelial damage: requirements for adherence and chemokine release.* Am J Physiol Cell Physiol, 2002. **283**(6): p. C1592-603.
29. Aiboshi, J., et al., *Blood transfusion and the two-insult model of post-injury multiple organ failure.* Shock, 2001. **15**(4): p. 302-6.
30. Silliman, C.C., et al., *Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase.* J Lab Clin Med, 1994. **124**(5): p. 684-94.
31. Silliman, C.C., et al., *Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium.* J Leukoc Biol, 2003. **73**(4): p. 511-24.
32. Silliman, C.C., et al., *The association of biologically active lipids with the development of transfusion-related acute lung injury: a retrospective study.* Transfusion, 1997. **37**(7): p. 719-26.
33. Stack, G., et al., *Cytokine generation in stored, white cell-reduced, and bacterially contaminated units of red cells.* Transfusion, 1995. **35**(3): p. 199-203.
34. Shanwell, A., et al., *Generation of cytokines in red cell concentrates during storage is prevented by prestorage white cell reduction.* Transfusion, 1997. **37**(7): p. 678-84.

35. Johnson, J.L., et al., *Alteration of the postinjury hyperinflammatory response by means of resuscitation with a red cell substitute.* J Trauma, 2003. **54**(1): p. 133-9; discussion 139-40.
36. Belizaire, R.M., et al., *Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation.* J Am Coll Surg, 2012. **214**(4): p. 648-55; discussion 656-7.
37. Kent, M.W., et al., *The pro-inflammatory potential of microparticles in red blood cell units.* Transfus Med, 2014. **24**(3): p. 176-81.
38. Graca-Souza, A.V., et al., *Neutrophil activation by heme: implications for inflammatory processes.* Blood, 2002. **99**(11): p. 4160-5.
39. Kono, M., et al., *Morphological and flow-cytometric analysis of haemin-induced human neutrophil activation: implications for transfusion-related acute lung injury.* Blood Transfus, 2013. **11**(1): p. 53-60.
40. Ohbuchi, A., et al., *Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon.* Biochem Biophys Rep, 2017. **11**: p. 147-153.
41. Kono, M., et al., *Heme-related molecules induce rapid production of neutrophil extracellular traps.* Transfusion, 2014. **54**(11): p. 2811-9.
42. Eichelbronner, O., et al., *Endotoxin promotes adhesion of human erythrocytes to human vascular endothelial cells under conditions of flow.* Crit Care Med, 2000. **28**(6): p. 1865-70.
43. Anniss, A.M. and R.L. Sparrow, *Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions.* Am J Hematol, 2007. **82**(6): p. 439-45.
44. Lentsch, A.B. and P.A. Ward, *Regulation of inflammatory vascular damage.* J Pathol, 2000. **190**(3): p. 343-8.
45. Peters, A.L., et al., *Transfusion of 35-Day Stored RBCs in the Presence of Endotoxemia Does Not Result in Lung Injury in Humans.* Crit Care Med, 2016. **44**(6): p. e412-9.
46. Singer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 801-10.
47. Kuijpers, T.W., et al., *Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation.* Blood, 1991. **78**(4): p. 1105-11.
48. Kuijpers, T.W., et al., *Combined immunodeficiency with severe inflammation and allergy caused by ARPC1B deficiency.* J Allergy Clin Immunol, 2017. **140**(1): p. 273-277 e10.
49. Schleimer, R.P. and B.K. Rutledge, *Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters.* J Immunol, 1986. **136**(2): p. 649-54.
50. Livaditi, O., et al., *Neutrophil CD64 expression and serum IL-8: sensitive early markers of severity and outcome in sepsis.* Cytokine, 2006. **36**(5-6): p. 283-90.
51. Takami, M., V. Terry, and L. Petruzzelli, *Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1.* J Immunol, 2002. **168**(9): p. 4559-66.

52. DiVietro, J.A., et al., *Immobilized IL-8 triggers progressive activation of neutrophils rolling in vitro on P-selectin and intercellular adhesion molecule-1.* J Immunol, 2001. **167**(7): p. 4017-25.
53. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
54. Rygard, S.L., et al., *Effects of shorter versus longer storage time of transfused red blood cells in adult ICU patients: a systematic review with meta-analysis and Trial Sequential Analysis.* Intensive Care Med, 2018. **44**(2): p. 204-217.
55. Chai-Adisaksopha, C., et al., *Mortality outcomes in patients transfused with fresher versus older red blood cells: a meta-analysis.* Vox Sang, 2017. **112**(3): p. 268-278.
56. Heddle, N.M., et al., *Red blood cell processing methods and in-hospital mortality: a transfusion registry cohort study.* Lancet Haematol, 2016. **3**(5): p. e246-54.
57. Peters, A.L., et al., *Transfusion of 35-day-stored red blood cells does not alter lipopolysaccharide tolerance during human endotoxemia.* Transfusion, 2017. **57**(6): p. 1359-1368.
58. Chasse, M., et al., *Association of Blood Donor Age and Sex With Recipient Survival After Red Blood Cell Transfusion.* JAMA Intern Med, 2016. **176**(9): p. 1307-14.

CHAPTER 7

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The effect of red cell transfusion on platelet function in critically ill

ABSTRACT

INTRODUCTION: Red blood cell (RBC) transfusion is associated with an increased risk of pro-thrombotic events, but the underlying mechanism is poorly understood. We hypothesized that RBC transfusion modulates platelet activity in critically ill patients with and without sepsis.

STUDY DESIGN AND METHODS: In a prospective cohort study, 37 critically ill patients receiving a single RBC unit to correct for anemia were sampled prior to and 1 hour after transfusion. Platelet exposure of P-selectin, CD63 and binding of PAC-1 as well as formation of platelet-leukocyte complexes were measured by flow cytometry. The ability of plasma from critically ill patients to induce *ex vivo* platelet aggregation was assessed by flow cytometry after incubation with platelets from a healthy donor.

RESULTS: RBC transfusion neither triggered the expression of platelet activation markers nor the formation of platelet-leukocyte complexes. Plasma from critically ill patients induced more spontaneous platelet aggregation prior to RBC transfusion compared to healthy controls, which was further augmented following RBC transfusion. Also collagen-induced platelet aggregation was already increased prior to RBC transfusion compared to healthy controls, and this response was unaffected by RBC transfusion. In contrast, ristocetin-induced platelet agglutination was decreased when compared to controls, suggesting impaired vWF-dependent platelet agglutination, even in the presence of high vWF levels. Following RBC transfusion, ristocetin-induced platelet agglutination further decreased. There were no differences between septic and non-septic recipients in all assays.

CONCLUSION: *Ex vivo* platelet aggregation is disturbed in the critically ill. Transfusion of a RBC unit may further increase the spontaneous platelet aggregatory response.

INTRODUCTION

Red blood cell (RBC) transfusions are often administrated to critically ill patients [1] to correct for anemia or to replace blood loss after acute bleeding. However, RBC transfusions are also associated with an increased risk for adverse effects, including thromboembolic events [2-9]. In patients with an acute coronary syndrome, RBC transfusion is associated with myocardial infarction [2-5]. In patients undergoing a surgical procedure, perioperative RBC transfusion is associated with stroke [6, 7], contributing to mortality [10].

The underlying mechanisms by which RBC transfusion increases the risk of arterial thrombosis may not merely be a haematocrit-mediated platelet margination towards the vessel wall [11], but may be due to platelet activation as well. In an *in vitro* transfusion model, RBC products induced platelet activation and aggregation [12]. In a flow model, RBC enhanced platelet aggregation [13], via both physical and chemical effects [14-16]. Both extracellular vesicles (EVs) as well as cell free hemoglobin, released upon hemolysis, can increase nitric oxide scavenging, resulting in vasoconstriction [17] and platelet activation [18, 19]. Also, adenosine diphosphate (ADP), released from activated/damaged RBCs, triggers platelet adhesion and aggregation *in vitro* [19]. Experimental models showed that both ADP and hemoglobin infusions in rats trigger platelet aggregation [19]. Besides arterial thromboembolic events, RBC transfusions are also associated with venous thromboembolic events. In a murine model, transfusion with murine RBCs resulted in an increased clot formation [20]. Furthermore, in a murine vascular thrombosis model, RBCs contribute to thrombus formation by mediating platelet adhesion to the intact endothelium [21].

In surgical patients, RBC transfusion is associated with an increased risk of postoperative venous thromboembolic events [8, 9]. Potential mechanisms include an increase in blood viscosity [2], a decrease in deformability of RBCs during storage [22] and the induction of shear stress by the release of potential pro-coagulant mediators [23]. EVs from RBC products have pro-coagulant properties by exposing phosphatidylserine (PS) [24] and can facilitate thrombin generation *in vitro* [25-27].

In critically ill patients, systemic inflammation is associated with enhanced coagulation and platelet activation [28, 29]. Also, thromboembolic events are frequent in this patient population [30] and associated with poor outcome [31].

This study aimed to investigate the effect of RBC transfusion on platelet activation and aggregation in critically ill septic and non-septic patients. We hypothesized that RBC transfusion in critically ill patients may induce platelet activation, in particular when systemic inflammation is present. First, platelet surface expression markers were measured in critically ill patients before and after receiving a RBC transfusion. Second, the formation of complexes between platelets and leukocytes was measured [32, 33]. Finally, the effect of plasma from the RBC recipients on the ability to form platelet aggregates was measured *ex vivo*, using platelets from healthy volunteers.

MATERIAL & METHODS

This prospective, observational cohort study adhered to the declaration of Helsinki and was approved by the Medical Ethical Committee of the Academic Medical Center (trialregister.nl NTR 6596, NL61833.018.017). Written informed consent was obtained from all patients (or from their representatives) and healthy controls.

Patients

Critically ill patients receiving a single RBC transfusion because of anemia, when their hemoglobin level was 7 g/dL, were eligible for the study. Patients were excluded when they were actively bleeding or when they received multiple RBC units or a RBC, plasma or thrombocyte transfusion in the last 24 hours prior to inclusion. Patients were subdivided into sepsis and non-sepsis at the time of the transfusion according to the SEPSIS-3 criteria [34]; which includes a Sequential Organ Failure Assessment (SOFA) score ⩾2 in combination with a suspected or proven infection which was treated with antibiotics. Citrate-anticoagulated blood samples were collected from septic and non-septic patients from an indwelling, non-heparinized arterial catheter prior to and 1 hour after administration of the RBC transfusion. Part of the whole blood samples was directly used for experiments, the rest was centrifuged (1500g for 20 minutes and 10.000g for 5 minutes) and the platelet poor plasma (PPP) was stored at -80°C until use.

Whole blood platelet activation markers

Citrated whole blood was collected prior to and 1 hour after the transfusion. Within 5 minutes after collection the blood was transferred to tubes containing Hepes buffer (pH 7.4, 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 20 mM Hepes, 5.5 mM glucose (Merck- Millipore, Burlington, MA) and 0.5% (w/v) human serum albumin (Brocacef, Maarsen, The Netherlands)) and the antibodies directed against activation markers. Platelet activation was measured before and

after addition of the platelet agonist thrombin receptor activating peptide (TRAP-6; 15μM; Bachem, Bubendorf, Switzerland) by flow cytometry, combining antibodies against a general antigen (anti-CD61-APC; Dako, Cambridge, United Kingdom) and their activation markers: P- selectin (translocation of P- selectin; anti-CD62p-PE; Beckman Coulter, Marseille, France), CD63 (a lysosomal membrane glycoprotein; anti-CD63-PE; Beckman Coulter) and PAC1- binding (fibrinogen binding site exposed on the activated form of the glycoprotein (GP) IIb/IIIa receptor; anti-PAC1-FITC; BD Bioscience, San Jose, CA). After gentle mixing and incubation for 30 minutes at room temperature in the dark the samples (total volume 55 μL) were fixed with 2.5 mL 0.3% paraformaldehyde-containing Hepes buffer. Fixed samples were measured on flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ) to determine the number of antibodies that bind to platelets and evaluated by both the percentage (%) of positive platelets and as mean fluorescence intensity (MFI). The threshold was set at 1% of the isotype control.

Circulating platelet-leukocyte complexes

Platelet-leukocyte complexes (PLC) are defined as monocytes, granulocytes or lymphocytes that are bound to platelets. Citrate-anticoagulated whole blood was collected prior to and 1 hour after the transfusion, and within 5 minutes after collection the blood was added to tubes containing Hepes and antibodies. The PLC were measured by using a platelet marker (anti-CD61-PerCP; BD Bioscience) in combination with a marker for monocytes (anti-CD14-PE; eBioscience, San Diego, CA), granulocytes (anti-CD66b-FITC; Beckman Coulter) or lymphocytes (anti-CD4-PE; eBioscience). After gentle mixing and incubation for 30 minutes at room temperature in the dark, the samples (total volume 55 μL) were fixed with 0.5 mL 0.3% paraformaldehyde-containing Hepes buffer. After 60 minutes of fixation, red blood cells were lysed by addition of distilled water [35] and samples were measured by flow cytometry. Leukocytes were identified by the characteristic side scatter and the pan leukocyte marker (anti-CD45-APC; BD Bioscience). Samples were analyzed by percentage CD61 positive cells within the population of monocytes, granulocytes and lymphocytes. The threshold of platelet-leukocyte binding was set at 1% of the isotype control.

Platelet aggregation test

The capacity of RBC transfusion to trigger platelet aggregation was assessed following *ex vivo* incubation of platelets from healthy volunteers with citrate plasma from critically ill patients collected both *before* and *after* transfusion, measured by flow cytometry [36-40]. This assay was chosen because it required less volume

sample and is faster to perform than the light transmission aggregometry. First, heparinized blood from healthy volunteers (blood group O) was centrifuged for 15 minutes at 210g and platelet-rich plasma (PRP) was collected. PRP was divided in two suspensions and stained with anti-CD31-APC (BD Bioscience) or anti-CD31-FITC (BD Bioscience). After incubation the stained populations were washed twice with sequestrine buffer (17.5 mM Na_2HPO_4, 8.9 mM Na_2EDTA, 154 mM $NaCl_2$, pH 6.9 containing 0.1% (w/v bovine albumin) (Sigma-Aldrich)) by 5 minutes at 2250g centrifugation and resuspended to a concentration of 40×10^6/mL in Hepes buffer. The differently labeled platelets from healthy volunteers were 1:1 mixed and subsequently incubated with 50% citrate plasma from transfused recipients and 20 µM PPACK (D- phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; Calbiochem, Darmstadt, Germany), while shaking at 700 rpm at 37 °C. After 25 minutes incubation 3 mM $CaCl_2$ was added and incubated extra for 5 minutes as done before [36, 40]. Platelet aggregation was induced using different stimuli. After taking the baseline sample, the platelet suspensions were on a shaker and activated with 10 µg/mL collagen (Agro-Bio, STAGO; Asnieres, France), 100ng/mL phorbol myristate acetate (PMA, Sigma-Aldrich; St Louis, MO) or 1.5 mg/mL ristocetin (Biopool, Trinity Biotech Plc, Bray, Co Wicklow, Ireland). Here, PMA was used because of the number of events measured on the flow cytometry was found to be higher compared to TRAP. This enhanced the gating and the assay's sensitivity. At different time points samples were taken stopping the reaction by addition to a 9× volume of 0.5% (v/v) formaldehyde (Polysciences Inc., Warrington, NJ, USA, methanol-free) in PBS. The collagen and PMA stimulation were measured over a time frame of 5 minutes and the ristocetin over 1 minute. Fixed samples were measured by flow cytometry (LSR+ HTS, BD Bioscience) within 60 minutes. For analysis a quadrant was set in box plot of the control samples without adding plasma, the percentage of double-colored platelets were used for measurement of platelet aggregation (described more in detail in [36]). Data are analyzed as Area Under the Curve (AUC). Von Willebrand Factor antigen (vWF:Ag) levels in the plasma were measured by ELISA with antibodies from DAKO (Copenhagen, Denmark).

Statistical analysis

Data were expressed as mean and SD (when normally distributed) or as median with interquartile range (IQR, when non-normally distributed). Comparisons between before and after transfusion were tested using the paired t-test (when data were normally distributed) or Wilcoxon Signed Ranks (when data were not normally distributed). For the comparisons between septic and non-septic patients the post transfusion change versus baseline (delta) was determined and tested by Mann Whitney

U test (when data were not normally distributed). Analyses were done by IBM SPSS Statistics version 24. P values <0.05 were considered statistically significant.

Power statement

The sample size of the study was based on the primary outcome (trialregister.nl NTR 6596, NL61833.018.017). For the present outcomes, the sample size used (17 vs 20) will have 80% power to detect an effect size of 0.95 using a t-test with a two-sided significance level of 0.05.

RESULTS

In total 37 patients were included, of which 17 patients were septic at the time of the transfusion. Due to logistical problems, measurement of activation markers was not done in 2 patients and PLC measurements were missing in 4 patients. The baseline characteristics are presented in Table 1. Non-septic patients were more often male. There were no differences in age, referral specialty, hemoglobin level (pre- and post transfusion) and storage time of the RBC between the septic and non-septic group. The platelet count did not differ before and after RBC transfusion.

Table 1. Critically ill patient characteristics.

	All patients (n = 37)	Septic patients (n = 17)	Non-septic patients (n = 20)	P value
Age (years)	61 [55-71]	63 [56-71]	60 [53-66]	0.357
Sex Male, n (%)	22 (59)	7 (41)	15 (75)	0.037
Specialty, n (%) Cardiology Cardiothoracic surgery Internal medicine Neurology Surgery Traumatology	 7 (19) 9 (24) 9 (24) 1 (3) 8 (22) 3 (8)	 2 (12) 3 (18) 5 (29) 0 (0) 6 (35) 1 (6)	 5 (25) 6 (30) 4 (20) 1 (5) 2 (10) 2 (10)	0.355
SOFA on transfusion day	7 [5-11]	8 [5-11]	7 [5-9]	0.341
RBC storage time, days	13 [6-22]	12 [4-22]	16 [6-23]	0.407
Hemoglobin (mmol/L) Pre transfusion Post transfusion	 4.1 [3.9-4.5] 5.0 [4.6-5.3]	 4.0 [3.8-4.4] 5.0 [4.5-5.3]	 4.3 [4.1-4.6] 5.0 [4.6-5.2]	 0.209 0.821

Table 1. Continued.

	All patients (n = 37)	Septic patients (n = 17)	Non-septic patients (n = 20)	P value
Platelet count (×10^9/L)				
Pre transfusion	156 [106-205]	174 [130-276]	134 [104- 199]	0.297
Post transfusion	183 [104-241]	207 [129-256]	150 [103- 211]	0.266
Leukocytes (×10^9/L)				
Pre transfusion	14.2 [9.5-17.9]	15.0 [9.9-18.9]	13.1 [10.7-17.0]	0.684
Post transfusion	15.3 [9.5-16.7]	15.4 [9.6-19.1]	15.3 [9.3-16.0]	0.330
Hospital mortality, n (%)	12 (32)	8 (47)	4 (20)	0.080

SOFA: Sequential Organ Failure Assessment, RBC: Red Blood Cell. Data are expressed as median with interquartile range [IQR].

The effect of RBC transfusion on whole blood platelet activation markers and platelet-leukocyte complexes (PLC)

Prior to RBC transfusion, platelets had a markedly high expression of CD63 even without additional stimulation with TRAP. RBC transfusion did not induce a change in the percentage and the mean fluorescence intensity (MFI) of platelets expressing P-selectin, CD63 or binding of PAC1 compared to pre-transfusion levels. These results were found with and without additional stimulation with TRAP (Figure 1), with the exception of a slight decrease of TRAP-induced binding of PAC1 after RBC transfusion (23.2% [9.6-44.6] vs 19.7% [7.0-42.1], $p<0.05$).

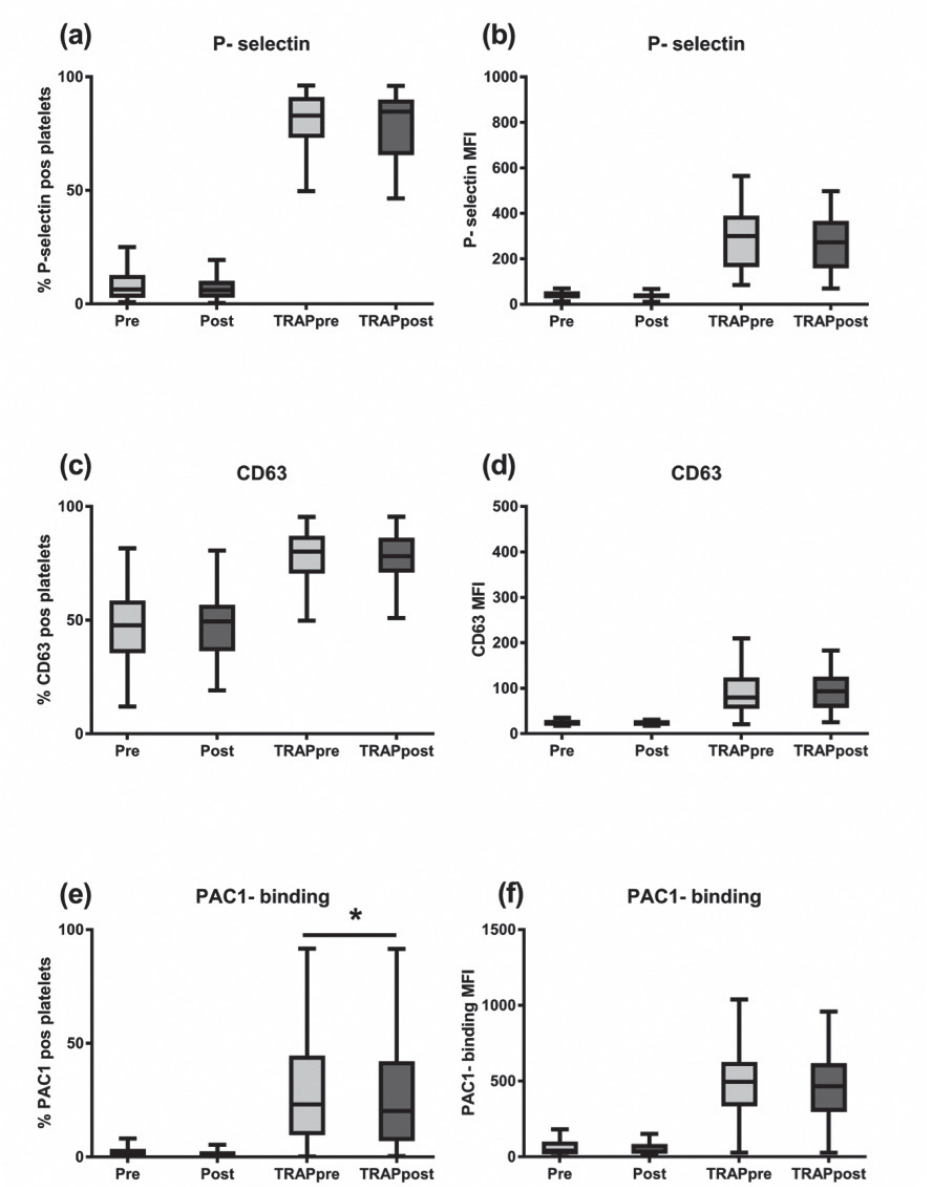

Figure 1. Flow cytometric determination of surface expression markers on resting and TRAP-stimulated platelets from critically ill patients who received a RBC transfusion. (a) Percentage (%) platelets expressing P-selectin and (b) mean fluorescence intensity (MFI) of the antibody binding P-selectin; (c) % platelets expressing CD63 and (d) MFI of the antibody binding CD63; (f) % of platelet binding PAC1 and (e) MFI of the antibody binding PAC1. Data are median [IQR]. *p<0.05, **p<0.005, ***p<0.001.

Also, after RBC transfusion, the formation of platelet-monocyte, platelet-granulocyte and platelet-lymphocyte complexes did not differ compared to pre-transfusion levels. The platelet cell surface activation markers and the number of PLC post transfusion did not differ between septic and non-septic recipients of a RBC transfusion (Figure 2).

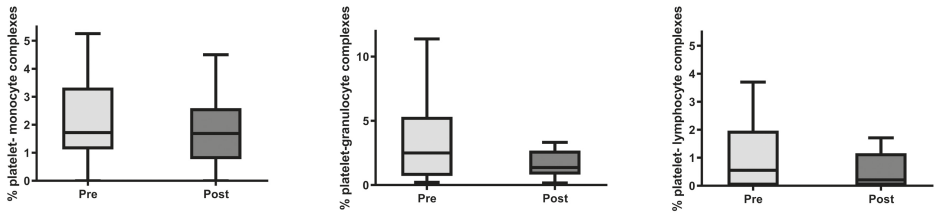

Figure 2. Flow cytometric determination of platelet-leukocyte complexes in critically ill patients who received a RBC transfusion. (a) Percentage (%) of platelet-monocyte complexes, (b) % of platelet-lymphocyte complexes, (c) % of platelet-granulocyte complexes. Data are median [IQR].

The effect of RBC transfusion on the ability of recipients to induce ex vivo platelet aggregation

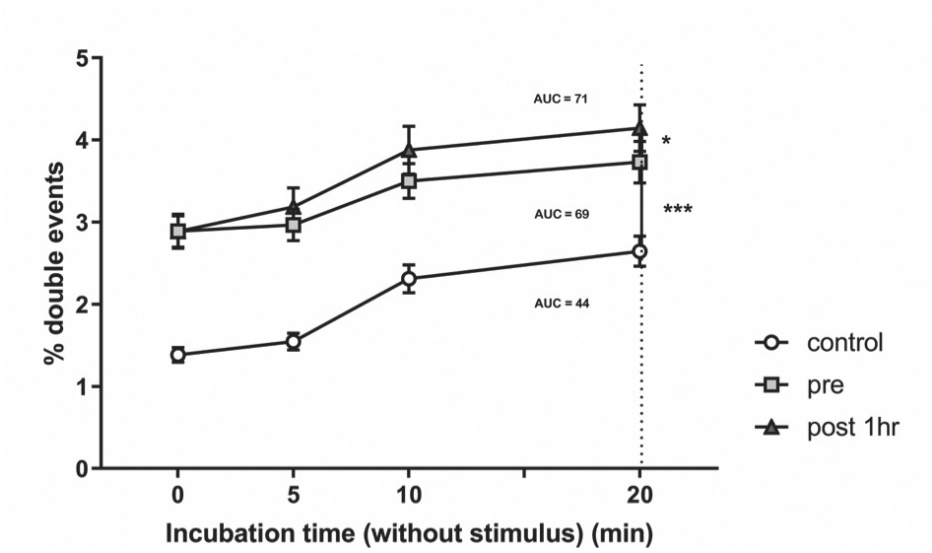

Figure 3. Flow cytometric determination of spontaneous platelet aggregation of platelets from healthy with plasma from critically ill patients before or after a RBC transfusion. Controls were incubated with plasma from healthy controls. Platelet aggregation was measured by flow cytometry, as determined by the percentage of double events. Time is incubation time. Data are Area under the Curve (AUC).

In the functional platelet aggregation assay, the capacity of plasma from the critically ill patients to induce *ex vivo* spontaneous platelet aggregation was already increased prior to RBC transfusion when compared to plasma from the healthy controls (AUC 69 [46-87] vs 44 [30-52], p<0.001, Figure 3). The capacity of patient plasma to induce platelet aggregates was further augmented following RBC transfusion when compared to pre-transfusion levels (AUC 71 [53-90] vs 69 [46-86], p<0.05, Figure 3). Also, collagen-induced platelet aggregation was increased in the presence of plasma from the critically ill patients when compared to control plasma (AUC 19 [15-25] vs 12 [11-14], p<0.001, Figure 4a), but this aggregation was not further augmented following RBC transfusion (AUC 18 [14-26]). In the PMA-induced platelet aggregation, there were no differences between plasma from the critically ill patients and healthy controls, and transfusion did not affect the PMA- induced platelet aggregation capacity of the plasma samples. In contrast, ristocetin-induced platelet agglutination was decreased in plasma from the critically ill patients compared to those of healthy controls (AUC 9 [6-12] vs 11 [9-14], p<0.05, Figure 4c), suggesting that vWF dependent platelet agglutination is impaired. This response was further decreased after RBC transfusion when compared to pre-transfusion levels (AUC 9 [6-11] vs AUC 9 [6-12], p<0.05, Figure 4c). Of note, compared to controls, the levels of vWF:Ag were increased in plasma from the critically ill patients (109% [46-204] vs 477% [170-1825], p<0.001). However, there were no differences in vWF:Ag between pre- and post-transfusion levels. In all assays used, the effect of the RBC transfusion on platelet aggregation response was not different between septic and non-septic patients.

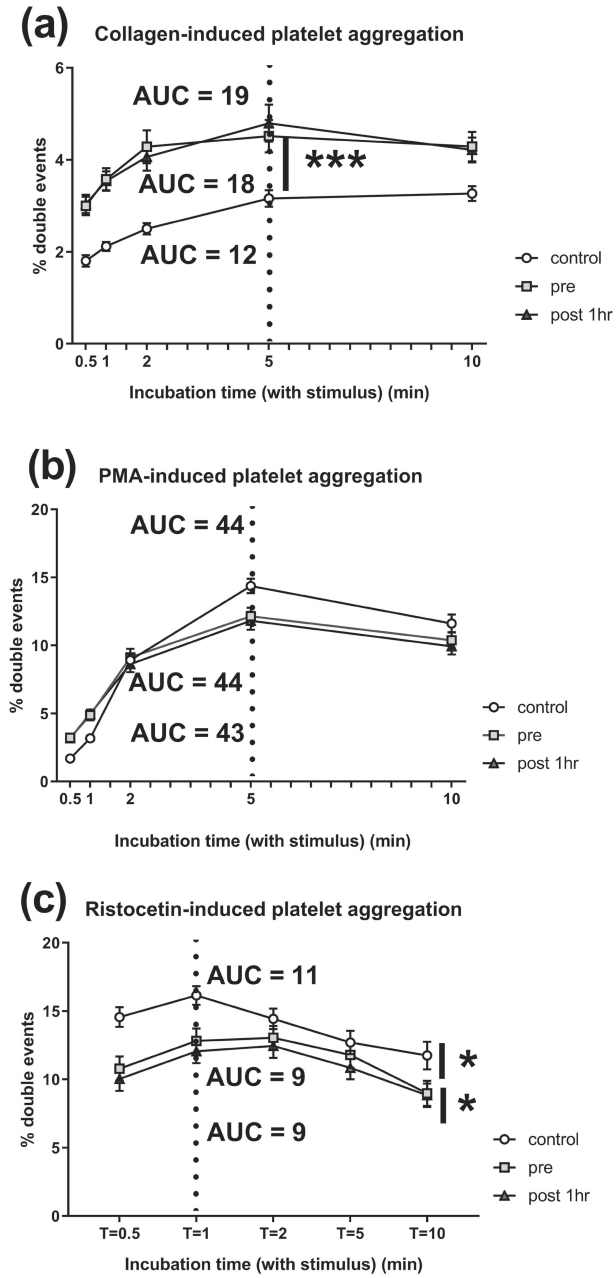

Figure 4. Flow cytometric determination of collagen-, PMA-, or ristocetin-induced platelet agglutination of platelets from healthy volunteers with plasma from critically ill patients before or after a RBC transfusion. Aggregation was measured by flow cytometry as percentage of double events with collagen stimulation (a), with PMA stimulation (b) and with ristocetin stimulation. Time is incubation with stimulus. Data are Area Under the Curve (AUC).

DISCUSSION

This study investigated the effect of a single RBC transfusion on platelet function in critically ill patients. The main conclusions are (1) a single unit of RBC transfusion does not induce expression of platelet activation markers or formation of platelet-leukocytes complexes. Furthermore, (2) the ability of critically ill patients to induce *ex vivo* platelet aggregation is disturbed compared to controls, and (3) RBC transfusion further augmented the spontaneous platelet aggregation response.

In this study, RBC transfusion did not induce expression of platelet activation markers in critically ill patients. Of note, we found a reduction in the percentage of platelets binding PAC1 after TRAP stimulation, which may indicate a reduced number of reactive platelets. However, MFI did not differ between groups. Thereby, we think this is a modest effect. Also, RBC transfusion did not result in the formation of platelet-leukocyte complexes in critically ill recipients, when compared to pre-transfusion values. Whether this relates to the pre-transfusion activation status remains to be determined, as we could not perform measurements in healthy controls receiving an RBC transfusion.

There are several explanations for the observed modest effect of the RBC transfusion on the activation markers and formation of complexes. First, to a certain extent, platelets of critically ill patients may already be activated [41-43]. Activated platelets can attach to the blood vessel wall, rendering them no longer measurable in our assays [44]. Also, unstimulated platelets in this cohort partially expressed CD63, indicating some activation. However, there were still robust responses in platelet activation after exogenous activation with TRAP, this would not be the case if platelets were preactivated. Second, the study could be underpowered to find a difference in activation markers. If not underpowered, the effects of RBC transfusion on platelet aggregation in this study may occur independent of expression of activation markers.

We show that the capacity of plasma of critically ill patients to induce platelet aggregation is disturbed when compared to healthy controls. The capacity to induce spontaneous aggregation was increased in plasma from the critically ill patients compared to controls. We hypothesize that this is related to an underlying inflammatory status. In line with this, *in vitro* platelet aggregation is increased in the presence of lipopolysaccharide (LPS) [45]. It has previously been demonstrated that plasma from septic patients increases the aggregation of platelets from healthy

volunteers *ex vivo* [41]. We built on these findings in this study, showing that both spontaneous as well as collagen-induced aggregation capacity of plasma is enhanced in critically ill patients when compared to controls. We hypothesize that several inflammatory conditions, including sepsis and trauma, may cause activation of the vascular endothelium [46-48] with disruption of the vessel wall and exposure of collagen [49, 50].

Notably, we found that platelet aggregation results vary depending on the stimulus used. In this study, plasma-induced platelet agglutination mediated by vWF was decreased in critically ill patients when compared to healthy controls. This was not due to low levels of vWF, as the level of vWF antigen was higher in critically ill patients when compared to controls, as found before [51]. An explanation may be stress-induced GPIb shedding, because ristocetin binds to GPIb as part of the vWF-receptor complex and spontaneous receptor shedding is frequently observed under stress [52]. Alternatively, the presence of abnormal vWF variants may have resulted in decreased vWF-mediated platelet agglutination. However, we think this is unlikely, because the binding in patients was overall low. We do not have an explanation for our finding that vWF-mediated platelet agglutination is decreased. Of note, the decrease in vWF mediated platelet agglutination was modest and may not reflect clinical significance.

RBC transfusion further aggravated the patient plasma induced disturbances in platelet aggregation capacity in critically ill recipients. Our findings confirm *in vitro* data, in which RBCs increased platelet aggregation after stimulation with weak agonists ADP and arachidonic acid [53]. In patients with acute coronary syndrome, RBC transfusion resulted in an increase in plasma-induced platelet aggregation after stimulation with ADP and TRAP compared with pre-transfusion levels, measured by light transmission aggregometry (LTA) [54]. In addition, in patients with chemotherapy-induced anemia, RBC transfusion was associated with increased fibrin formation with impaired clot strength, measured by thromboelastography, which was more present in the patients who received fresh RBCs [55]. The clinical relevance of this finding is unknown. Notably, critically ill patients often receive RBC transfusions [1] while having low platelet counts with a tendency to bleed. Theoretically, our results may suggest that RBC transfusion increases the bleeding risk in these patients by further derangement of platelet aggregation. On the other hand, our data could also suggest that in patients who need RBC transfusions in the intensive care unit, an active approach to detect (micro) thrombotic events should be employed. Of note, however, we did not find a decrease in platelet counts after

RBC transfusion, so even the plasma from the critically patients gives an increased aggregation of platelets from a healthy donor, the effect on the platelets of the critically patients is unclear.

It has been suggested previously that the underlying inflammatory condition may influence the effects of the RBC transfusion. *In vitro* under flow, both endotoxin and TNF-a can promote the adherence of donor RBCs to the endothelium [56, 57]. Although we could not see differences between septic and non-septic patients, the number of patients we studied was limited and might have been too small to detect differences.

Our study had several limitations. As previously mentioned, activated platelets might sequestrate from the circulation and in this respect not show up in our platelet activation or PLC assay. For logistical reasons we had to fix the samples, which might affect the measurements. Also we were not able to perform aggregation assays or studies in flow models with the platelets derived from the patients themselves. Furthermore, we did not investigate a control group in the platelet activation and PLC assay. In addition, blood samples from patients and controls differed, as arterial samples were used in the patient population and venous samples were used in the controls. Notwithstanding this, the method of processing was the same and patients served as their own control by comparing pre- and post-transfusion measurements. General limitations were that our study design is limited by time points and the moderate number of patients studied. In this respect, critically ill patients in particular are notably heterogeneous. Moreover, only the effect of a single RBC transfusion episode was monitored, the lack of effect may be due to the fact that a single RBC is under the detection limit of RBC effects on circulating platelets based on the volume of circulating blood. However, studying transfusion of one RBC unit was deliberately chosen since a single RBC transfusion to correct anemia is common practice in the ICU [58]. A final limitation is that we can not exclude that some of the effects is a result of volume loading instead of a specific effect of RBC transfusion.

CONCLUSION

In conclusion, this study found that RBC transfusion in critically ill patients is associated with mixed patient plasma induced effects on platelet aggregation. Future studies are needed to investigate which factors mediate the enhanced platelet aggregation after RBC transfusion, and if there are possibilities to eliminate this effect by adjustment of processing methods [59].

REFERENCES

1. Vincent, J.L., et al., *Worldwide audit of blood transfusion practice in critically ill patients.* Crit Care, 2018. **22**(1): p. 102.
2. Rao, S.V., et al., *Relationship of blood transfusion and clinical outcomes in patients with acute coronary syndromes.* JAMA, 2004. **292**(13): p. 1555-62.
3. Wu, W.C., et al., *Blood transfusion in elderly patients with acute myocardial infarction.* N Engl J Med, 2001. **345**(17): p. 1230-6.
4. Mincu, R.I., T. Rassaf, and M. Totzeck, *Red blood cell transfusion in patients with ST-elevation myocardial infarction-a meta-analysis of more than 21,000 patients.* Neth Heart J, 2018. **26**(9): p. 454-460.
5. Chatterjee, S., et al., *Association of blood transfusion with increased mortality in myocardial infarction: a meta-analysis and diversity-adjusted study sequential analysis.* JAMA Intern Med, 2013. **173**(2): p. 132-9.
6. Mariscalco, G., et al., *Red blood cell transfusion is a determinant of neurological complications after cardiac surgery.* Interact Cardiovasc Thorac Surg, 2015. **20**(2): p. 166-71.
7. Brascia, D., et al., *Impact of transfusion on stroke after cardiovascular interventions: Meta-analysis of comparative studies.* J Crit Care, 2017. **38**: p. 157-163.
8. Goel, R., et al., *Association of Perioperative Red Blood Cell Transfusions With Venous Thromboembolism in a North American Registry.* JAMA Surg, 2018. **153**(9): p. 826-833.
9. Nilsson, K.R., et al., *Association between venous thromboembolism and perioperative allogeneic transfusion.* Arch Surg, 2007. **142**(2): p. 126-32; discussion 133.
10. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
11. Andrews, D.A. and P.S. Low, *Role of red blood cells in thrombosis.* Curr Opin Hematol, 1999. **6**(2): p. 76-82.
12. Silvain, J., et al., *Impact of red blood cell transfusion on platelet activation and aggregation in healthy volunteers: results of the TRANSFUSION study.* Eur Heart J, 2010. **31**(22): p. 2816-21.
13. Reimers, R.C., S.P. Sutera, and J.H. Joist, *Potentiation by red blood cells of shear-induced platelet aggregation: relative importance of chemical and physical mechanisms.* Blood, 1984. **64**(6): p. 1200-6.
14. Goldsmith, H.L., et al., *Physical and chemical effects of red cells in the shear-induced aggregation of human platelets.* Biophys J, 1995. **69**(4): p. 1584-95.
15. Joist, J.H., J.E. Bauman, and S.P. Sutera, *Platelet adhesion and aggregation in pulsatile shear flow: effects of red blood cells.* Thromb Res, 1998. **92**(6 Suppl 2): p. S47-52.
16. Aarts, P.A., et al., *Blood platelets are concentrated near the wall and red blood cells, in the center in flowing blood.* Arteriosclerosis, 1988. **8**(6): p. 819-24.
17. Donadee, C., et al., *Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion.* Circulation, 2011. **124**(4): p. 465-76.

18. Kim-Shapiro, D.B., J. Lee, and M.T. Gladwin, *Storage lesion: role of red blood cell breakdown*. Transfusion, 2011. **51**(4): p. 844-51.
19. Helms, C.C., et al., *Mechanisms of hemolysis-associated platelet activation*. J Thromb Haemost, 2013. **11**(12): p. 2148-54.
20. Walton, B.L., et al., *Elevated hematocrit enhances platelet accumulation following vascular injury*. Blood, 2017. **129**(18): p. 2537-2546.
21. Barr, J.D., et al., *Red blood cells mediate the onset of thrombosis in the ferric chloride murine model*. Blood, 2013. **121**(18): p. 3733-41.
22. Berezina, T.L., et al., *Influence of storage on red blood cell rheological properties*. J Surg Res, 2002. **102**(1): p. 6-12.
23. Twomley, K.M., S.V. Rao, and R.C. Becker, *Proinflammatory, immunomodulating, and prothrombotic properties of anemia and red blood cell transfusions*. J Thromb Thrombolysis, 2006. **21**(2): p. 167-74.
24. Devalet, B., et al., *Application of a clot-based assay to measure the procoagulant activity of stored allogeneic red blood cell concentrates*. Blood Transfus, 2018. **16**(2): p. 163-172.
25. Sweeney, J., N. Kouttab, and J. Kurtis, *Stored red blood cell supernatant facilitates thrombin generation*. Transfusion, 2009. **49**(8): p. 1569-79.
26. Rubin, O., et al., *Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation*. Transfusion, 2013. **53**(8): p. 1744-54.
27. Bouchard, B.A., et al., *Microparticles formed during storage of red blood cell units support thrombin generation*. J Trauma Acute Care Surg, 2018. **84**(4): p. 598-605.
28. de Stoppelaar, S.F., C. van 't Veer, and T. van der Poll, *The role of platelets in sepsis*. Thromb Haemost, 2014. **112**(4): p. 666-77.
29. Mavrommatis, A.C., et al., *Coagulation system and platelets are fully activated in uncomplicated sepsis*. Crit Care Med, 2000. **28**(2): p. 451-7.
30. Cook, D., et al., *Venous thromboembolic disease: an observational study in medical-surgical intensive care unit patients*. J Crit Care, 2000. **15**(4): p. 127-32.
31. Hirsch, D.R., E.P. Ingenito, and S.Z. Goldhaber, *Prevalence of deep venous thrombosis among patients in medical intensive care*. JAMA, 1995. **274**(4): p. 335-7.
32. Zarbock, A., R.K. Polanowska-Grabowska, and K. Ley, *Platelet-neutrophil-interactions: linking hemostasis and inflammation*. Blood Rev, 2007. **21**(2): p. 99-111.
33. Rinder, H.M., et al., *Dynamics of leukocyte-platelet adhesion in whole blood*. Blood, 1991. **78**(7): p. 1730-7.
34. Singer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)*. JAMA, 2016. **315**(8): p. 801-10.
35. Kageyama, K., et al., *Increased platelet, leukocyte, and endothelial cell activity are associated with increased coagulability in patients after total knee arthroplasty*. J Thromb Haemost, 2007. **5**(4): p. 738-45.
36. De Cuyper, I.M., et al., *A novel flow cytometry-based platelet aggregation assay*. Blood, 2013. **121**(10): p. e70-80.
37. van de Vijver, E., et al., *Defects in Glanzmann thrombasthenia and LAD-III (LAD-1/v) syndrome: the role of integrin beta1 and beta3 in platelet adhesion to collagen*. Blood, 2012. **119**(2): p. 583-6.

38. Meinders, M., et al., *Repercussion of Megakaryocyte-Specific Gata1 Loss on Megakaryopoiesis and the Hematopoietic Precursor Compartment.* PLoS One, 2016. **11**(5): p. e0154342.
39. Vinholt, P.J., et al., *Measurement of platelet aggregation, independently of patient platelet count: a flow-cytometric approach.* J Thromb Haemost, 2017. **15**(6): p. 1191-1202.
40. Baker-Groberg, S.M., et al., *Assessment of neonatal platelet adhesion, activation, and aggregation.* J Thromb Haemost, 2016. **14**(4): p. 815-27.
41. Gawaz, M., et al., *Platelet function in septic multiple organ dysfunction syndrome.* Intensive Care Med, 1997. **23**(4): p. 379-85.
42. Lundahl, T.H., et al., *Activated platelets and impaired platelet function in intensive care patients analyzed by flow cytometry.* Blood Coagul Fibrinolysis, 1996. **7**(2): p. 218-20.
43. Salat, A., et al., *Changes of platelet surface antigens in patients suffering from abdominal septic shock.* Thromb Res, 1999. **95**(6): p. 289-94.
44. Russwurm, S., et al., *Platelet and leukocyte activation correlate with the severity of septic organ dysfunction.* Shock, 2002. **17**(4): p. 263-8.
45. Salat, A., et al., *Endotoxin enhances in vitro platelet aggregability in whole blood.* Thromb Res, 1999. **93**(3): p. 145-8.
46. Shapiro, N.I., et al., *The association of endothelial cell signaling, severity of illness, and organ dysfunction in sepsis.* Crit Care, 2010. **14**(5): p. R182.
47. Hack, C.E. and S. Zeerleder, *The endothelium in sepsis: source of and a target for inflammation.* Crit Care Med, 2001. **29**(7 Suppl): p. S21-7.
48. Greven, J., et al., *Update on the role of endothelial cells in trauma.* Eur J Trauma Emerg Surg, 2018. **44**(5): p. 667-677.
49. Kumar, P., et al., *Molecular mechanisms of endothelial hyperpermeability: implications in inflammation.* Expert Rev Mol Med, 2009. **11**: p. e19.
50. Gaddnas, F., et al., *Markers of collagen synthesis and degradation are increased in serum in severe sepsis: a longitudinal study of 44 patients.* Crit Care, 2009. **13**(2): p. R53.
51. Kawecki, C., P.J. Lenting, and C.V. Denis, *von Willebrand factor and inflammation.* J Thromb Haemost, 2017. **15**(7): p. 1285-1294.
52. Chen, Z., et al., *Shear-induced platelet receptor shedding by non-physiological high shear stress with short exposure time: glycoprotein Ibalpha and glycoprotein VI.* Thromb Res, 2015. **135**(4): p. 692-8.
53. Bochsen, L., et al., *The influence of platelets, plasma and red blood cells on functional haemostatic assays.* Blood Coagul Fibrinolysis, 2011. **22**(3): p. 167-75.
54. Silvain, J., et al., *Impact of red blood cell transfusion on platelet aggregation and inflammatory response in anemic coronary and noncoronary patients: the TRANSFUSION-2 study (impact of transfusion of red blood cell on platelet activation and aggregation studied with flow cytometry use and light transmission aggregometry).* J Am Coll Cardiol, 2014. **63**(13): p. 1289-1296.
55. Roeloffzen, W.W., et al., *Effects of red blood cells on hemostasis.* Transfusion, 2010. **50**(7): p. 1536-44.

56. Anniss, A.M. and R.L. Sparrow, *Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions.* Am J Hematol, 2007. **82**(6): p. 439-45.
57. Eichelbronner, O., et al., *Endotoxin promotes adhesion of human erythrocytes to human vascular endothelial cells under conditions of flow.* Crit Care Med, 2000. **28**(6): p. 1865-70.
58. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
59. Glenister, K.M. and R.L. Sparrow, *Level of platelet-derived cytokines in leukoreduced red blood cells is influenced by the processing method and type of leukoreduction filter.* Transfusion, 2010. **50**(1): p. 185-9.

CHAPTER 8

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Transfusion of 35 days stored red blood does not alter lipopolysaccharide tolerance during human endotoxemia

ABSTRACT

BACKGROUND: Transfusion-related immunomodulation (TRIM) encompasses immunosuppressive and pro-inflammatory effects induced by red blood cell (RBC) transfusion. Changes that occur during storage in the RBC product have been hypothesized to underlie TRIM, mediated by tolerance of toll-like receptors (TLR). We investigated whether transfusion of 35-day-stored autologous RBCs alters cytokine production in response to stimulation with lipopolysaccharide (LPS) or lipotheic acid (LTA), in a clinically relevant model of endotoxemia.

STUDY DESIGN AND METHODS: Eighteen volunteers received 2ng/kg LPS intravenously, followed by normal saline or 2- or 35 day-stored autologous RBC transfusion. Before LPS, before transfusion and 6 hours after transfusion blood was collected to measure cytokine gene expression. Whole blood was used for *ex vivo* stimulation with LPS and LTA, after which cytokine levels were measured with enzyme-linked immunosorbent assay.

RESULTS: *In vivo* LPS induced a biphasic response in cytokine mRNA with peak values 2 hours after LPS infusion. Storage time of RBC transfusion did not influence cytokine mRNA levels. *In vivo* infusion of LPS resulted in tolerance for *ex vivo* stimulation with LPS and LTA. However, transfusion of either fresh or stored RBCs did not further affect the capacity to produce cytokines after *ex vivo* stimulation.

CONCLUSION: In a clinically relevant model of human endotoxemia, autologous transfusion of 35 days stored RBCs does not influence cytokine mRNA levels nor does it change the capacity of leukocytes in whole blood to produce cytokines after *ex vivo* stimulation with LPS or LTA.

INTRODUCTION

Red blood cell (RBC) transfusions are administered to improve tissue oxygenation and can be lifesaving, especially during severe hemorrhaging. However, transfusion has also been related serious to adverse events leading to increased morbidity and mortality, especially in critically ill patients [1, 2]. Several randomized trials suggest that a restrictive transfusion policy results in a better outcome than a liberal transfusion policy [3]. However, evidence on the deleterious properties of RBC transfusion is conflicting. In a set of heterogeneous trials (e.g. varying transfusion thresholds, patient populations, transfusion products) restrictive transfusion was associated with a reduced number of infectious complications [4], even though no association could be found with mortality or overall morbidity [5] suggesting that transfusion related immunomodulation (TRIM) may play a role. TRIM encompasses immunosuppressive and pro-inflammatory effects induced by RBC transfusion. The effect was first described in 1973 in a study demonstrating increased graft survival following renal transplantation in multiple transfused patients [6]. Other effects attributed to TRIM are the higher recurrence rates of cancer after transfusion and decreased recurrence of auto-immune diseases [7]. The factors in transfusion that mediate TRIM are unknown. The presence of donor white blood cells (WBCs) and time-dependent changes related to storage of blood products, have been hypothesized to be related to TRIM [7]. Storage of RBCs induces the RBC "storage lesion", which results in declining product quality during shelf-life [8]. The storage lesion has been implicated in transfusion-induced adverse events [9, 10]. *In vitro* stored RBC units suppresses monocyte function [11, 12] but RBC transfusion has also been reported to enhance the release of danger signal High Mobility Group Box 1 (HMGB1) in mice. HMGB1 can initiate and sustain the inflammatory response through toll-like receptor (TLR) 2 and TLR4, which supports the hypothesis that storage of RBCs has pro-inflammatory effects [13].

On the other hand, clinical studies that compared fresh products with standard time stored products did not detect a decrease in mortality or morbidity after transfusion of fresh products [14, 15]. However, these studies did not investigate maximum storage time and other observational studies did report an effect of storage time [16]. Therefore, we investigated whether transfusion of maximum stored autologous RBCs results in immunomodulation *in vivo*. We used a randomized controlled trial in healthy volunteers to investigate the hypothesis that 35 day stored autologous leukoreduced RBC transfusion would result in lipopolysaccharide (LPS) tolerance of TLR2 and TLR4 to respond to stimulation (tolerance) compared to 2 day stored

autologous RBC transfusion. Among others, patients suffering from sepsis [17], systemic inflammatory syndrome [18], trauma [19] and major surgery [20] also experience TLR LPS tolerance. Therefore, we used a model of human inflammation induced by intravenous (IV) injection of LPS to enhance translatability of our trial to clinical practice.

MATERIAL & METHODS

All procedures have been reviewed and approved by the Academic Medical Center Medical Ethical Committee and are according to the Declaration of Helsinki including Good Clinical Practice. The study has been registered at the Dutch Trial Register (NTR4455). All enrolled volunteers provided written informed consent before enrollment.

Subject and design

This study is part of a larger study on the influence of storage time of RBCs on transfusion related acute lung injury (TRALI) [21]. In short, a total of 18 healthy volunteers, ages 18 to 35 years old, were included in our open label randomized controlled study. Volunteers were screened at the Academic Medical Center. To be included an unremarkable medical history, as well as physical examination was required. Participants were not allowed to participate in another intervention trial during the course of our study.

Randomization and blood donation

Volunteers were randomly assigned into one of three groups: 6 volunteers received 2-day-stored (fresh) autologous RBC transfusion, 6 received 35-day-stored (stored) autologous RBC transfusion, and 6 volunteers were infused with 350 mL of NaCl 0.9% as endotoxemic control. Depending on group allocation, the volunteers donated one unit of blood 2 or 35 days before the study day. We used 35-day-stored products as shelf life has been maximized to 35 days in The Netherlands. Half of the endotoxemic control group donated 2 days before the study day, the other half 35 days before the experiment. The donated whole blood was processed to one unit RBCs according to Sanquin protocols: whole blood (target volume, 500 mL) was collected in bottom-and-top systems with 70 mL of citrate-phosphate-dextrose (CPD) and placed on butane-1,4-diol cooling plates until the following morning (16-22 h). The units where then centrifuged (RC-12BP, Sorvall, Kendro, 4793g, accumulated centrifugal effect 9×10^7) and separated into a RBC unit, a unit of plasma, and a buffy coat of 50 ± 5 mL with a hematocrit of 42 ± 6 % using a separator (CompoMat

G5, Fresenius). Saline, adenine, glucose and mannitol storage medium (SAG-M) (110 mL) was added to the red cell concentrate and the RBC product was leukoreduced by filtration, with remaining leukocytes below 1 × 10^6/unit. The products were cooled down to below 6°C within 30 hours after collection and then stored in polyvinyl-chloride-di-ethyl-hexyl-phtalate containers at 2 to 6°C for 2 or 35 days.

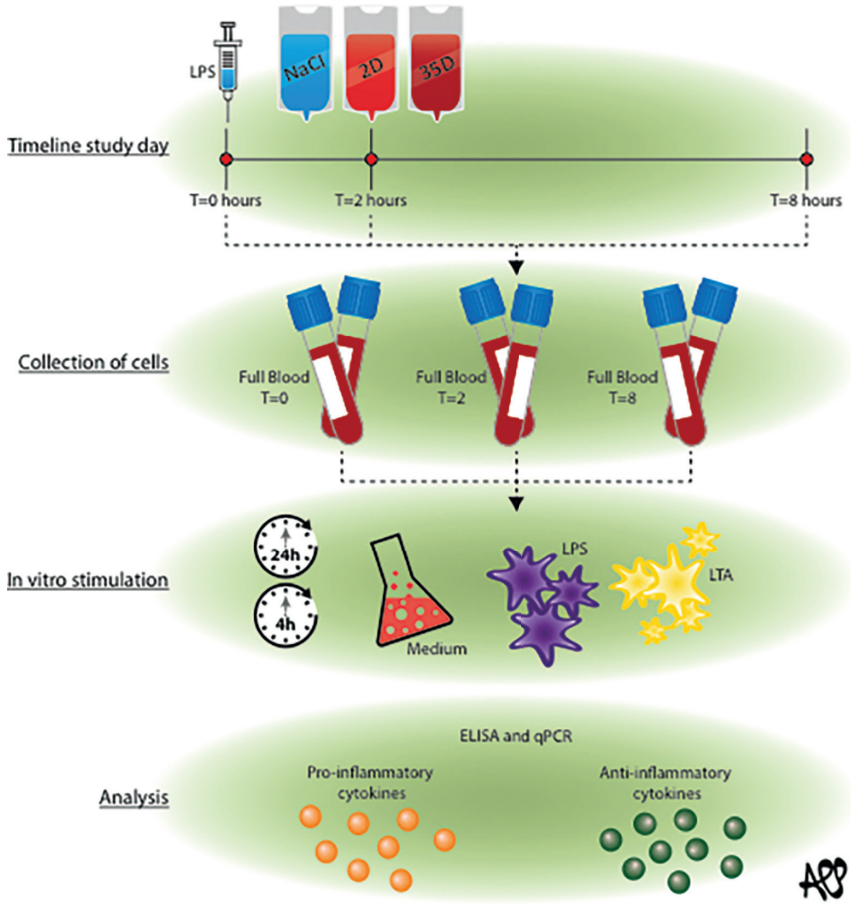

Figure 1. Set-up of experiments.
During the study day healthy volunteers received LPS at T=0, followed by transfusion or saline infusion at T=2 and conclusion of the experiment at T=8. This was followed by stimulation of whole blood with LPS, LTA or medium as control. In the final phase of the experiment ELISA and qPCR were performed to investigate cytokine responses.

Experiment

On the day of the experiment, volunteers were admitted to the Intensive Care Unit (Figure 1). At T=0 hours all volunteers received an infusion of 2 ng/kg LPS (National Institute of Health Clinical Center) as described before [22-24]. Two hours (T=2) after infusion of LPS the intervention group received an autologous transfusion of either 'fresh' or 'stored' RBCs. The experiment ended 8 hours after infusion of LPS. At this time point all LPS symptoms had abated and this time point corresponded with the harvesting of tissues in animal models in which a detrimental effect of transfusion was detected [25, 26]. The volunteers in the control group received an equivalent volume of saline (endotoxemic control). Arterial blood was collected in a lithium heparin coated tube and in a tube (PAXgene, PreAnalytiX) at baseline (T=0 hours), 2-hours after infusion of LPS but before transfusion (T=2 hours) and 8 hours after infusion of LPS (T=8 hours). The PAXgene tubes were stored without any additional pre-processing at -80°C until further analysis.

Qualitative polymerase chain reaction

Whole blood in the PAXgene tubes was defrosted on a roller mixer and allowed to rotate for an additional 6 hours. RNA was isolated with the blood RNA Kit (PAXgene, PreAnalytiX) according to manufacturer's protocols. RNA yield was measured using UV-Vis Spectrophotometer (NanoDrop 2000, Thermo Scientific). cDNA synthesis was performed with equal RNA-input using a cDNA the Synthesis Kit (SensiFAST, Bioline), according to manufacturer's protocols.

Quantitative polymerase chain reaction (qPCR) was performed using a real- time PCR system (Lightcycler 480, Roche) with master mix (SensiFAST, Bioline) and 50 ng of primer (Sigma Aldrich). Intron spanning primer sequences and annealing temperatures are given in supplemental table 1. Primer sequences for interleukin (IL)-8, IL-10 and tumor necrosis factor (TNF)α were published previously [27-29]. Primer sequences for IL1β, IL-6 and β-actin were derived from the Harvard Primer Bank (Primer-Bank IDs: 27894305c1, 224831235c1 and 4501885a1, respectively). The qPCR programs was denaturation for 5 minutes at 95°C, 45 to 55 cycles of 5 seconds at 95°C, and 10 seconds annealing at 65°C; followed by 15 seconds at 72°C. Quantification was performed using software (LinReg). The mean efficiency was calculated for each assay, and samples that had a deviation of >5% were excluded. Calculated values were normalized by the geometric mean of the reference gene values (β -actin and EF1α1).

Ex vivo stimulation

Ex vivo stimulation was used to investigate the effect of storage time of RBC transfusion on cytokine production in whole blood. All experiments were performed in duplicate. For the various time points, half a milliliter of heparinized whole blood was suspended in 0.5 mL RPMI-L-glut (Gibco, Thermo Fisher Scientific, Waltham, ME, USA) in a sterile 24 well cell culture plate. The suspensions were stimulated *ex vivo* with either LPS 100 ng/ml (E. coli, O111:B4, ultrapure, Sigma, St. Louis, MO, USA), 10 µg/ml lipotheic acid (LTA; Invivogen) or solely RPMI-L-glutamine as control as described [17, 22]. LPS is the endotoxin of Gram-negative bacteria and a TLR4 ligand. Similarly, LTA is major constituent of the cell wall of Gram-positive bacteria and ligand of the TLR2. We used LPS and LTA to investigate the hypothesis that stored transfusion products induce tolerance to these two TLR ligands. Cultures were incubated for 4 or 24 hours. After stimulation the samples were centrifuged (1500g, 5 min, 4°C) and the supernatant was stored at -80°C until further analysis.

Enzyme-linked immunosorbent assay

Standard sandwich enzyme-linked immunosorbent assays (ELISA) were used to quantify interleukin (IL)-1β, IL-6, IL-8, IL-10, tissue necrosis factor (TNF)α and transforming growth-factor (TGF)-β according to manufacturer's protocols (eBioscience). ELISA was performed on all samples of the stimulation assays and on the supernatant of the EDTA anticoagulated samples. Two samples were included on each ELISA plate to account for interplate variability.

Supernatants from *ex-vivo* stimulated WBCs in whole blood, incubated with LPS or LTA for 4 or 24 hours, were analyzed in batch after completion of the study. The pattern in 4-hour stimulated WBCs in whole blood was similar to 24-hour stimulations. We therefore only present the data from 24-hour stimulated samples in this paper.

Power calculation

This study was a secondary investigation of a study on the effect of transfusion on pulmonary function and was initially powered to detect differences in pulmonary inflammation [21]. We performed a second power calculation to investigate whether it could also be used to investigate the effect of transfusion on *ex vivo* stimulation with LPS. We based our calculations on a previous publication that investigated the effect of RBC storage time on cytokine production by monocytes *in vitro*. In this study TNFα was 48,525 +/- 7,832 pg/mL in controls. Stored RBCs decreased monocyte TNFα production approximately 50% with a standard deviation of 7.5%

[12]. Based on these numbers we needed to include 4 volunteers to detect a similar signal with an alpha of 0.005 and a beta of 0.90. However, *in vivo* studies usually result in smaller differences. We therefore also calculated the smallest detectable effect. With 6 volunteers, an alpha of 0.05 and a beta of 0.80 we are able to detect a difference of 15% between our groups with a standard deviation of 9%.

Statistical analysis

Data was inspected for normality using histograms and density plots. As this inspection showed no Gaussian distributions we used a Wilcoxon sign rank test to compare T=0 (baseline) with T=2 (pre-transfusion) and to compare cytokines levels after LPS or LTA incubation with the negative control. Differences at T=8 (post-transfusion) and the delta between T=2 and T=8 between treatment groups were investigated with a Kruskal-Wallis test. All statistical analyses were performed in computer software (R version 3.1.2, R-core team). Double-sided p values of less than 0.05 were considered to be a statistically significant. As this was considered an explorative study, we did not correct for multiple testing. Samples with values below the detection limit of the assay were expressed as equivalent to the detection limit and compared to expression as equivalent to 0. This did not change the results of our study and therefore we used expression as equivalent to the detection limit for all statistical analyses.

RESULTS

We screened 22 volunteers and included 18 subjects all of whom completed the study according to protocol. The participants had the expected response to LPS and developed tachycardia, decreased blood pressure, tachypnea and fever 1.5 hours after infusion. Symptoms abated 8 hours after infusion. Laboratory measurements at the routine clinical laboratory showed leukocytosis [21].

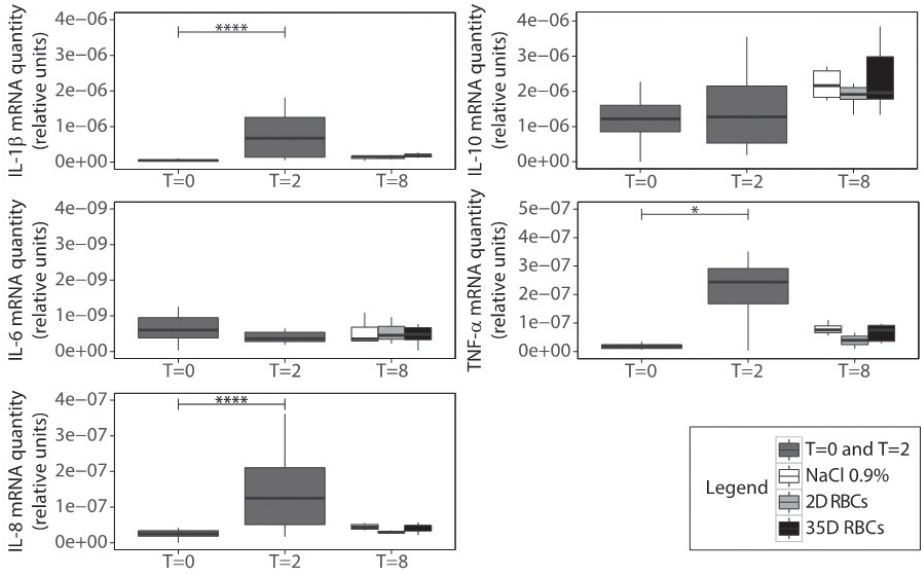

Figure 2. mRNA quantification of cytokines in plasma of volunteers.
At T=0 volunteers received LPS, at T=2 volunteers received saline, 2D stored RBCs or 35D stored RBCs. IL: interleukin, LPS: lipopolysaccharide, RBCs: red blood cells. *p<0.05, **** p<0.0001. Boxes represent the inter-quartile range [IQR] with median. The upper and lower whisker extends to 1.5 * IQR.

Transfusion of 35 days stored RBCs does not result in changed cytokine gene expression in unstimulated blood

We first investigated whether transfusion of stored RBCs influenced the level of cytokines proteins in plasma following IV LPS challenge. These were samples collected from the healthy volunteers and analyzed for cytokine levels in the plasma without any additional *ex vivo* stimulation. All cytokines followed a typical biphasic pattern as expected after LPS infusion but storage time of RBCs did not change the levels compared to fresh RBCs or saline infusion (supplemental figure 1) [21]. We then investigated whether transfusion of stored RBCs affected cytokine gene expression in these samples. LPS induced a biphasic response in IL-1β, IL-8 and TNFα mRNA with peak values 2 hours after LPS infusion ($p<0.0001$, $p<0.0001$ and $p<0.05$ for IL-1β, IL-8 and TNFα, respectively; Figure 2). IL-6 mRNA levels did not change during the experiment in accordance with a previously published report [30], and IL-10 increased gradually with maximum levels at T=8 ($p<0.01$ T=8 compared to T=0; Figure 2). In summary, LPS infusion resulted in a LPS-typical biphasic pattern, but no influence of the transfusion of stored or fresh RBCs was seen on pro- or anti-inflammatory cytokine protein and gene levels.

Lipopolysaccharide results in LPS tolerance

In vivo LPS infusion leads to tolerance to *ex vivo* stimulation of whole blood with TLR ligands, a phenomenon known as LPS-tolerance [31]. We first confirmed that volunteers developed LPS tolerance during endotoxemia. Samples obtained at T=0 were collected before *in vivo* infusion of LPS. At T=0 *ex vivo* incubation with LPS or LTA resulted in release of IL-1β, IL-6, IL-8, IL-10, TNFα and TGF-β compared to incubation in medium alone ($p<0.001$ for all cytokines for both LPS and LTA; Figure 3). At T=2, 2 hours after *in vivo* infusion of LPS, IL-1β, IL-6, IL-8, IL-10 and TNFα did not reach the detection limit of the assays in most samples ($p<0.0001$ compared to T=0; Figure 3), indicative of a clear reduced response to stimulation with LPS and LTA during endotoxemia. LTA stimulation had a similar, although less pronounced, effect (IL-1β $p<0.05$, IL-6 $p<0.0001$, IL-8 no change, IL-10 $p<0.01$ and TNFα $p<0.01$; Figure 3). TGF-β levels were not affected by LPS and LTA.

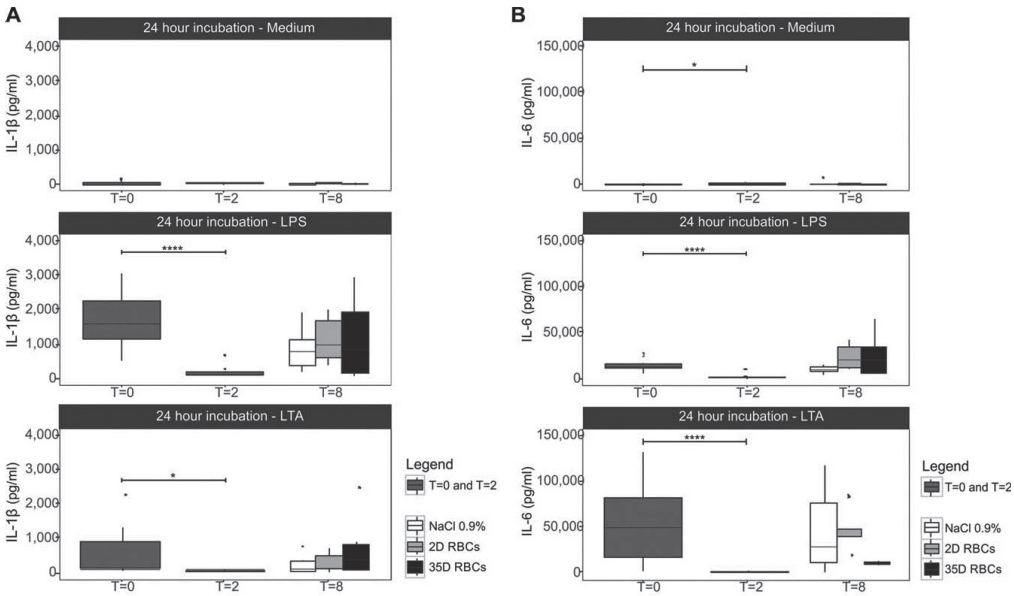

Figure 3. Intravenous LPS reduced (A) IL-1β, (B) IL-6, (C) IL-8, (D) IL-10, (E) TNFα and TGF-β production after *ex vivo* stimulation with LPS or LTA. (F) IV LPS did not change TGF- β production after ex vivo stimulation with LPS and LTA. Volunteers were received LPS (2 ng/kg) at T=0 hours and received either 35 days stored autologous RBC transfusion, 2 days stored autologous RBC transfusion or saline as endotoxemic control at T-2 hours. Transfusion did not affect cytokine levels. *p<0.05; ****p<0.0001. Boxes represent the inter-quartile range [IQR] with median. The upper and lower whisker extends from the hinge to 1.5 * IQR

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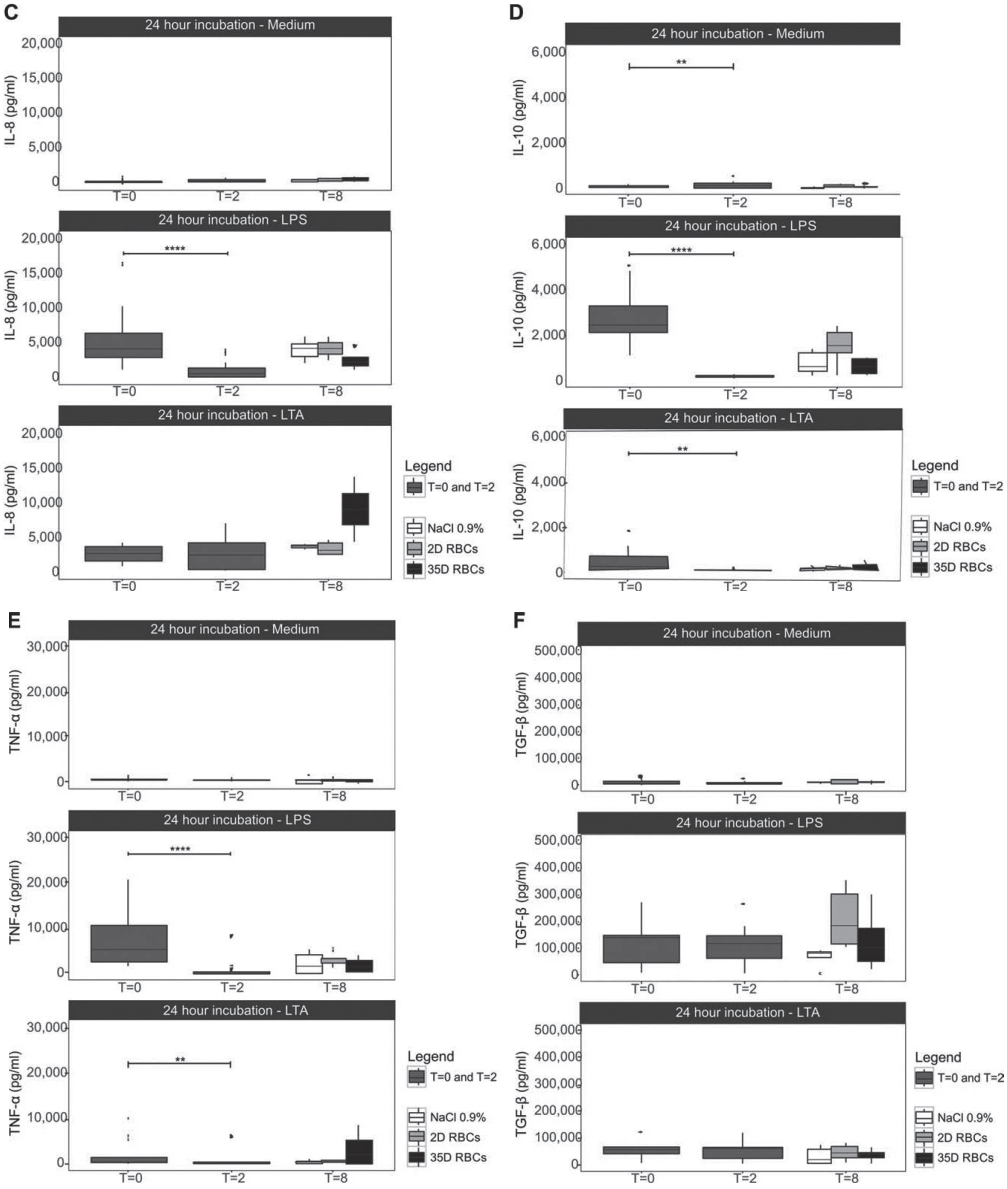


Figure 3. continued.

Transfusion of stored RBCs does not alter the ex vivo response of whole blood to LPS or LTA compared to transfusion of fresh RBCs or endotoxemic control

After establishing that *in vivo* LPS resulted in LPS tolerance, we investigated whether stored RBC transfusion influenced the response of whole blood to *ex vivo* stimulation with LPS and LTA compared to transfusion of fresh RBCs or saline infusion. At T=8, 8 hours after *in vivo* LPS-infusion and 6 hours after transfusion of fresh or stored RBCs, cytokine levels returned to baseline in the *ex vivo* stimulation experiments. We compared the increment in cytokine levels in each group from T=2 to T=8 and compared the levels between the three groups at T=8 to establish the influence of RBC transfusion. *Ex vivo* stimulation with LPS did not result in changed cytokine levels between transfusion of stored RBCs, transfusion of fresh RBCs or endotoxemic control at T=8. The increment from T=2 to T=8 did also not differ between the intervention groups. Stimulation with LTA produced similar results. We did not detect any differences in cytokine expression between T=2 and T=8, nor did the groups differ at T=8 (Figure 3). However, although the difference did not reach statistical significance, IL-6 response to LTA stimulation appeared to be reduced in the stored group with a concurrent decrease in IL-8 response (Figure 3B and 3C). As the responses of IL-6 and IL-8, which are both pro-inflammatory, go in opposite directions and the results from 4 hour stimulations did not show similar patterns in these *ex vivo* stimulations (data not shown), we concluded that these are a result of assay variation.

DISCUSSION

In this study we investigated the effect of storage time of red blood cell transfusion products on the response of whole blood on *ex vivo* stimulation with LPS and LTA in a human model of endotoxemia. We found that 1) Transfusion of 35-day-stored RBCs does not result in changed cytokine gene expression compared to fresh RBCs; 2) LPS results in LPS tolerance; 3) Transfusion of stored RBCs *in vivo* does not result in altered cytokine levels after *ex vivo* stimulation of whole blood compared to transfusion of fresh RBCs or endotoxemic control.

The patterns of cytokine expressions we detected after *in vivo* exposure to LPS, and additional stimulation with LPS or LTA *ex vivo*, is in accordance with other investigations with human endotoxemia [30-32]. However, our data argues against our hypothesis that transfusion of stored RBCs alters the response of whole blood to additional *ex vivo* stimulation with LPS or LTA. In our study, the cytokine levels before and after *ex vivo* stimulation were similar in all three groups. This is not

in line with previous studies. *In vitro* stored RBCs induce immunosuppression in whole blood and isolated monocytes stimulated with LPS [11, 33, 34]. Moreover, in pediatric intensive care patients transfusion of stored RBCs was found to result in lower TNFα production after *ex vivo* LPS stimulation of whole blood compared to transfusion of fresh RBCs [35].

There are several explanations for our findings. First, it is possible that children are more susceptible to the effects of RBC storage time than adult patients. However, it is also possible that the adverse effects that have been attributed to stored transfusion products have been overestimated. The evidence that transfusion of products with increased storage time can have detrimental effects is largely derived from observational studies which report conflicting evidence [9]. The pediatric study may have suffered from its observational design: the decision to transfuse in this study was made by the treatment team, which may have resulted in inclusion bias [35]. Moreover, several recent randomized controlled trials that investigated the effect of RBC storage time on morbidity and mortality did not detect any detrimental effects of longer RBC shelf life both in adult and pediatric patients [14, 15, 36]. Another explanation for our findings may be that both *in vitro* studies and *in vivo* studies that report a harmful effect of storage time have made use of allogeneic products. It is possible that interactions between donor and recipient and the use of animals and *in vitro* techniques, and not storage time, are responsible for the effects detected in these studies [11-13, 35, 37]. Indeed, several autologous human volunteer studies did not detect a role for storage time in the pathogenesis of transfusion-related adverse effects [38, 39]. These studies investigated RBC storage time in healthy volunteers, which limits comparability to clinical practice. However, in the present study we were able to overcome this limitation by using LPS endotoxemia as a model for sepsis and still were not able to confirm the hypothesis that stored RBCs have immunomodulatory effects.

Our results may also have been influenced by RBC production and storage techniques. The quality of RBCs during storage is influenced by production protocols and storage solutions [40-44]. There is limited evidence of what the effect is of these different techniques on RBC function, but *in vitro* studies indicated that storage solution influences immunomodulatory effect of stored RBCs [12]. Another factor that can influence transfusion product quality are donor related factors. Increasing evidence indicates that donor genetic traits and lifestyle can influence product quality and perhaps also mediate adverse effects of transfusion [45]. The fact that we only included healthy volunteers might thus even have led to a “donor bias”.

It is also possible that the RBC transfusions have immunomodulatory effects that cannot be detected by investigating response to *ex vivo* stimulation of whole blood with LPS or LTA. An *in vitro* study detected changed expression of TLR4 gene expression in WBCs after exposure of whole blood to stored RBCs [46]. In another study healthy volunteers received a stored autologous transfusion to investigate diagnostic techniques to detect autologous blood doping. In this study proteomics revealed increased expression of TLR4, TLR5, and TLR6 in T-lymphocytes [47]. HLA-DR expression is another potentially interesting marker. HLA-DR is an antigen presenting molecule that is expressed by monocytes. During immunoparalysis in for example sepsis but also LPS endotoxemia, monocyte HLA-DR is down regulated [48, 49]. It may be worthwhile to use these techniques to investigate the effect of transfusion in patients or in endotoxemia.

Our study has several limitations. First, we used an autologous transfusion model. Although this facilitated investigation of the isolated effect of storage time, it decreases comparability with clinical practice. Moreover, it cannot be excluded that storage time and allogeneic properties of the transfusion product have synergistic effects and that stored allogeneic products thus are able to induce adverse effects. Another limiting factor is that we sampled blood up to 8 hours after LPS infusion, corresponding to 6 hours after transfusion. It is possible that this time-window was too short to detect an effect of the interventions. Although most studies showed an effect within 6 hours of transfusion, in an earlier study in which healthy volunteers received a stored autologous transfusion, blood was sampled after 72 and 96 hours. In these samples down regulation of lymphocyte TLR was detected accompanied by an adaptive immune response [47]. This study did not investigate time points before 72 hours after transfusion and we cannot exclude that sampling later than 8 hours after LPS infusion in our study would have resulted in evidence supporting immunomodulation. In our study we used whole blood for *ex vivo* stimulation with LPS or LTA. However, it is possible that different types of WBCs behave differently in response to transfusion or that responsive immune cells reside outside the blood stream (e.g. spleen or liver). These cells cannot be investigated with stimulations of whole blood. It may be worthwhile if future studies investigate the effect of RBC transfusion on subsets of immune cells, instead of whole blood. Finally we cannot exclude that a more potent inflammatory state is required to be susceptible for RBC-induced immunomodulation. LPS endotoxemia does not equal sepsis, even though LPS infusion successfully induced systemic inflammatory response syndrome in our healthy volunteers. It is possible that more severe disease, including non-infectious pathology, activates other inflammatory pathways in which the

patient is susceptible for immunomodulation by transfusion. Moreover, our healthy volunteers did not suffer from anemia during our study. We were thus not able to investigate whether anemia alters the response to RBC transfusion.

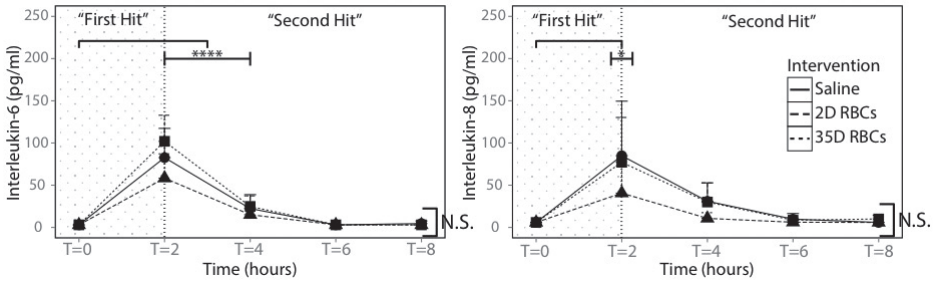
CONCLUSION

In conclusion, we investigated whether transfusion of 35-day-stored RBCs changed cytokine mrRNA levels or induced tolerance of the TLR2 or TLR4 for stimulation in a human autologous transfusion model of endotoxemia. Storage time of RBC transfusion did not influence cytokine mRNA levels nor did it affect the reaction of TLR2 or TLR4 to stimulation with LPS or LTA. In our healthy human volunteer autologous transfusion study, transfusion of stored RBCs did not affect cytokine production after *ex vivo* LPS stimulation.

Suplemmental

Supplemental Table 1. Primer sequences and annealing temperatures.

Gene	Primer sequence: forward	Primer sequence: backward	Annealing temperature
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA	65
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	65
IL-8	TTG GCA GCC TTC CTG AT T	AAC TTC TCC ACA ACC CTC TG	65
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	65
TNFα	GCGTGGAGCTGAGAGATAACC	GATCCCAAAGTAGACCTGCCC	65
β-act	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	65
EF1α1	TTTTCGCAACGGGTTTGCC	TTGCCCGAATCTACGTGTCC	65



Supplemental Figure 1. Plasma protein levels of interleukin-6 and interleukin-8 after *in vivo* LPS infusion and transfusion of 2 days or 35 days stored red blood cells or saline infusion as control. Transfusion had no effect on plasma cytokine levels.

REFERENCES

1. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
2. Tinmouth, A., et al., *Clinical consequences of red cell storage in the critically ill.* Transfusion, 2006. **46**(11): p. 2014-27.
3. Salpeter, S.R., J.S. Buckley, and S. Chatterjee, *Impact of more restrictive blood transfusion strategies on clinical outcomes: a meta-analysis and systematic review.* Am J Med, 2014. **127**(2): p. 124-131 e3.
4. Rohde, J.M., et al., *Health care-associated infection after red blood cell transfusion: a systematic review and meta-analysis.* JAMA, 2014. **311**(13): p. 1317-26.
5. Holst, L.B., et al., *Restrictive versus liberal transfusion strategy for red blood cell transfusion: systematic review of randomised trials with meta-analysis and trial sequential analysis.* BMJ, 2015. **350**: p. h1354.
6. Opelz, G., et al., *Effect of blood transfusions on subsequent kidney transplants.* Transplant Proc, 1973. **5**(1): p. 253-9.
7. Vamvakas, E.C. and M.A. Blajchman, *Transfusion-related immunomodulation (TRIM): an update.* Blood Rev, 2007. **21**(6): p. 327-48.
8. Hess, J.R., *Measures of stored red blood cell quality.* Vox Sang, 2014. **107**(1): p. 1-9.
9. Lelubre, C. and J.L. Vincent, *Relationship between red cell storage duration and outcomes in adults receiving red cell transfusions: a systematic review.* Crit Care, 2013. **17**(2): p. R66.
10. Alexander, P.E., et al., *Transfusion of fresher versus older red blood cells in hospitalized patients: a systematic review and meta-analysis.* Blood, 2015.
11. Muszynski, J.A., et al., *Supernatants from stored red blood cell (RBC) units, but not RBC-derived microvesicles, suppress monocyte function in vitro.* Transfusion, 2015. **55**(8): p. 1937-45.
12. Muszynski, J., et al., *Immunosuppressive effects of red blood cells on monocytes are related to both storage time and storage solution.* Transfusion, 2012. **52**(4): p. 794-802.
13. Qing, D.Y., et al., *Red blood cells induce necroptosis of lung endothelial cells and increase susceptibility to lung inflammation.* Am J Respir Crit Care Med, 2014. **190**(11): p. 1243-54.
14. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
15. Steiner, M.E., et al., *Effects of red-cell storage duration on patients undergoing cardiac surgery.* N Engl J Med, 2015. **372**(15): p. 1419-29.
16. Muszynski, J.A., et al., *Transfusion-related immunomodulation: review of the literature and implications for pediatric critical illness.* Transfusion, 2016.
17. Wiersinga, W.J., et al., *Immunosuppression associated with interleukin-1R-associated-kinase-M upregulation predicts mortality in Gram-negative sepsis (melioidosis).* Crit Care Med, 2009. **37**(2): p. 569-76.

18. Cavaillon, J.M., et al., *Immunodepression in sepsis and SIRS assessed by ex vivo cytokine production is not a generalized phenomenon: a review.* J Endotoxin Res, 2001. **7**(2): p. 85-93.
19. Majetschak, M., et al., *The extent of traumatic damage determines a graded depression of the endotoxin responsiveness of peripheral blood mononuclear cells from patients with blunt injuries.* Crit Care Med, 1999. **27**(2): p. 313-8.
20. Kleinschmidt, S., et al., *Proinflammatory cytokine gene expression in whole blood from patients undergoing coronary artery bypass surgery and its modulation by pentoxifylline.* Shock, 1998. **9**(1): p. 12-20.
21. Peters, A.L., et al., *Transfusion of 35-Day Stored RBCs in the Presence of Endotoxemia Does Not Result in Lung Injury in Humans.* Crit Care Med, 2016. **44**(6): p. e412-9.
22. Lankelma, J.M., et al., *Antibiotic-induced gut microbiota disruption during human endotoxemia: a randomised controlled study.* Gut, 2016.
23. Stegenga, M.E., et al., *Hyperglycemia enhances coagulation and reduces neutrophil degranulation, whereas hyperinsulinemia inhibits fibrinolysis during human endotoxemia.* Blood, 2008. **112**(1): p. 82-9.
24. van Zoelen, M.A., et al., *Expression and role of myeloid-related protein-14 in clinical and experimental sepsis.* Am J Respir Crit Care Med, 2009. **180**(11): p. 1098-106.
25. Vlaar, A.P., et al., *Supernatant of stored platelets causes lung inflammation and coagulopathy in a novel in vivo transfusion model.* Blood, 2010. **116**(8): p. 1360-8.
26. Vlaar, A.P., et al., *Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model.* Anesthesiology, 2010. **113**(1): p. 92-103.
27. Bouaboula, M., et al., *Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control.* J Biol Chem, 1992. **267**(30): p. 21830-8.
28. Hanley, P.J., et al., *Extracellular ATP induces oscillations of intracellular Ca2+ and membrane potential and promotes transcription of IL-6 in macrophages.* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9479-84.
29. Sharif, O., et al., *Transcriptional profiling of the LPS induced NF-kappaB response in macrophages.* BMC Immunol, 2007. **8**: p. 1.
30. Marsik, C., et al., *Endotoxemia enhances expression of the signaling receptor (GP130) on protein and molecular level.* Clin Immunol, 2005. **114**(3): p. 293-8.
31. de Vos, A.F., et al., *In vivo lipopolysaccharide exposure of human blood leukocytes induces cross-tolerance to multiple TLR ligands.* J Immunol, 2009. **183**(1): p. 533-42.
32. van der Poll, T., et al., *Changes in endotoxin-induced cytokine production by whole blood after in vivo exposure of normal humans to endotoxin.* J Infect Dis, 1996. **174**(6): p. 1356-60.
33. Mynster, T., *Effects of red cell storage and lysis on in vitro cytokine release.* Transfus Apher Sci, 2001. **25**(1): p. 17-23.
34. Biedler, A.E., et al., *Impact of alloantigens and storage-associated factors on stimulated cytokine response in an in vitro model of blood transfusion.* Anesthesiology, 2002. **97**(5): p. 1102-9.
35. Muszynski, J.A., et al., *Red blood cell transfusion and immune function in critically ill children: a prospective observational study.* Transfusion, 2014.

36. Fergusson, D.A., et al., *Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants. The ARIPI randomized trial.* Jama-Journal of the American Medical Association, 2012. **308**(14): p. 1443-1451.
37. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
38. Weiskopf, R.B., et al., *Fresh and stored red blood cell transfusion equivalently induce subclinical pulmonary gas exchange deficit in normal humans.* Anesth Analg, 2012. **114**(3): p. 511-9.
39. Berra, L., et al., *Transfusion of stored autologous blood does not alter reactive hyperemia index in healthy volunteers.* Anesthesiology, 2012. **117**(1): p. 56-63.
40. Veale, M.F., G. Healey, and R.L. Sparrow, *Effect of additive solutions on red blood cell (RBC) membrane properties of stored RBCs prepared from whole blood held for 24 hours at room temperature.* Transfusion, 2011. **51 Suppl 1**: p. 25S-33S.
41. Sparrow, R.L., et al., *In vitro measures of membrane changes reveal differences between red blood cells stored in saline-adenine-glucose-mannitol and AS-1 additive solutions: a paired study.* Transfusion, 2014. **54**(3): p. 560-8.
42. Sparrow, R.L. and K.S. Chan, *Microparticle content of plasma for transfusion is influenced by the whole blood hold conditions: pre-analytical considerations for proteomic investigations.* J Proteomics, 2012. **76 Spec No.**: p. 211-9.
43. Bakkour, S., et al., *Manufacturing method affects mitochondrial DNA release and extracellular vesicle composition in stored red blood cells.* Vox Sang, 2016.
44. Bicalho, B., A.S. Pereira, and J.P. Acker, *Buffy coat (top/bottom)- and whole-blood filtration (top/top)-produced red cell concentrates differ in size of extracellular vesicles.* Vox Sang, 2015. **109**(3): p. 214-20.
45. Tzounakas, V.L., et al., *Donor-variation effect on red blood cell storage lesion: A close relationship emerges.* Proteomics Clin Appl, 2016.
46. Escobar, G.A., et al., *Stored packed red blood cell transfusion up-regulates inflammatory gene expression in circulating leukocytes.* Ann Surg, 2007. **246**(1): p. 129-34.
47. Pottgiesser, T., et al., *Gene expression in the detection of autologous blood transfusion in sports--a pilot study.* Vox Sang, 2009. **96**(4): p. 333-6.
48. Fijen, J.W., et al., *Leukocyte activation and cytokine production during experimental human endotoxemia.* Eur J Intern Med, 2000. **11**(2): p. 89-95.
49. Monneret, G., et al., *Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock.* Intensive Care Med, 2006. **32**(8): p. 1175-83.

CHAPTER 9

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Summary and discussion of this thesis

SUMMARY

In this thesis the effect of red blood cell (RBC) transfusion on the host immune response in critically ill patients was investigated.

The specific research questions were:

(1) Which mediators in the RBC transfusion bag affect the host immune response after transfusion?
(2) What effect does RBC transfusion have on different immune cells?
(3) What is the role of the underlying immune status on the host response after transfusion?
(4) What is the effect of storage time of the RBC unit on the immune response after transfusion?

Chapter 2 gives an overview of the current knowledge of the ability of extracellular vesicles (EVs) to induce a host response. This review describes that all kinds of cells are able to vesiculate and that EVs can induce a myriad of pro- and anti-inflammatory responses. Notably, pro-inflammatory effects were most obvious. Therefore, we hypothesized that EVs may play a role in mediating an exaggerated host response following RBC transfusion.

Chapter 3 gives an overview of the knowledge of non-antibody mediated transfusion-related acute lung injury (TRALI). This review focused on the soluble mediators and the morphological and functional changes of the donor RBCs that accumulate during storage. The implicated mediators, like bioactive lipids, soluble CD40 ligand and EVs have been shown to induce TRALI in 'two- hit' animal models, suggesting that these mediators are involved in the onset of TRALI. Also, in these models, it was shown that the underlying inflammatory condition of the recipient predisposes to the onset of TRALI. These findings are in line with the fact that critically ill patients have an increased risk to develop TRALI after RBC transfusion, as these patients often have an underlying inflammatory condition [1, 2]. Regarding the impact of storage time, it appears that increased storage time increases the risk of TRALI in pre-clinical studies. However, this finding is contradictory to clinical studies, which have shown no difference in the occurrence of pulmonary complications with the use of fresh versus stored RBCs.

The role of EVs in mediating adverse effects of RBC transfusion was further assessed in **Chapter 4**. It was hypothesized that EVs derived from donor RBCs can activate endothelial cells *in vitro* trough activation of host immune cells. Incubation of endothelial cells with EVs from the transfusion bag induced upregulation of adhesion molecules, which occurred in the presence of monocytes but not in the presence of granulocytes. In addition, EVs from RBCs are phagocytosed, in part via complement receptor 3. EVs from RBCs can also induce endothelial shedding of von Willebrand factor (vWF) directly. Storage time of the RBC-derived EVs did not play a role in this study. In conclusion, EVs from stored RBC induce upregulation of adhesion molecules. This may be relevant, as adherence of RBCs and other cells to the microvasculature may be one of the mechanisms involved in the association between RBC transfusion and organ failure [3]. Whether this is truly a mechanism of transfusion mediated organ failure remains to be determined in clinical studies.

Besides EVs, iron may play a role as a modulator of the host immune response after transfusion. RBC transfusion results in iron loading, which is associated both with an increased risk for nosocomial infection and with organ failure due to oxidative stress. In **Chapter 5** the effect of RBC transfusion on iron metabolism was investigated. In a prospective cohort study of critically ill patients, it was found that transfusion of 1 unit of RBCs transiently increased the serum iron levels, but not the transferrin saturation, suggesting that RBC transfusion does not increase iron availability for erythropoiesis. In addition, the iron level was back to baseline 24 hours after transfusion. RBC transfusion increased hepcidin levels. Hepcidin levels may increase due to an inflammatory stimulus, but in this study IL-6 levels decreased after transfusion, thus an increase in hepcidin is more likely to be the result of high iron levels after clearance of the donor RBCs. No correlation between storage time of the RBCs and changes in iron variables was found. Taken together, the pro-inflammatory effects of RBC transfusion in critically ill patients may not be mediated by an increase in iron levels, because the increase is transient and the transferrin saturation does not increase. Also, an RBC transfusion may not beneficially alter iron metabolism.

Regarding effector cells of the effects of RBC transfusion, neutrophils may be one of the immune cells involved in the immune response after RBC transfusion. In **Chapter 6** the effect of RBC transfusion on neutrophil function was investigated in both an endotoxemia model and in a cohort of critically ill patients. Neutrophil activation *ex vivo* was assessed by measuring the reactive oxygen species (ROS) production and adhesion of neutrophils from healthy volunteers after incubation

with the plasma from the endotoxemic volunteers and from the critically ill patients. In the human endotoxemia model, RBC transfusion increased the ability of plasma to induce neutrophil adhesion. No differences were found between plasma from the volunteers who received fresh RBCs compared to plasma from volunteers who received stored RBCs. In the critically ill patients, the adhesion of neutrophils *ex vivo* increased after transfusion, which was more pronounced after incubation with plasma from septic patients, compared to plasma from non-septic patients. This suggests that the inflammatory status of the recipient plays a role in inducing neutrophil activation after RBC transfusion. Storage time also seems to influence the neutrophil function; the ROS production by neutrophils increased after incubation with plasma obtained after transfusion of fresh RBCs but not after standard issued RBCs. In conclusion, RBC transfusion induces changes in the plasma of the recipient that are associated with increased neutrophil adhesion in both a model of human endotoxemia and in critically ill patients with sepsis. Therefore, neutrophils may be activated to play a role in mediating host response following RBC transfusion.

It is well established that RBC transfusion can have an effect on neutrophils [4, 5]. However, much less is known about the effects of RBC transfusions on platelet function. As RBC transfusions are associated with an increased risk for thromboembolic events, we hypothesized that platelets are affected by RBC transfusions as well. Also, we hypothesized that the response of platelets is related to the underlying inflammatory status of the recipient. In **Chapter 7** the effect of RBC transfusion on platelet activity in critically ill patients was investigated. The ability of plasma from critically ill patients and healthy controls to induce *ex vivo* platelet aggregation was assessed by flow cytometry after incubation with platelets from a healthy donor. It was found that already at baseline, prior to transfusion, plasma derived from critically ill patients induced more spontaneous platelet aggregation compared to healthy controls. Following RBC transfusion, the increased spontaneous platelet aggregation response *ex vivo* was further augmented. On the other hand, the vWF-mediated platelet agglutination *ex vivo* decreased after incubation of plasma from the critically ill compared to controls and was further reduced after RBC transfusion. This indicates that RBC transfusion may indirectly affect platelet function in critically ill patients. Whether these findings represent mechanisms underlying the association between RBC transfusions and thromboembolic events remains to be determined.

The condition of the recipient may modulate the effect of a RBC transfusion on patient outcome, including the risk of developing a nosocomial infection. Previous

studies have suggested that transfusion is associated with increased risks of infection, a phenomenon that is part of an entity called transfusion related immunomodulation (TRIM). This may occur in particular with products that have been stored for a longer time. However, observational clinical studies that suggested this association may be hampered by bias [6]. LPS tolerance, defined as a blunted inflammatory response of whole blood to *ex vivo* stimulation with toll like receptor (TLR) ligands, could be a mechanism that leads to an increased risk of infections after RBC transfusion. Therefore, we examined in **Chapter 8** whether transfusion of a maximum stored autologous RBC transfusion results in LPS tolerance compared to a fresh autologous RBC transfusion. In this study, the storage time of RBCs did not influence the cytokine response by white blood cells in whole blood after stimulation with LPS or LTA *ex vivo*. This suggests that storage time does not affect the immunosuppression after RBC transfusion.

DISCUSSION OF THIS THESIS

Does transfusion related immunomodulation (TRIM) exist?

The results of this thesis suggest that RBC transfusion has an impact on the host immune response. A TRIM effect has been described in many experimental studies published previously. We extend these data with clinical studies. We found that RBC transfusion is associated with a pro-inflammatory response of monocytes and neutrophils. It is very difficult to demonstrate causality in a non-randomized setting. In our observational studies, we were not able to prove a causality, certainly not in an ICU setting where many confounders can play a role, including disease severity, the underlying disease and treatment strategies. However, the results of this thesis underline the existence of TRIM for the following reasons. First, the results found in a heterogeneous cohort of critically ill patients corresponded more or less to results found in the endotoxemia model, which is a controlled reproducible model without confounders. In addition, in the studies of critically ill patients, mediators or RBC effects were compared pre- and post-RBC transfusion, limiting the amount of variables that could have influenced the results. Finally, the findings are consistent with earlier experimental work, as well as with a previous clinical study showing that RBC transfusion is associated with a decrease in pro-inflammatory cytokines after transfusion [7].

In this discussion, the 4 questions asked are elaborated upon, with an outlook on the future.

1) Which mediators in the RBC transfusion bag affect the host immune response after transfusion?

Washing the product reduces the organ injury in a rat transfusion model [8], suggesting a role for mediators in the supernatant. EVs from all kinds of cells are able to induce a host response [9]. Earlier work showed that *in vitro*, EVs from stored RBCs induced a strong dose-dependent inflammatory host response [10]. In line with that, we showed that EVs from RBCs can induce upregulation of adhesion molecules on endothelial cells and can be procoagulant by secretion of vWF. This can lead to adhesion of endogenous circulating cells, as well donor RBC to the vessel after transfusion, impairing the microcirculation and resulting in organ damage. Therefore, we think that EVs from RBCs are involved in the host response after transfusion. Although it has yet to be demonstrated in a clinical setting, an animal study has shown that donor RBCs adhere to the endothelium of the microcirculation [11].

Increased iron levels after RBC transfusions have been associated with pro-inflammatory effects in experimental studies [12, 13]. Whether iron is involved in the host response is not clear from our studies. In our study of critically ill patients, a single RBC transfusion resulted in a transient increase of the iron level. This is in line with earlier published studies of healthy volunteers [14, 15]. Also, RBC transfusion did not increase IL-6 levels. Therefore, we do not expect a major role for iron in the host response following transfusion.

(2) What effect does RBC transfusion have on different immune cells?

Several immune cells have been said to be affected by transfusion. Donor RBC-derived EVs have been found to interact with monocytes *in vitro*. Following incubation with EVs from RBCs, monocytes secrete pro-inflammatory cytokines and chemokines [16]. This corresponds with our data, which show that EVs from RBCs were phagocytized by monocytes and induce upregulation of adhesion molecules on endothelial cells only when monocytes were added. Besides monocytes, we also showed that RBC transfusion may impact neutrophil function. This corresponds to TRALI, where neutrophils interact with the endothelium, mediating the endothelial damage in the lung [5]. Therefore, the response of monocytes as well as neutrophils may be modified by RBC transfusion. It is not entirely clear what the clinical consequences of these effects are. Possibly, RBC transfusion activates immune cells, which may result in inflammation and ultimately in organ damage. This should be

further investigated. In addition, it is important to find out which factors in the transfusion product mediates activation of immune cells.

Besides immune cells, RBC transfusion may also have an effect on platelets. Platelets can aggregate after activation and interact with neutrophils [17]. Platelet-neutrophil interactions are associated with increased vascular permeability and the occurrence of organ injury, in particular lung injury [18]. In accordance, observational studies have shown an association between antiplatelet drugs and reduced organ failure and mortality in the critically ill [19, 20]. Besides the interaction with neutrophils, activated platelets may aggregate and contribute to microthrombi. This thesis describes an increased spontaneous platelet aggregation in critically ill patients after RBC transfusion in an *ex vivo* study design. An altered platelet function after RBC transfusion is also found in healthy volunteers [21] and in patients with acute coronary syndrome [22]. Therefore, it can be speculated that RBC transfusion augments platelet activation and aggregation, which may lead to microthrombi formation. Microthrombi may damage the endothelium and clot the microcirculation, which may lead to organ failure. Whether this is the case, must be investigated. Future studies should also focus on which factors mediate the altered platelet function.

(3) What is the role of the underlying immune status in the recipient on the host response after transfusion?

The underlying inflammatory status plays a role in the immune effects of RBC transfusion. Plasma from septic patient induced more neutrophil adhesion after RBC transfusion compared to non-septic patients. With regard to the effect of RBC transfusion on platelets, there were no differences between septic and non-septic patients. However, critically ill patients already induced more platelet aggregation at baseline compared to plasma from healthy controls. Following transfusion, the ability of critically ill patients to induce platelet aggregation *ex vivo* increased even further. We assume it is possible that ICU patients run a higher risk for RBC induced host response, due to an already activated immune status. This is in line with the fact that critically ill patients are at increased risk for developing TRALI [2].

(4) What is the effect of storage time of the RBC unit on the immune response after transfusion?

In the endotoxemia model, no effect of storage time was found on inflammation and coagulations markers. In this standardized model, autologous RBCs were used, which is not in line with general practice. However, confounders are excluded and the maximum of storage time was used. Therefore, it can be concluded that soluble

autologous factors that accumulate in the product during storage do not cause harm. As a critical note, it is possible that a more pronounced underlying inflammation is necessary to find a difference, since LPS-induced endotoxemia is not the same as sepsis. In critically ill patients, it was remarkable that after transfusion of fresh blood products, the neutrophil ROS production was increased compared to transfusion of blood that had been stored longer. However, several randomized controlled trials showed that the use of fresh RBC is not inferior to the outcome compared to standard issued RBCs [23-25]. Whether transfusion of fresh RBCs are related to an adverse outcome, such as a more pronounced immune response, than longer stored RBCs is an angle that not has been taken often. Storage time had no effect in sub-studies of the larger trials that specifically looked at the inflammation and coagulation parameters after transfusion [7, 26, 27]. However, studies on the immune response after transfusion usually have a different primary endpoint, so these studies could be underpowered.

IMPLICATIONS OF FINDINGS AND FUTURE OUTLOOK

1. Optimize the practice

Transfusion practice. It is important that physicians realize that RBC transfusions can be accompanied by an exaggerated host immune response, with adverse effects for the recipient. Earlier studies showed that liberal transfusions in critically ill patients resulted in an increased morbidity and mortality compared to restrictive transfusions [28, 29]. The finding that RBC transfusions can be harmful has resulted in a change in transfusion policy of the ICU. A restrictive transfusion trigger with a hemoglobin level of 7 g/dL has been widely adopted in the clinical practice. Even though the decision to give a transfusion must not only be based on the hemoglobin level, but also the clinical condition of the recipient, some physicians still give transfusions at hemoglobin levels of 10 g/dL. In addition, the transfusion practice between individual physicians [30, 31] and different institutes [32] also varies. Increasing the awareness of physicians of the adverse effects of RBC transfusions may lead to less unnecessary transfusions, resulting in lower costs and less adverse effects for the patients. Note however, that not all critically ill patients benefit from a restrictive transfusion policy. In patients with cardiovascular diseases [33] and oncologic patients it was found that a restrictive transfusion policy is associated with higher mortality [34, 35] or increased risk of acute coronary syndrome [33].

Specify patient populations at risk. Critically ill patients may have an increased risk for the adverse effects of RBC transfusion. It has not yet been established in this

heterogeneous group whether some patients are more at risk than others. In this thesis, after transfusion, the increase in adhesion of neutrophils *ex vivo* was more present in septic patients than in non-septic patients. Although we found no difference in the effect of transfusion on platelet activity between the two groups, this cannot be excluded due to possible underpowering. Therefore, patients with a strong inflammatory response may be more susceptible to adverse effects of RBC transfusion, which may alter the risk-benefit of RBC transfusions. In line with this, it has been shown that sepsis can induce red cell dysfunction (termed SIRD), which impairs oxygen delivery of RBCs to tissues [36]. Thus, it can be speculated that septic patients may benefit from a more restrictive policy. It would be interesting to investigate which populations are more at risk of developing an altered immune host response after RBC transfusion, because the response may be related to organ failure. Given that multiple organ failure is an important complication in critically ill patients [37], it is important to gain more insight into how to protect certain patients against the adverse effects of transfusion. Whether the transfusion policy can be adjusted by lowering the transfusion trigger in case of inflammation or anti-inflammatory pre-treatment, which may also be accompanied by side effects, needs to be investigated further.

2. Optimize the RBC products

Washing. Given that soluble mediators in the supernatant of RBCs, like EVs, may be involved in the host response after transfusion, it could be beneficial to remove these mediators by washing the RBC unit directly prior to transfusion. It has been demonstrated *in vitro* that after washing the RBC unit, the amount of EVs decreases compared to standard unwashed RBCs [38, 39]. Note however, that the level of cell free hemoglobin was higher in the supernatant of the washed RBCs [38, 40], suggesting that the washing process may result in hemolytic injury of the RBCs [39]. The possible damage may depend on the washing device used [39]. However, the changes in the amount of EVs and free hemoglobin were not found in the plasma of the recipient after transfusion [38], so the question remains whether these changes in EVs and free hemoglobin are clinically relevant. Another *in vitro* study showed that washing RBCs abrogated the effect of RBCs on neutrophil priming [41]. In animal models it is already shown that washing the RBCs attenuates inflammation and organ injury after transfusion [42, 43]. Most clinical research about washing RBCs is carried out in cardiac surgery patients, probably because washing devices are already used during surgery for autologous RBCs [44]. In a randomized controlled trial in pediatric cardiac surgery patients, washing RBCs resulted in a reduction of inflammation and a non-significant trend in reduced mortality [45]. Contradictory,

in a Cochrane review, no differences in length of stay at the hospital, mechanical ventilation and mortality was found between preterm infants who received washed RBCs compared to unwashed RBCs [45]. However the level of evidence is low, because this review is based on a small single study. In a randomized controlled trial with adult cardiac surgery patients, transfusion of washed RBCs decreased the mortality compared to patients who received unwashed RBCs [46]. However, in the plasma of adult patients who underwent cardiac surgery, no differences in platelet, leukocytes or endothelial activation, parameters of iron metabolism or oxidative stress are found in recipients who received washed RBCs compared to the recipients who received unwashed RBCs [38]. Several studies in different patient populations about the role of washed RBCs related to outcome after transfusion are underway and will provide more insight into the clinical effects of washing [47]. Even though washing RBCs may be beneficial and no clinical adverse effects of washing are reported, it is also time consuming and expensive. Additional research in critically ill patients is necessary before adjustments can be made in practice. In the future, more attention should be paid to the relationship between storage and processing of the RBCs and the immune response following transfusion.

Of note, besides washing, different storage solutions (i.e. additive solutions) and processing methods to produce a RBC unit are used worldwide, whereby the interaction and activation of the immune system after transfusion could be different.

Additive solutions. Storage solutions can influence the function and survival (e.g. hemolysis, shedding of EVs and adhesion to endothelium) of stored RBCs differently [48-51]. *In vitro*, the amount of EVs is lower in leukoreduced RBCs stored in additive solutions PAGGSM and Erythrosol-4 compared to SAGM, the standard additive solution in Europe [49, 50]. Also compared to AS-1, the standard additive solution used in the USA, RBCs stored in SAG-M became more adherent and shed more EVs *in vitro* [48, 51]. However, besides the additive solution there are also differences between the USA and Europe in preparation methods [52], so this study is not entirely translatable. More importantly, whether this also has clinical consequences needs further investigation in a clinical setting.

Processing methods. The quality of the product depends on the processing methods, as the processing methods may have an effect on changes of the RBC membrane. *In vitro*, an increased level of EVs is found in the RBC unit after whole blood filtration [52] compared to buffy coat filtration [53]. Also, the level of EVs and DNA are higher in non-leukoreduced RBCs compared to leukoreduced RBCs *in vitro* [54]. Besides the

amount of EVs, the processing methods may also affect the characteristics of the EVs [54, 55]. The processing methods can also influence the number of residual leukocytes after leukoreduction, which can lead to accumulation of pro-inflammatory cytokines. Processing the RBCs within 8 hours results in less residual leukocytes compared to processing the RBCs after 24 hours *in vitro* [52]. Also *in vitro*, supernatants from whole blood filtrated RBCs have a pro-inflammatory effect on monocytes, an effect that was not seen with red cell filtered RBCs [55]. This may also have clinical consequences, because in a retrospective cohort study in-hospital patients who received a RBC transfusion, transfusion with fresh whole blood filtrated RBCs was associated with mortality, which was not found after transfusion of red cell filtered units [56]. It must be further investigated whether the processing methods also have a potential effect on outcome in critically ill patients.

Donor characteristics. More attention should also be paid to donor specific factors (sex, lifestyle, age) that may contribute to the storage quality [57, 58]. In a retrospective observational study, transfusion of RBCs from younger and female donors or products with antigens were associated with increased mortality [59, 60]. We think it is important to identify the donor characteristics that influence the quality of the RBC units and may have an impact on the host response and then to take this into account when selecting the donors.

Storage time. In transfusion medicine, the question whether storage time is related to adverse outcome has led to a surge of research. The results of these studies are not unambiguous, even though the large randomized studies showed no differences in outcome related to storage time [23-25]. Of note, these studies compared fresh RBCs (< eight days) with standard issued RBCs (median of twenty-one days), so not the maximum storage time. These studies hardly take into account the differences in the product due to additive solutions, processing methods and donor characteristics, which may have confounded the results. Another reason to investigate the effect of storage time further, is that there are signals that fresh RBCs may be associated with adverse outcome. Supernatant from RBCs filtered from fresh whole blood are associated with an augmented inflammatory response, measured by IL-8 production by unstimulated monocytes *in vitro* [55]. A meta-analysis of clinical trials also found that transfusion of fresh RBCs may be related to adverse effects, with an increased mortality in patients who received fresher RBCs compared to those who received older RBCs [61]. In this thesis, we found that the plasma from critically ill patients who received fresh RBCs induced more ROS production by neutrophils than the plasma of recipients who received standard issued RBCs. Therefore, it is

possible that fresh RBCs may have a more pronounced effect on the host response after transfusion, possibly via activation of monocytes or neutrophils, which should be investigated further. Then. it is especially interesting to find out what factor in these fresh products triggers the host response following transfusion.

IN CONCLUSION

In this thesis we investigated the effect of RBC transfusion on the host immune response. We conclude that RBC transfusions in critically ill patients are associated with an inflammatory and procoagulant host response. Activated neutrophils, monocytes, platelets and endothelium cells are likely to be involved in this complex response, which seems stronger when an inflammatory status is present in the recipient. We hypothesize that RBC-derived EVs are potent mediators of the association between RBC transfusion and the host response. There are multiple opportunities for further improvement of clinical practice as well as of the RBC product, with the aim to reduce immune modulating effects of RBC transfusion.

REFERENCES

1. Gajic, O., et al., *Transfusion-related acute lung injury in the critically ill: prospective nested case-control study.* Am J Respir Crit Care Med, 2007. **176**(9): p. 886-91.
2. Vlaar, A.P. and N.P. Juffermans, *Transfusion-related acute lung injury: a clinical review.* Lancet, 2013. **382**(9896): p. 984-94.
3. Spinella, P.C., et al., *Properties of stored red blood cells: understanding immune and vascular reactivity.* Transfusion, 2011. **51**(4): p. 894-900.
4. Thomas, G.M., et al., *Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice.* Blood, 2012. **119**(26): p. 6335-43.
5. Shaz, B.H., S.R. Stowell, and C.D. Hillyer, *Transfusion-related acute lung injury: from bedside to bench and back.* Blood, 2011. **117**(5): p. 1463-71.
6. van de Watering, L. and C. Biomedical Excellence for Safer Transfusion, *Pitfalls in the current published observational literature on the effects of red blood cell storage.* Transfusion, 2011. **51**(8): p. 1847-54.
7. Norris, P.J., et al., *Influence of blood storage age on immune and coagulation parameters in critically ill transfused patients.* Transfusion, 2019. **59**(4): p. 1223-1232.
8. Vlaar, A.P., et al., *Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model.* Anesthesiology, 2010. **113**(1): p. 92-103.
9. Hezel, M.E.V., et al., *The Ability of Extracellular Vesicles to Induce a Pro-Inflammatory Host Response.* Int J Mol Sci, 2017. **18**(6).
10. Straat, M., et al., *Extracellular Vesicles from Red Blood Cell Products Induce a Strong Pro-Inflammatory Host Response, Dependent on Both Numbers and Storage Duration.* Transfus Med Hemother, 2016. **43**(4): p. 302-305.
11. Chin-Yee, I.H., et al., *Transfusion of stored red blood cells adhere in the rat microvasculature.* Transfusion, 2009. **49**(11): p. 2304-10.
12. Hod, E.A. and S.L. Spitalnik, *Stored red blood cell transfusions: Iron, inflammation, immunity, and infection.* Transfus Clin Biol, 2012. **19**(3): p. 84-9.
13. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
14. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
15. Peters, A.L., et al., *Transfusion of 35-day stored red blood cells does not result in increase of plasma non-transferrin bound iron in human endotoxemia.* Transfusion, 2017. **57**(1): p. 53-59.
16. Danesh, A., et al., *Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses in vitro.* Blood, 2014. **123**(5): p. 687-96.
17. Lisman, T., *Platelet-neutrophil interactions as drivers of inflammatory and thrombotic disease.* Cell Tissue Res, 2018. **371**(3): p. 567-576.

18. Zarbock, A. and K. Ley, *The role of platelets in acute lung injury (ALI).* Front Biosci (Landmark Ed), 2009. **14**: p. 150-8.
19. Erlich, J.M., et al., *Prehospitalization antiplatelet therapy is associated with a reduced incidence of acute lung injury: a population-based cohort study.* Chest, 2011. **139**(2): p. 289-295.
20. Kor, D.J., et al., *Association of prehospitalization aspirin therapy and acute lung injury: results of a multicenter international observational study of at-risk patients.* Crit Care Med, 2011. **39**(11): p. 2393-400.
21. Silvain, J., et al., *Impact of red blood cell transfusion on platelet activation and aggregation in healthy volunteers: results of the TRANSFUSION study.* Eur Heart J, 2010. **31**(22): p. 2816-21.
22. Silvain, J., et al., *Impact of red blood cell transfusion on platelet aggregation and inflammatory response in anemic coronary and noncoronary patients: the TRANSFUSION-2 study (impact of transfusion of red blood cell on platelet activation and aggregation studied with flow cytometry use and light transmission aggregometry).* J Am Coll Cardiol, 2014. **63**(13): p. 1289-1296.
23. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
24. Steiner, M.E., et al., *Effects of red-cell storage duration on patients undergoing cardiac surgery.* N Engl J Med, 2015. **372**(15): p. 1419-29.
25. Fergusson, D.A., et al., *Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial.* JAMA, 2012. **308**(14): p. 1443-51.
26. Spinella, P.C., et al., *Effects of blood storage age on immune, coagulation, and nitric oxide parameters in transfused patients undergoing cardiac surgery.* Transfusion, 2019. **59**(4): p. 1209-1222.
27. Kor, D.J., et al., *Fresh red blood cell transfusion and short-term pulmonary, immunologic, and coagulation status: a randomized clinical trial.* Am J Respir Crit Care Med, 2012. **185**(8): p. 842-50.
28. Hebert, P.C., et al., *A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group.* N Engl J Med, 1999. **340**(6): p. 409-17.
29. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
30. Edwards, J., et al., *Patient blood transfusion management: discharge hemoglobin level as a surrogate marker for red blood cell utilization appropriateness.* Transfusion, 2012. **52**(11): p. 2445-51.
31. Frank, S.M., et al., *Variability in blood and blood component utilization as assessed by an anesthesia information management system.* Anesthesiology, 2012. **117**(1): p. 99-106.
32. Bennett-Guerrero, E., et al., *Variation in use of blood transfusion in coronary artery bypass graft surgery.* JAMA, 2010. **304**(14): p. 1568-75.
33. Docherty, A.B. and T.S. Walsh, *Anemia and blood transfusion in the critically ill patient with cardiovascular disease.* Crit Care, 2017. **21**(1): p. 61.

34. Patel, N.N., et al., *Indications for red blood cell transfusion in cardiac surgery: a systematic review and meta-analysis.* Lancet Haematol, 2015. **2**(12): p. e543-53.
35. Fominskiy, E., et al., *Liberal transfusion strategy improves survival in perioperative but not in critically ill patients. A meta-analysis of randomised trials.* Br J Anaesth, 2015. **115**(4): p. 511-9.
36. Xue Lin, S.R., David Timm, d'Alessandro Angelo, Prakash Jaya, Ernst Melanie, Julie Prior, Shah Shivam, Jackie Wu, Julie Reisz, and Allan Doctor, *Sepsis Induced Red Cell Dysfunction (SiRD): Physiology and Mechanisms.* Blood, 2017.
37. Afessa, B., et al., *Systemic inflammatory response syndrome, organ failure, and outcome in critically ill obstetric patients treated in an ICU.* Chest, 2001. **120**(4): p. 1271-7.
38. Wozniak, M.J., et al., *Randomized trial of red cell washing for the prevention of transfusion-associated organ injury in cardiac surgery.* Br J Anaesth, 2017. **118**(5): p. 689-698.
39. Bennett-Guerrero, E., et al., *Randomized study of washing 40- to 42-day-stored red blood cells.* Transfusion, 2014. **54**(10): p. 2544-52.
40. Harm, S.K., et al., *Haemolysis and sublethal injury of RBCs after routine blood bank manipulations.* Transfus Med, 2012. **22**(3): p. 181-5.
41. Biffl, W.L., et al., *Plasma from aged stored red blood cells delays neutrophil apoptosis and primes for cytotoxicity: abrogation by poststorage washing but not prestorage leukoreduction.* J Trauma, 2001. **50**(3): p. 426-31; discussion 432.
42. Cortes-Puch, I., et al., *Washing older blood units before transfusion reduces plasma iron and improves outcomes in experimental canine pneumonia.* Blood, 2014. **123**(9): p. 1403-11.
43. Baek, J.H., et al., *Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy.* J Clin Invest, 2012. **122**(4): p. 1444-58.
44. Murphy, G.J., et al., *Trial protocol for a randomised controlled trial of red cell washing for the attenuation of transfusion-associated organ injury in cardiac surgery: the REDWASH trial.* Open Heart, 2016. **3**(1): p. e000344.
45. Cholette, J.M., et al., *Washing red blood cells and platelets transfused in cardiac surgery reduces postoperative inflammation and number of transfusions: results of a prospective, randomized, controlled clinical trial.* Pediatr Crit Care Med, 2012. **13**(3): p. 290-9.
46. Jy, W., et al., *Transfusion with Washed vs. Unwashed Packed Red Cells in Coronary Artery Bypass Graft (CABG) Surgery: Major Outcome Differences.* Blood, 2014. **124**(21): p. 2887-2887.
47. Schmidt, A.E., Majed A Refaai, Scott A. Kirkley and Neil Blumberg, *Proven and potential clinical benefits of washing red blood cells before transfusion: current perspectives.* International Journal of Clinical Transfusion Medicine, 2016.
48. Sparrow, R.L., et al., *In vitro measures of membrane changes reveal differences between red blood cells stored in saline-adenine-glucose-mannitol and AS-1 additive solutions: a paired study.* Transfusion, 2014. **54**(3): p. 560-8.

49. Veale, M.F., G. Healey, and R.L. Sparrow, *Effect of additive solutions on red blood cell (RBC) membrane properties of stored RBCs prepared from whole blood held for 24 hours at room temperature.* Transfusion, 2011. **51 Suppl 1**: p. 25S-33S.
50. Zehnder, L., et al., *Erythrocyte storage in hypertonic (SAGM) or isotonic (PAGGSM) conservation medium: influence on cell properties.* Vox Sang, 2008. **95**(4): p. 280-7.
51. Hess, J.R., et al., *Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions.* Transfusion, 2009. **49**(12): p. 2599-603.
52. Radwanski, K., et al., *The effects of red blood cell preparation method on in vitro markers of red blood cell aging and inflammatory response.* Transfusion, 2013. **53**(12): p. 3128-38.
53. Bicalho, B., A.S. Pereira, and J.P. Acker, *Buffy coat (top/bottom)- and whole-blood filtration (top/top)-produced red cell concentrates differ in size of extracellular vesicles.* Vox Sang, 2015. **109**(3): p. 214-20.
54. Bakkour, S., et al., *Manufacturing method affects mitochondrial DNA release and extracellular vesicle composition in stored red blood cells.* Vox Sang, 2016. **111**(1): p. 22-32.
55. Almizraq, R.J., et al., *Blood manufacturing methods affect red blood cell product characteristics and immunomodulatory activity.* Blood Adv, 2018. **2**(18): p. 2296-2306.
56. Heddle, N.M., et al., *Red blood cell processing methods and in-hospital mortality: a transfusion registry cohort study.* Lancet Haematol, 2016. **3**(5): p. e246-54.
57. Tzounakas, V.L., et al., *Donor variation effect on red blood cell storage lesion: a multivariable, yet consistent, story.* Transfusion, 2016. **56**(6): p. 1274-86.
58. Tzounakas, V.L., et al., *Donor-variation effect on red blood cell storage lesion: A close relationship emerges.* Proteomics Clin Appl, 2016. **10**(8): p. 791-804.
59. Chasse, M., et al., *Association of Blood Donor Age and Sex With Recipient Survival After Red Blood Cell Transfusion.* JAMA Intern Med, 2016. **176**(9): p. 1307-14.
60. Chasse, M., et al., *Effect of Blood Donor Characteristics on Transfusion Outcomes: A Systematic Review and Meta-Analysis.* Transfus Med Rev, 2016. **30**(2): p. 69-80.
61. Chai-Adisaksopha, C., et al., *Mortality outcomes in patients transfused with fresher versus older red blood cells: a meta-analysis.* Vox Sang, 2017. **112**(3): p. 268-278.

CHAPTER 10

Nederlandse samenvatting en discussie
List of publications
PhD portfolio
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Acknowledgement

SAMENVATTING EN DISCUSSIE

In dit proefschrift zijn de effecten onderzocht van een rode bloedcel(RBC)-transfusie op de immuunrespons van ernstig zieke patiënten.

De specifieke onderzoeksvragen waren:

(1) Welke mediatoren in de RBC-transfusiezak hebben invloed op de immuunreactie in de ontvanger na transfusie?
(2) Welk effect heeft een RBC-transfusie op verschillende immuuncellen in de ontvanger?
(3) Wat is de rol van de onderliggende immuunstatus van de ontvanger op de immuunreactie, na een RBC-transfusie?
(4) Wat is het effect van de opslagduur van de RBC-transfusiezak op de immuunreactie, na een RBC-transfusie?

Hoofdstuk 2 bevat een samenvatting van de bestaande literatuur over het vermogen van extracellulaire vesikels (EV's), om een immuunreactie te induceren bij de gastheer. Deze review beschrijft dat allerlei soorten cellen in staat zijn om te vesiculeren. Deze EV's kunnen een groot aantal pro- en anti-inflammatoire reacties induceren, echter lijken de pro- inflammatoire effecten de overhand te nemen. Daarmee veronderstelden we dat EV's een rol kunnen spelen bij een immuunreactie in de gastheer na een RBC-transfusie.

Hoofdstuk 3 bevat een samenvatting van de bestaande literatuur over niet-antilichaam gemedieerde transfusie-gerelateerde acute longschade (TRALI). Deze review concentreerde zich op de oplosbare mediatoren en de morfologische en functionele veranderingen van de donor rode bloedcellen die ontstaan tijdens opslag. In diermodellen kunnen mediatoren, zoals bioactieve lipiden, CD40 ligand en EV's TRALI induceren, wat suggereert dat deze mediatoren betrokken zijn in het ontstaan van TRALI. Ook werd in deze modellen aangetoond dat een onderliggende ontsteking in de ontvanger predisponeert tot het ontstaan van TRALI. Dit komt overeen met het feit dat ernstig zieke patiënten een verhoogd risico hebben om TRALI te ontwikkelen na een RBC-transfusie, omdat deze patiënten vaak een onderliggende inflammatoire aandoening hebben [1, 2]. Wat betreft de invloed van de opslagduur van de rode bloedcellen op het ontstaan van TRALI, lijkt het er in preklinische studies op dat een langere opslagduur het risico op TRALI verhoogt. Echter is deze bevinding in tegenspraak met klinische onderzoeken, die geen verschillen

laten zien in het optreden van long-gerelateerde complicaties na transfusie met verse vergeleken met langer bewaarde rode bloedcellen.

De rol van EV's bij het ontstaan van de nadelige effecten van transfusie werd verder onderzocht in **Hoofdstuk 4.** Onze hypothese was dat EV's uit RBC-transfusieproducten endotheelcellen kunnen activeren door activatie van immuuncellen van de ontvanger. Incubatie van endotheelcellen met EV's uit de transfusiezak resulteerde in verhoogde expressie van adhesiemoleculen *in vitro*, dit gebeurde alleen in de aanwezigheid van monocyten. Deze opregulatie werd niet gevonden in de aanwezigheid van neutrofielen. Bovendien worden EV's uit de transfusiezak, gedeeltelijk via complement 3, gefagocyteerd door monocyten. Verder induceerden de EV's uit de transfusiezak, zonder tussenkomst van immuuncellen, de endotheliale secretie van von Willebrandfactor (vWF). De opslagduur van de transfusieproducten waaruit de EV's werden geïsoleerd, speelde geen rol in deze studie. Concluderend, EV's uit een RBC-transfusiezak kunnen opregulatie van adhesiemoleculen op de endotheelcellen induceren. Dit kan relevant zijn, aangezien de adhesie van rode bloedcellen of andere cellen aan de microvasculatuur een mechanisme kan zijn dat betrokken is bij de associatie tussen RBC-transfusie en orgaanfalen [3]. Of dit een mechanisme is van transfusie-gemedieerd orgaanfalen moet verder onderzocht worden in klinische onderzoeken.

Naast EV's kan ijzer een rol spelen als mediator in de immuunreactie van de ontvanger na transfusie. RBC-transfusie kan resulteren in ijzerbelasting, wat zowel geassocieerd is met een verhoogde kans op nosocomiale infecties als met een verhoogde kans op orgaanfalen, als gevolg van oxidatieve stress. In **Hoofdstuk 5** werd het effect van RBC-transfusie op het ijzermetabolisme onderzocht. In een prospectieve cohortstudie met ernstig zieke patiënten werd gevonden dat transfusie van één RBC-eenheid het serum ijzer tijdelijk verhoogt, maar niet de verzadiging van transferrine, wat suggereert dat een RBC-transfusie de ijzerbeschikbaarheid voor de erytropoëse niet verhoogt. Bovendien daalde het serum ijzer vierentwintig uur na de transfusie terug naar het niveau van voor de transfusie. RBC-transfusie verhoogde ook de hepcidine-niveaus. Het hepcidine-niveau kan toenemen als gevolg van een inflammatoire stimulus, maar in deze studie was de IL-6 verlaagd na transfusie. Daarom is de toename van hepcidine waarschijnlijk het gevolg van het verhoogde serum-ijzer na de klaring van de donor rode bloedcellen. Er werd geen verband gevonden tussen de opslagduur van de donor rode bloedcellen en de veranderingen in de ijzervariabelen. Alles bij elkaar genomen: de pro- inflammatoire effecten van een RBC-transfusie bij ernstig zieke patiënten worden waarschijnlijk

niet gemedieerd door een toename in de ijzerbelasting, omdat de toename voorbijgaand is en de verzadiging van transferrine niet is toegenomen. Verder zou het kunnen dat het ijzermetabolisme niet per se gunstig wordt beïnvloed door een rode bloedceltransfusie.

Neutrofielen zijn mogelijk één van de gastheercellen die betrokken zijn bij de immuunreactie die na een RBC-transfusie kan ontstaan. In **Hoofdstuk 6** werd het effect van een RBC-transfusie op neutrofielen onderzocht, zowel in een endotoxinemie-model als in een cohort van ernstig zieke patiënten. Activatie van neutrofielen *ex vivo* werd beoordeeld door de reactive oxygen species (ROS)-productie en adhesie van neutrofielen van gezonde mensen te meten na incubatie van plasma van beide cohorten. In het endotoxinemie-model, waar vrijwilligers na LPS-toediening een autologe RBC-transfusie kregen, was het vermogen van plasma van de vrijwilligers om adhesie van neutrofielen te induceren verhoogd na een RBC-transfusie. Er werden geen verschillen gevonden in neutrofiel-activatie tussen vrijwilligers die een vers (twee dagen opgeslagen) rode bloedcelproduct toegediend kregen in vergelijking met vrijwilligers die een oud (vijfendertig dagen opgeslagen) product kregen.

Bij de ernstig zieke patiënten nam de adhesie van neutrofielen *ex vivo* toe na RBC-transfusie. De toename was meer uitgesproken in septische patiënten vergeleken met niet-septische patiënten, wat de suggestie wekt dat de ontstekingsstatus van de ontvanger een rol speelt in het induceren van neutrofiel-activatie na transfusie. Bij de ernstig zieke patiënten lijkt de opslagduur van de RBC-transfusiezak ook de neutrofielenfunctie te beïnvloeden; de ROS-productie van neutrofielen nam toe na incubatie met plasma dat verzameld is nadat een transfusie met verse rode bloedcellen was gegeven, maar niet na een transfusie met standaard bewaarde rode bloedcellen. Concluderend, RBC-transfusie induceert veranderingen in het plasma van de ontvanger die geassocieerd zijn met een verhoogde adhesie van neutrofielen in zowel een humaan endotoxinemie-model als bij ernstig zieke patiënten met sepsis. Daarom spelen geactiveerde neutrofielen mogelijk een rol bij het veroorzaken van een immuunreactie bij de gastheer na een RBC-transfusie.

Het is duidelijk vastgesteld dat een RBC-transfusie effect kan hebben op de neutrofielen [4, 5]. Er is echter veel minder bekend over de effecten van een RBC-transfusie op de bloedplaatjesfunctie. Omdat RBC-transfusies geassocieerd zijn met een verhoogd risico op trombo-embolische aandoeningen, hebben we verondersteld dat bloedplaatjes ook betrokken zijn bij de immuunreactie na transfusie. Ook in

dit hoofdstuk onderzochten we de hypothese dat de reactie van bloedplaatjes na transfusie gerelateerd is aan de onderliggende ontstekingsstatus van de ontvanger. In **Hoofdstuk 7** werd het effect van een RBC-transfusie op bloedplaatjesactivatie onderzocht in ernstig zieke patiënten. Het vermogen van plasma van ernstig zieke patiënten om aggregatie van bloedplaatjes *ex vivo* te induceren werd gemeten door middel van flow cytometrie na incubatie met bloedplaatjes van gezonde mensen. Nog voordat de transfusie gegeven werd, gaf het plasma van ernstig zieke patiënten al meer spontane plaatjesaggregatie dan plasma van gezonde controles. Na een RBC-transfusie nam deze spontane plaatjesaggregatie nog verder toe. Aan de andere kant, was de door vWF gemedieerde plaatjesaggregatie *ex vivo* juist verminderd na incubatie van plasma van ernstig zieke patiënten vergeleken met gezonde controles en nam verder af na transfusie. Dit geeft aan dat een RBC-transfusie de bloedplaatjesfunctie in ernstig zieke patiënten beïnvloedt. Of deze bevindingen een mogelijk onderliggend mechanisme zijn voor de transfusie geassocieerde thromboembolische aandoeningen zou verder onderzocht moeten worden.

De conditie van de ontvanger heeft mogelijk invloed op het ontstaan van nadelige effecten na een rode bloedcel transfusie, zoals ook op het risico op het ontstaan van nosocomiale infecties. Eerdere studies hebben laten zien dat rode bloedcel transfusies geassocieerd zijn met een verhoogd risico op infecties, een fenomeen dat deel uitmaakt van een immunologisch respons na transfusie, ook wel transfusie gemedieerde immunomodulatie (TRIM) genoemd. Dit lijkt vooral voor te komen bij langer opgeslagen transfusieproducten. Echter observationele studies die deze associatie laten zien, zijn mogelijk vertekend door bias [6]. Immuuntolerantie, gedefinieerd als een verminderde immuunreactie van afweercellen na een tweede blootstelling aan bacteriedeeltjes, zou een mechanisme kunnen zijn dat leidt tot een verhoogd risico op infecties na een RBC-transfusie. Daarom hebben we in **Hoofdstuk 8** onderzocht of transfusie van oude autologe rode bloedcellen (vijfendertig dagen opgeslagen) in een endotoxinemie-model resulteert in immuuntolerantie in vergelijking met een verse autologe rode bloedcellen (twee dagen opgeslagen) transfusie. In deze studie had de opslagduur van de rode bloedcellen geen invloed op de cytokine-respons van de afweercellen in volbloed *in vitro* na stimulatie met bacteriedeeltjes. Dit suggereert dat er geen effect van opslagduur is op de activiteit van de afweercellen na een transfusie met rode bloedcellen in een endotoxinemie-model.

DISCUSSIE

Bestaat transfusie gemedieerde immunomodulatie (TRIM) eigenlijk wel?

Resultaten van dit proefschrift suggereren dat een RBC-transfusie een impact heeft op de immuunreactie van de ontvanger. In eerder gepubliceerde experimentele onderzoeken zijn vele TRIM-effecten beschreven. Wij vullen deze onderzoeken aan met klinische data. We vonden dat een RBC-transfusie geassocieerd is met een pro- inflammatoire reactie van monocyten en neutrofielen in de ontvanger. Het is erg lastig om causaliteit aan te tonen in een niet- gerandomiseerde setting. In onze observationele onderzoeken zijn we niet in staat om causaliteit te bewijzen, zeker niet in een ICU-omgeving waar vele confounders een rol kunnen spelen, waaronder de onderliggende ziekte, de ernst van de ziekte en de invloed van behandelingen. De resultaten van dit proefschrift onderstrepen echter het bestaan van TRIM om de volgende redenen. Ten eerste kwamen de resultaten gevonden in een heterogeen cohort van ernstig zieke patiënten min of meer overeen met resultaten gevonden in het endotoxinemie-model met gezonde vrijwilligers, dat een gecontroleerd reproduceerbaar model is zonder confounders. Bovendien werd in het cohort met de ernstig zieke patiënten, de meting na de transfusie altijd vergeleken met een meting voordat de transfusie gegeven was, waardoor de resultaten zo min mogelijk werden beïnvloed door andere variabelen. Ten slotte zijn de bevindingen consistent met eerder experimenteel werk, evenals met een andere klinische studie die aantoont dat een RBC-transfusie geassocieerd is met een toename van de coagulatiefactoren en een afname van pro- inflammatoire cytokines na transfusie [7].

In deze discussie worden de vier gestelde vragen uitgewerkt, met een vooruitblik op de toekomst.

(1) Welke mediatoren in de RBC-transfusiezak hebben invloed op de immuunreactie in de ontvanger na transfusie?

In een rat-transfusiemodel resulteert wassen van het transfusieproduct in minder orgaanbeschadiging [8], wat suggereert dat mediatoren in het supernatant een rol spelen. EV's van allerlei soorten cellen zijn in staat om een reactie in de gastheer te induceren [9]. Eerder *in vitro*-werk toonde aan dat EV's uit een rode bloedcel transfusiezak een sterk dosisafhankelijke inflammatoir response kunnen induceren [10]. In overeenstemming met deze resultaten hebben we laten zien dat EV's uit een RBC-transfusiezak een verhoogde expressie van adhesie moleculen kunnen induceren op endotheelcellen en procoagulant kunnen zijn door secretie van vWF. Dit kan leiden tot het plakken van zowel lichaamseigen circulerende cellen als ook

van donor rode bloedcellen aan het bloedvat na een transfusie, waardoor de microcirculatie verstoord kan raken, met orgaanschade als gevolg. Daardoor denken wij dat EV's uit een rode bloedcel transfusiezak betrokken zijn bij een immuunreactie in de ontvanger na transfusie. Hoewel het nog aangetoond moet worden in een klinische omgeving, heeft een dierstudie aangetoond dat donor rode bloedcellen inderdaad hechten aan het endotheel van de microcirculatie [11].

Verhoogde ijzerniveaus na een RBC-transfusie zijn in experimentele onderzoeken in verband gebracht met pro-inflammatoire effecten [12, 13]. Of ijzer betrokken is in de immuunreactie van de ontvanger is niet duidelijk in onze studies. In onze studie met ernstig zieke patiënten resulteerde een RBC-transfusie in een tijdelijke verhoging van het ijzer. Dit komt overeen met eerder gepubliceerde studies met gezonde vrijwilligers [14, 15]. Ook leidde een RBC-transfusie niet tot een verhoging van IL-6. Daarom verwachten we dat ijzer geen belangrijke rol speelt tijdens de immuunreactie van de ontvanger na transfusie.

(2) Welk effect heeft een RBC-transfusie op verschillende immuuncellen in de ontvanger?

Van verschillende type immuuncellen wordt verondersteld dat ze betrokken zijn bij de reactie in de ontvanger na een transfusie. EV's uit een RBC-transfusiezak bleken *in vitro* interactie te hebben met monocyten. Na incubatie van EV's scheiden monocyten pro- inflammatoire cytokines en chemokines uit [16]. Dit komt overeen met onze data, die laten zien dat EV's uit een transfusiezak door monocyten gefagocyteerd worden en daarnaast een opregulatie van adhesiemoleculen op endotheelcellen kunnen induceren, alleen in de aanwezigheid van monocyten.

Naast op de monocytenfunctie hebben we ook aangetoond dat een rode bloedcel transfusie invloed kan hebben op de functie van neutrofielen. Dit komt overeen met TRALI, waar neutrofielen de interactie aan gaan met het endotheel, waardoor endotheelschade in de longen wordt veroorzaakt [5]. Dus zowel de status van monocyten als neutrofielen kan veranderen door een RBC-transfusie. Het is niet helemaal duidelijk hoe deze resultaten zich vertalen naar de kliniek. Het kan verondersteld worden dat immuuncellen geactiveerd worden door een RBC-transfusie en vervolgens kunnen zorgen voor endotheelschade met orgaanschade als gevolg. Dit zou verder onderzocht moeten worden. Daarnaast is het belangrijk om erachter te komen welke factoren in het transfusieproduct de activering van immuuncellen bewerkstelligen.

Naast op de immuuncellen, kan een RBC-transfusie ook invloed hebben op de bloedplaatjesfunctie. Bloedplaatjes kunnen, na activatie, aggregeren en interactie hebben met neutrofielen [17]. De bloedplaatjes- neutrofiel interactie is geassocieerd met een verhoogde vasculaire permeabiliteit en het ontstaan van orgaanfalen, in het bijzonder van de longen [18]. Daar aan toegevoegd, observationele studies laten een associatie zien tussen plaatjesremmers en verminderd orgaanfalen en mortaliteit in ernstig zieke patiënten [19, 20]. Naast de interactie met neutrofielen, kunnen geactiveerde bloedplaatjes ook aggregeren en bijdragen aan microthrombi. Dit proefschrift beschrijft een verhoogde spontane aggregatie van bloedplaatjes *ex vivo* na een RBC-transfusie bij ernstig zieke patiënten. Ook bij gezonde vrijwilligers [21] en in patiënten met acuut coronair syndroom [22] wordt een veranderde functie van de bloedplaatjes gevonden na een RBC-transfusie. Daarom kan gespeculeerd worden dat een RBC-transfusie de activatie en aggregatie van bloedplaatjes versterkt, wat kan leiden tot de vorming van microthrombi. Deze microthrombi vorming kan zorgen voor endotheelschade en stolling in de microcirculatie, wat kan leiden tot orgaanfalen. Of dit het geval is moet worden onderzocht. Ook moeten toekomstige studies zich richten op welke factoren in de RBC-transfusiezak deze afwijking in de bloedplaatjes functie veroorzaken.

(3) Wat is de rol van de onderliggende immuunstatus van de ontvanger op de immuunreactie na RBC-transfusie?

De onderliggende inflammatoire status speelt een rol bij de immunologische effecten van een RBC-transfusie. Plasma van septisch patiënten verhoogde de adhesie van neutrofielen meer na een RBC-transfusie dan het plasma van niet-septische patiënten. Met betrekking tot het effect van een RBC-transfusie op de bloedplaatjes waren er geen verschillen tussen septische en niet-septische patiënten. Plasma van ernstig zieke patiënten induceerde echter, nog voor de transfusie was gegeven, meer aggregatie *ex vivo* van bloedplaatjes dan plasma van gezonde controles. Na de RBC-transfusie nam de plaatjesaggregatie *ex vivo* zelfs nog verder toe in ernstig zieke patiënten. We nemen aan dat ernstig zieke patiënten mogelijk een verhoogd risico hebben op een immuunreactie na transfusie, vanwege een reeds geactiveerde immuunstatus. Dit komt overeen met het feit dat ernstig zieke patiënten een verhoogd risico hebben op het krijgen van TRALI [2].

(4) Wat is het effect van de opslagduur van de RBC-transfusiezak op de immuunreactie na transfusie?

In het endotoxinemie-model werd geen effect van opslagtijd gevonden op de ontstekings- en stollingsmarkers. In dit gestandaardiseerde model werden autologe

RBC-producten gebruikt, wat niet in lijn is met de algemene praktijk. Echter confounders zijn uitgesloten in dit model, waardoor het mogelijk was om het effect van de maximale opslagtijd van de rode bloedcellen op inflammatie te onderzoeken. Er zou geconcludeerd kunnen worden dat autologe mediatoren die zich in het product ophopen tijdens de opslag geen verhoogde inflammatie induceren. Echter het zou kunnen dat een meer uitgesproken onderliggende ontsteking noodzakelijk is om een effect te kunnen vinden van de opslagduur, omdat de door LPS-geïnduceerde endotoxinemie niet hetzelfde is als sepsis.

In het onderzoek met ernstig zieke patiënten hadden we de opmerkelijke bevinding dat na transfusie van verse bloedproducten de ROS-productie van neutrofielen was toegenomen, in vergelijking met de transfusie van langer bewaard bloed. Verschillende gerandomiseerde onderzoeken hebben echter aangetoond dat er geen verschil in uitkomst is na gebruik van verse rode bloedcellen in vergelijking met standaard bewaarde rode bloedcellen [23-25]. Of transfusie van verse rode bloedcellen gerelateerd is aan een nadelige uitkomst, zoals een meer uitgesproken immuunrespons, ten opzichte van langer opgeslagen rode bloedcellen, is een hypothese die niet vaak is verondersteld. De opslagduur van rode bloedcellen had geen effect in deelstudies van de grotere studies met ernstig zieke patiënten, die specifiek keken naar de ontsteking- en coagulatieparameters na transfusie [7, 26, 27]. Echter hadden deze studies meestal een andere primaire uitkomst dan de immuunreactie na transfusie en zouden dan ook underpowered kunnen zijn.

IMPLICATIES EN TOEKOMSTPERSPECTIEVEN

1. Optimaliseer de praktijk

Transfusiepraktijk. Het is belangrijk dat artsen zich realiseren dat een RBC-transfusie gepaard kan gaan met een overdreven immuunreactie, met nadelige effecten in de ontvanger als gevolg. Eerdere studies hebben aangetoond dat een liberaal transfusiebeleid in ernstig zieke patiënten kan resulteren in meer morbiditeit en mortaliteit in vergelijking met een restrictief transfusiebeleid [28, 29]. De bevinding dat een RBC-transfusie ook schadelijk kan zijn, heeft geresulteerd in wijzigingen van het transfusiebeleid op de intensive care. Een restrictieve transfusietrigger bij een hemoglobinelevel van 7 g/dl (gelijk aan 4.3 mmol/l) wordt in de klinische praktijk op grote schaal toegepast. Hoewel een beslissing om een transfusie te geven niet alleen gebaseerd moet zijn op het hemoglobinegehalte, maar ook op de klinische conditie van de ontvanger, geven sommige artsen nog steeds transfusies bij een hemoglobinelevel van 10 g/ dl en is er nog steeds veel variatie tussen zowel

individuele artsen [30, 31] als verschillende instituten [32]. Het vergroten van het bewustzijn van artsen over de nadelige effecten van een RBC-transfusie kan resulteren in minder onnodige transfusies. Dit kan leiden tot lagere kosten en minder nadelige effecten voor de patiënten. Wel is het belangrijk om ook bewust te zijn van het feit dat niet alle patiënten baat hebben bij een restrictief beleid. Bij patiënten met hart- en vaatziekten [33] en oncologische patiënten werd vastgesteld dat een restrictief transfusiebeleid geassocieerd is met een hogere mortaliteit [34, 35] of een verhoogd risico op een acuut coronair syndroom [33].

Specificeer patiëntenpopulaties die risico lopen. Ernstig zieke patiënten kunnen een verhoogd risico hebben op de nadelige effecten van een RBC-transfusie. Het is nog niet vastgesteld of binnen deze heterogene groep sommige patiënten meer risico lopen dan anderen. In dit proefschrift was de toename van de adhesie van de neutrofielen *ex vivo* meer aanwezig in septische patiënten dan in niet-septische patiënten. Hoewel we geen verschil vonden in het effect van transfusie op de bloedplaatjesactiviteit tussen septische en niet-septische patiënten, kan het niet worden uitgesloten vanwege mogelijke underpowering. Patiënten met een onderliggende ontsteking zijn mogelijk gevoeliger voor de nadelige effecten van een RBC-transfusie, wat de verhouding tussen de risico's en de voordelen van een RBC-transfusie veranderd. In het verlengde hiervan: sepsis kan rode cel dysfunctie induceren, waardoor de zuurstofafgifte aan de weefsels wordt bemoeilijkt [36]. Daarom kunnen we veronderstellen dat septische patiënten meer baat kunnen hebben bij een restrictief beleid. Het zou interessant zijn om te onderzoeken welke populaties meer risico lopen op een immuunreactie na een RBC-transfusie, omdat de immuunreactie gerelateerd kan zijn aan orgaanfalen. Aangezien multi-orgaanfalen een ernstige en veel voorkomende complicatie is bij ernstig zieke patiënten [37], is het belangrijk om meer inzicht te krijgen in hoe bepaalde patiënten beschermd kunnen worden tegen de nadelige effecten van transfusie. Mogelijk kan, in het geval van een onderliggende ontsteking, het transfusiebeleid worden aangepast door de transfusietrigger te verlagen of een ontstekingsremmend medicijn te geven. Dat zou echter ook gepaard kunnen gaan met bijwerkingen, dus dat zou verder onderzocht moeten worden.

2. Optimaliseer het RBC-product

Wassen van het product. Oplosbare mediatoren in het supernatant, zoals EV's van het rode bloedcelproduct, lijken betrokken te zijn bij een immuunreactie in de ontvanger. Daarom zou het nuttig kunnen zijn om deze mediatoren te verwijderen door het product te wassen voordat de transfusie gegeven wordt. *In vitro* is aangetoond dat na het wassen van het product het aantal EV's afneemt in vergelijking met producten

die niet gewassen zijn [38, 39]. Echter nam de hoeveelheid vrij hemoglobine in het supernatant van het product toe [38, 40], wat suggereert dat het wassen mogelijk gepaard gaat met hemolyse van de rode bloedcellen [39]. De mogelijke schade aan de rode bloedcellen is afhankelijk van het apparaat dat gebruikt wordt om de cellen te wassen [39]. De veranderingen in EV's en vrij hemoglobine die *in vitro* werden gevonden, werden echter niet terug gezien in het plasma van de ontvanger na transfusie [38], dus de vraag is of deze veranderingen klinisch relevant zijn. Een andere *in vitro* studie toonde aan dat het wassen van de rode bloedcellen het vermogen om neutrofielen te primen deed verminderen [41]. In diermodellen is al aangetoond dat het wassen van het RBC-product de ontsteking en orgaanschade na een transfusie vermindert [42, 43]. Klinische studies die kijken naar het effect van het wassen van de rode bloedcellen worden meestal gedaan in patiënten die een hartoperatie ondergaan, waarschijnlijk omdat de apparaten waar de cellen mee gewassen worden, al in gebruik zijn tijdens de operatie voor autologe rode bloedcellen [44]. In een gerandomiseerde studie met kinderen die een hartoperatie ondergingen resulteerde het wassen van de rode bloedcellen in een vermindering van de ontsteking en was er een niet-significante trend in afname van mortaliteit [45].

Daarentegen werd in een Conchrane review geen verschil gevonden in de opnameduur, mechanische beademing en mortaliteit tussen prematuren die gewassen rode bloedcellen ontvingen in vergelijking met ongewassen rode bloedcellen [45]. Het bewijsniveau van deze Cochrane review was echter laag, omdat deze beoordeling gebaseerd is op één studie van een beperkte omvang. In een gerandomiseerde studie met volwassen patiënten die hartchirurgie ondergingen, zorgde transfusie met gewassen rode bloedcellen voor een lagere mortaliteit vergeleken met patiënten die ongewassen rode bloedcellen kregen [46]. Echter in het plasma van volwassen hartchirurgie patiënten werden geen verschillen gevonden in de activatie van bloedplaatjes, leukocyten of endotheelcellen, in parameters van het ijzermetabolisme of in oxidatieve stress tussen ontvangers die gewassen rode bloedcellen kregen en ontvangers die ongewassen rode bloedcellen ontvingen [38]. Momenteel lopen er meerdere studies in verschillende patiëntenpopulaties die kijken naar het effect van gewassen rode bloedcellen op uitkomst na transfusie. Deze studies zullen meer informatie geven over de klinische relevantie van wassen [47]. Ook al lijkt het wassen van rode cellen gunstig te zijn voor de patiënt en zijn er geen klinische nadelige effecten op dit moment bekend, is het ook tijdrovend en duur. Aanvullend onderzoek in ernstig zieke patiënten is nodig voordat er aanpassingen kunnen volgen in de praktijk. In de toekomst zou meer aandacht moeten worden besteed

aan de relatie tussen de immuunreactie in de ontvanger na transfusie en de opslag en verwerking van rode bloedcellen.

Het is belangrijk om te realiseren dat, naast het wel of niet wassen van het rode bloedcel product, transfusiestudies door verschillen in bewaarvloeistoffen en verwerkingsmethodes van het rode bloedcelproduct wereldwijd beïnvloed kunnen worden. Dit maakt vergelijken lastig.

Bewaarvloeistoffen. Bewaarvloeistoffen hebben invloed op de functie en overleving (bijvoorbeeld de hemolyse, de secretie van EV's en de adhesie aan het endotheel) van opgeslagen rode bloedcellen [48-51]. In een transfusiezak waar rode bloedcellen zijn opgeslagen in PAGGSM en Erythrosol-4 is het aantal EV's lager dan in een zak met bewaarvloeistof SAG-M, wat de standaard bewaarvloeistof is in Europa [49, 50]. Ook vergeleken met AS-1, de standaard bewaarvloeistof in de Verenigde Staten, zijn rode bloedcellen die opgeslagen waren in SAG-M meer adherent en scheiden meer EV's uit *in vitro* [48, 51]. Echter zijn er naast de bewaarvloeistoffen ook andere verschillen in de bereiding van het transfusieproduct tussen de VS en Europa [52], waardoor deze studie moeilijk vertaalbaar is. Dat maakt de resultaten van deze studie moeilijk te generaliseren. En nog belangrijker, of dit ook klinische gevolgen heeft zou verder onderzocht moeten worden.

Verwerkingmethodes. De kwaliteit van het transfusieproduct hangt af van de verwerkingsmethoden, omdat de verwerkingsmethoden kunnen zorgen voor veranderingen van het membraan van de rode bloedcellen. In een transfusiezak is het aantal EV's hoger na volbloed-filtratie [52] vergeleken met na *buffy coat*-filtratie [53]. Ook is het aantal EV's en DNA hoger in een RBC-product waar de leukocyten niet uit gefilterd zijn, vergeleken met een product na leukocytenfiltratie [54]. Naast de hoeveelheid EV's, kan de verwerkingsmethode ook invloed hebben op de karakteristieken van de EV's [54, 55]. De verwerkingsmethode kan ook invloed hebben op het aantal resterende leukocyten na leukoreductie. Dit kan leiden tot ophoping van pro- inflammatoire cytokines. Wanneer de rode bloedcellen binnen acht uur worden verwerkt, leidt dit tot minder resterende leukocyten dan wanneer het product na vierentwintig uur wordt verwerkt [52]. Ook het supernatant van volbloed-gefilterde rode bloedcellen heeft een pro- inflammatoir effect op monocyten *in vitro*. Dit effect werd niet gezien na incubatie met het supernatant van RBC-gefilterde rode bloedcellen [55]. Mogelijk is dit klinisch van betekenis, want in een retrospectief cohortstudie met ziekenhuispatiënten die een RBC-transfusie kregen was een transfusie met verse volbloed-gefilterde rode bloedcellen geassocieerd met mortaliteit, dit

effect werd niet gevonden na transfusie van RBC-gefilterde eenheden [56]. Het zou verder onderzocht moeten worden of de verwerkingsmethoden ook een potentieel effect hebben op de uitkomst na transfusie bij ernstig zieke patiënten.

Donorkarakteristieken. Ook zou er meer aandacht besteed moeten worden aan donor-specifieke factoren, zoals geslacht, leefstijl en leeftijd, die mogelijk invloed hebben op de kwaliteit van opgeslagen rode bloedcellen [57, 58]. In een retrospectieve observationele studie werd transfusie van rode bloedcellen van jongeren en vrouwelijke donoren of producten met antigenen geassocieerd met een verhoging in mortaliteit [59, 60]. Wij denken dat het belangrijk is om die donorkarakteristieken te identificeren, die de kwaliteit van opgeslagen RBC-producten beïnvloeden. Daarmee kan vervolgens rekening gehouden worden bij het selecteren van de donoren.

Opslagduur. In de transfusiegeneeskunde zijn er vele onderzoeken gedaan naar de vraag of de opslagduur van de RBC-producten gerelateerd is aan een slechte uitkomst. De resultaten van deze onderzoeken zijn niet eenduidig, maar de grote gerandomiseerde onderzoeken laten geen verschillen zien in uitkomst met betrekking tot opslagduur [23-25]. Echter worden in deze studies verse rode bloedcellen (korter dan acht dagen opgeslagen) vergeleken met standaard uitgegeven rode bloedcellen (mediaan van eenentwintig dagen) en dus niet de maximale bewaartijd. Ook houden deze studies weinig rekening met de verschillen in het product als gevolg van het gebruik van verschillende bewaarvloeistoffen, verwerkingsmethodes en donorkarakteristieken, die mogelijk de resultaten hebben beïnvloed. Een andere reden om het effect van opslagduur nog verder te onderzoeken, is dat er aanwijzingen zijn dat transfusie van verse rode bloedcellen geassocieerd kunnen zijn met een nadelige uitkomst. Supernatant van verse volbloed-gefilterde rode bloedcellen is geassocieerd met een verhoogde ontstekingsreactie, gemeten door de IL-8 productie van niet gestimuleerde monocyten *in vitro* [55]. Een meta-analyse van klinische studies laat zien dat een transfusie met verse rode bloedcellen gerelateerd kan zijn een verhoogde kans op mortaliteit in vergelijking met patienten die een transfusie kregen met langer bewaarde rode bloedcellen [61]. In dit proefschrift hebben we gevonden dat plasma van ernstig zieke patiënten die verse rode bloedcellen kregen een hogere neutrofiel ROS-productie induceerde vergeleken met patiënten die standaard uitgegeven rode bloedcellen kregen. Dit suggereert dat een transfusie van verse rode bloedcellen een meer uitgesproken effect heeft op de immuunreactie van de ontvanger. Mogelijk via activering van monocyten of neutrofielen. Dit moet verder onderzocht worden. Dan is het vooral interessant

om uit te zoeken welke factor in deze verse RBC-producten een rol speelt bij de immuunreactie.

CONCLUSIE

In dit proefschrift hebben we het effect van een RBC-transfusie op de immuunreactie van de ontvanger onderzocht. We concluderen dat RBC-transfusies bij ernstig zieke patiënten geassocieerd zijn met een inflammatoir en een procoagulante immuunreactie bij de ontvanger. Geactiveerde neutrofielen, monocyten, bloedplaatjes en endotheelcellen zijn betrokken bij deze complexe reactie, die meer uitgesproken is wanneer er reeds een ontstekingsreactie in de ontvanger aanwezig is ten tijde van de transfusie. We veronderstellen dat EV's uit een RBC-product krachtige mediatoren zijn in de associatie tussen RBC-transfusies en een immuunreactie bij de ontvanger. Er zijn meerdere mogelijkheden voor verdere verbetering van de klinische praktijk en van het RBC-product, met als doel de immuunmodulerende effecten van een RBC-transfusie te verminderen.

REFERENCES

1. Gajic, O., et al., *Transfusion-related acute lung injury in the critically ill: prospective nested case-control study.* Am J Respir Crit Care Med, 2007. **176**(9): p. 886-91.
2. Vlaar, A.P. and N.P. Juffermans, *Transfusion-related acute lung injury: a clinical review.* Lancet, 2013. **382**(9896): p. 984-94.
3. Spinella, P.C., et al., *Properties of stored red blood cells: understanding immune and vascular reactivity.* Transfusion, 2011. **51**(4): p. 894-900.
4. Thomas, G.M., et al., *Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice.* Blood, 2012. **119**(26): p. 6335-43.
5. Shaz, B.H., S.R. Stowell, and C.D. Hillyer, *Transfusion-related acute lung injury: from bedside to bench and back.* Blood, 2011. **117**(5): p. 1463-71.
6. van de Watering, L. and C. Biomedical Excellence for Safer Transfusion, *Pitfalls in the current published observational literature on the effects of red blood cell storage.* Transfusion, 2011. **51**(8): p. 1847-54.
7. Norris, P.J., et al., *Influence of blood storage age on immune and coagulation parameters in critically ill transfused patients.* Transfusion, 2019. **59**(4): p. 1223-1232.
8. Vlaar, A.P., et al., *Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model.* Anesthesiology, 2010. **113**(1): p. 92-103.
9. Hezel, M.E.V., et al., *The Ability of Extracellular Vesicles to Induce a Pro-Inflammatory Host Response.* Int J Mol Sci, 2017. **18**(6).
10. Straat, M., et al., *Extracellular Vesicles from Red Blood Cell Products Induce a Strong Pro-Inflammatory Host Response, Dependent on Both Numbers and Storage Duration.* Transfus Med Hemother, 2016. **43**(4): p. 302-305.
11. Chin-Yee, I.H., et al., *Transfusion of stored red blood cells adhere in the rat microvasculature.* Transfusion, 2009. **49**(11): p. 2304-10.
12. Hod, E.A. and S.L. Spitalnik, *Stored red blood cell transfusions: Iron, inflammation, immunity, and infection.* Transfus Clin Biol, 2012. **19**(3): p. 84-9.
13. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
14. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
15. Peters, A.L., et al., *Transfusion of 35-day stored red blood cells does not result in increase of plasma non-transferrin bound iron in human endotoxemia.* Transfusion, 2017. **57**(1): p. 53-59.
16. Danesh, A., et al., *Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses in vitro.* Blood, 2014. **123**(5): p. 687-96.
17. Lisman, T., *Platelet-neutrophil interactions as drivers of inflammatory and thrombotic disease.* Cell Tissue Res, 2018. **371**(3): p. 567-576.

18. Zarbock, A. and K. Ley, *The role of platelets in acute lung injury (ALI).* Front Biosci (Landmark Ed), 2009. **14**: p. 150-8.
19. Erlich, J.M., et al., *Prehospitalization antiplatelet therapy is associated with a reduced incidence of acute lung injury: a population-based cohort study.* Chest, 2011. **139**(2): p. 289-295.
20. Kor, D.J., et al., *Association of prehospitalization aspirin therapy and acute lung injury: results of a multicenter international observational study of at-risk patients.* Crit Care Med, 2011. **39**(11): p. 2393-400.
21. Silvain, J., et al., *Impact of red blood cell transfusion on platelet activation and aggregation in healthy volunteers: results of the TRANSFUSION study.* Eur Heart J, 2010. **31**(22): p. 2816-21.
22. Silvain, J., et al., *Impact of red blood cell transfusion on platelet aggregation and inflammatory response in anemic coronary and noncoronary patients: the TRANSFUSION-2 study (impact of transfusion of red blood cell on platelet activation and aggregation studied with flow cytometry use and light transmission aggregometry).* J Am Coll Cardiol, 2014. **63**(13): p. 1289-1296.
23. Lacroix, J., et al., *Age of transfused blood in critically ill adults.* N Engl J Med, 2015. **372**(15): p. 1410-8.
24. Steiner, M.E., et al., *Effects of red-cell storage duration on patients undergoing cardiac surgery.* N Engl J Med, 2015. **372**(15): p. 1419-29.
25. Fergusson, D.A., et al., *Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial.* JAMA, 2012. **308**(14): p. 1443-51.
26. Spinella, P.C., et al., *Effects of blood storage age on immune, coagulation, and nitric oxide parameters in transfused patients undergoing cardiac surgery.* Transfusion, 2019. **59**(4): p. 1209-1222.
27. Kor, D.J., et al., *Fresh red blood cell transfusion and short-term pulmonary, immunologic, and coagulation status: a randomized clinical trial.* Am J Respir Crit Care Med, 2012. **185**(8): p. 842-50.
28. Hebert, P.C., et al., *A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group.* N Engl J Med, 1999. **340**(6): p. 409-17.
29. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
30. Edwards, J., et al., *Patient blood transfusion management: discharge hemoglobin level as a surrogate marker for red blood cell utilization appropriateness.* Transfusion, 2012. **52**(11): p. 2445-51.
31. Frank, S.M., et al., *Variability in blood and blood component utilization as assessed by an anesthesia information management system.* Anesthesiology, 2012. **117**(1): p. 99-106.
32. Bennett-Guerrero, E., et al., *Variation in use of blood transfusion in coronary artery bypass graft surgery.* JAMA, 2010. **304**(14): p. 1568-75.
33. Docherty, A.B. and T.S. Walsh, *Anemia and blood transfusion in the critically ill patient with cardiovascular disease.* Crit Care, 2017. **21**(1): p. 61.

34. Patel, N.N., et al., *Indications for red blood cell transfusion in cardiac surgery: a systematic review and meta-analysis.* Lancet Haematol, 2015. **2**(12): p. e543-53.
35. Fominskiy, E., et al., *Liberal transfusion strategy improves survival in perioperative but not in critically ill patients. A meta-analysis of randomised trials.* Br J Anaesth, 2015. **115**(4): p. 511-9.
36. Xue Lin, S.R., David Timm, d'Alessandro Angelo, Prakash Jaya, Ernst Melanie, Julie Prior, Shah Shivam, Jackie Wu, Julie Reisz, and Allan Doctor, *Sepsis Induced Red Cell Dysfunction (SiRD): Physiology and Mechanisms.* Blood, 2017.
37. Afessa, B., et al., *Systemic inflammatory response syndrome, organ failure, and outcome in critically ill obstetric patients treated in an ICU.* Chest, 2001. **120**(4): p. 1271-7.
38. Wozniak, M.J., et al., *Randomized trial of red cell washing for the prevention of transfusion-associated organ injury in cardiac surgery.* Br J Anaesth, 2017. **118**(5): p. 689-698.
39. Bennett-Guerrero, E., et al., *Randomized study of washing 40- to 42-day-stored red blood cells.* Transfusion, 2014. **54**(10): p. 2544-52.
40. Harm, S.K., et al., *Haemolysis and sublethal injury of RBCs after routine blood bank manipulations.* Transfus Med, 2012. **22**(3): p. 181-5.
41. Biffl, W.L., et al., *Plasma from aged stored red blood cells delays neutrophil apoptosis and primes for cytotoxicity: abrogation by poststorage washing but not prestorage leukoreduction.* J Trauma, 2001. **50**(3): p. 426-31; discussion 432.
42. Cortes-Puch, I., et al., *Washing older blood units before transfusion reduces plasma iron and improves outcomes in experimental canine pneumonia.* Blood, 2014. **123**(9): p. 1403-11.
43. Baek, J.H., et al., *Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy.* J Clin Invest, 2012. **122**(4): p. 1444-58.
44. Murphy, G.J., et al., *Trial protocol for a randomised controlled trial of red cell washing for the attenuation of transfusion-associated organ injury in cardiac surgery: the REDWASH trial.* Open Heart, 2016. **3**(1): p. e000344.
45. Cholette, J.M., et al., *Washing red blood cells and platelets transfused in cardiac surgery reduces postoperative inflammation and number of transfusions: results of a prospective, randomized, controlled clinical trial.* Pediatr Crit Care Med, 2012. **13**(3): p. 290-9.
46. Jy, W., et al., *Transfusion with Washed vs. Unwashed Packed Red Cells in Coronary Artery Bypass Graft (CABG) Surgery: Major Outcome Differences.* Blood, 2014. **124**(21): p. 2887-2887.
47. Schmidt, A.E., Majed A Refaai, Scott A. Kirkley and Neil Blumberg, *Proven and potential clinical benefits of washing red blood cells before transfusion: current perspectives.* International Journal of Clinical Transfusion Medicine, 2016.
48. Sparrow, R.L., et al., *In vitro measures of membrane changes reveal differences between red blood cells stored in saline-adenine-glucose-mannitol and AS-1 additive solutions: a paired study.* Transfusion, 2014. **54**(3): p. 560-8.

49. Veale, M.F., G. Healey, and R.L. Sparrow, *Effect of additive solutions on red blood cell (RBC) membrane properties of stored RBCs prepared from whole blood held for 24 hours at room temperature.* Transfusion, 2011. **51 Suppl 1**: p. 25S-33S.
50. Zehnder, L., et al., *Erythrocyte storage in hypertonic (SAGM) or isotonic (PAGGSM) conservation medium: influence on cell properties.* Vox Sang, 2008. **95**(4): p. 280-7.
51. Hess, J.R., et al., *Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions.* Transfusion, 2009. **49**(12): p. 2599-603.
52. Radwanski, K., et al., *The effects of red blood cell preparation method on in vitro markers of red blood cell aging and inflammatory response.* Transfusion, 2013. **53**(12): p. 3128-38.
53. Bicalho, B., A.S. Pereira, and J.P. Acker, *Buffy coat (top/bottom)- and whole-blood filtration (top/top)-produced red cell concentrates differ in size of extracellular vesicles.* Vox Sang, 2015. **109**(3): p. 214-20.
54. Bakkour, S., et al., *Manufacturing method affects mitochondrial DNA release and extracellular vesicle composition in stored red blood cells.* Vox Sang, 2016. **111**(1): p. 22-32.
55. Almizraq, R.J., et al., *Blood manufacturing methods affect red blood cell product characteristics and immunomodulatory activity.* Blood Adv, 2018. **2**(18): p. 2296-2306.
56. Heddle, N.M., et al., *Red blood cell processing methods and in-hospital mortality: a transfusion registry cohort study.* Lancet Haematol, 2016. **3**(5): p. e246-54.
57. Tzounakas, V.L., et al., *Donor variation effect on red blood cell storage lesion: a multivariable, yet consistent, story.* Transfusion, 2016. **56**(6): p. 1274-86.
58. Tzounakas, V.L., et al., *Donor-variation effect on red blood cell storage lesion: A close relationship emerges.* Proteomics Clin Appl, 2016. **10**(8): p. 791-804.
59. Chasse, M., et al., *Association of Blood Donor Age and Sex With Recipient Survival After Red Blood Cell Transfusion.* JAMA Intern Med, 2016. **176**(9): p. 1307-14.
60. Chasse, M., et al., *Effect of Blood Donor Characteristics on Transfusion Outcomes: A Systematic Review and Meta-Analysis.* Transfus Med Rev, 2016. **30**(2): p. 69-80.
61. Chai-Adisaksopha, C., et al., *Mortality outcomes in patients transfused with fresher versus older red blood cells: a meta-analysis.* Vox Sang, 2017. **112**(3): p. 268-278.

CURRICULUM VITAE

Maike van Hezel was born on the 5th of April in 1987 in Groningen, the Netherlands. She obtained pre- university education (VWO) at Zernike College in Haren (Gn). In addition to school, she participated in many sports, on a high level. For example, she joined in the Dutch gymnastics championship, a national training camp with windsurfing and KNLTB tennis selection trainings. After her pre-university education, she had a gap year and went on a trip for six months through New Zealand and Australia.

In 2006 she started to study medicine, at the University of Amsterdam. During her doctorate degree, in 2010, she went to Cape Town (South Africa) to do her scientific internship at the Stellenbosch University, at the department of Pediatrics of the Tygerberg Hospital, under supervision of Prof. Kruger. After this internship followed a year of travelling around the world to surf.

In her final master internships, an eldest residency was performed at the department of Internal Medicine at Tergooi, in Hilversum. Her final master internship of choice was at the department of the Intensive Care, at the Academic Medical Center (AMC), in Amsterdam. Due to curiosity for anesthesiology she decided to do an extra internship of choise at the department of Anesthesiology, at the AMC, in Amsterdam.

In 2013 she started her PhD in both the Department of the Intensive Care (AMC, Amsterdam) and the Department of Blood Cell Research (Sanquin, Amsterdam), under supervision of Prof. Juffermans, Prof. Schultz and R. van Bruggen PhD. Unfortunately the PhD period was slightly different than normal, due to a surfing accident, with a recovery period longer than expected. However, she finished her thesis about the host immune response after RBC transfusion in critically ill patients. She is also back on track with surfing, she even surfed a barrel last summer.

LIST OF PUBLICATIONS

- **Van Hezel ME**, van Manen L, Boshuizen M, Straat M, De Cuyper IM, Beuger B, Nieuwland R, Tanck MWT, de Korte D, Zwaginga JJ, van Bruggen B, Juffermans NP. The effect of red blood cell transfusion on platelet function in critically ill patients. Thrombosis Research 2019 Dec; 184: 115-221

- **Van Hezel ME**, Boshuizen M, Peters AL, Straat M, Vlaar APJ, Spoelstra- de Man AME, Tanck MWT, Tool ATJ, Beuger B, Kuijpers T, Juffermans NP, van Bruggen B. Red blood cell transfusion results in adhesion of neutrophils in human endotoxemia and in critically ill patients with sepsis. Transfusion 2019.

- Boshuizen M, **Van Hezel ME**, van Manen L, Straat M, Somsen YBO, Spoelstra-de Man AME, Blumberg N, van Bruggen R, Juffermans NP. The effect of red blood cell transfusion on iron metabolism in critically ill patients. Transfusion 2018 Dec 31; 10.1111/trf.15127.

- **Van Hezel ME**, Nieuwland R, van Bruggen R, Juffermans NP. The Ability of Extracellullar Vesicles to Induce a Pro- Inflammatory Host Response. Int J Mol Sci. 2017 Jun 16;18(6).

- **Van Hezel ME**, Peters AL, Klanderman RB, Tuip- de Boer AM, Wiersinga WJ, Van der Spek AH, van Bruggen R, de Korte D, Juffermans NP, Vlaar APJ. Transfusion of 35- day stored red blood cells does not alter lipopolysaccharide tolerance during human endotoxemia. Transfusion 2017 Jun; 57 (6):1359- 1368.

- **Van Hezel ME**, Straat M, Bing A, Tuip- de Boer A, Weber N, Nieuwland R, van Bruggen R, Juffermans NP.Monocyte- mediated activation of endothelial cells occurs only after binding to extracellular vesicles from red cell products, a process mediated by β- integrin. Transfusion. 2016 Dec;56(12):3012-3020.

- Peters AL, **van Hezel ME**, Cortjens B, Tuip- de Boer AM, van Bruggen R, de Korte D, Jonkers RE, Bonta PI, Zeerleder SS, Lutter R, Juffermans NP, Vlaar AP. Transfusion of 35- day stored RBCs in the presence of endotoxemia does not result in lung injury in humans. Crit Care Med. 2016 Jun; 44(6):e412-9.

- Peters AL, **van Hezel ME**, Juffermans NP, Vlaar AP. **Pathogenesis of non-antibody mediated transfusion- related acute lung injury from bench to bedside.** Blood Rev. 2015 Jan;29(1):51-61.

PHD PORTFOLIO

PhD student : M.E. van Hezel

PhD period : Nov 2013 – Dec 2018

PhD supervisors : Prof. dr. N.P. Juffermans and Dr. R. van Bruggen

General courses	**Year**	**ECTS**
AMC World of Science		0.7
BROK (Basiscursus Regelgeving Klinische Onderzoek)		0.9
Informed Consent		0.3
Evidenced based searching		0.1
Laboratory Animals (Article 9)		3.9
Searching for a Systematic Review		0.1
Practical Biostatistics		1.1
Systematic Reviews		0.3
Sanquin Science Course		0.4
Presentations		
"Microparticles from red blood cell transfusion products induce a strong inflammatory host response", Poster presentation, ISICEM, Brussel, Belgium	2016	0.5
"*Transfusion result in priming and adhesion of neutrophils in human endotoxemia*	2018	0.5
Conferences		
International Symposium on Intensive Care and Emergency Medicine (ISICEM), 2018 *as well as in a cohort of critically ill patients*", Poster presentation, ISICEM, Paris, France		0.75

General courses	Year	ECTS
Other activities		
Intensive Care meeting	2013-2015	12
Intensive Care Journal club	2013-2015	4
Laboratory Experimental Intensive Care and Anaesthesiology research meeting	2013-2015	12
Sanquin research meeting	2013-2015	12
Sanquin Journal club	2013-2015	4
Sanquin Landsteiner lectures	2013-2015	12
Red Cell Meeting	2013-2015	4
Product Proces Ontwikkeling Sanquin (PPO)	2013-2015	4

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