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Erythrocyte-Derived Microvesicles Amplify Systemic Inflammation by Thrombin-Dependent Activation of Complement

Daniel Zecher, Arun Cumpelik, Jürg A. Schifferli

Objective—Transfusion of aged blood has been associated with increased morbidity and mortality in critically ill patients. During storage, erythrocytes release increasing numbers of microvesicles (red blood cell–derived microvesicles [RBC-MV]). We hypothesized that RBC-MV mediate some of the deleterious effects of aged blood transfusions.

Approach and Results—We established a murine transfusion model using RBC-MV purified from aged mouse erythrocytes. Injection of RBC-MV into healthy mice had no effect. However, they aggravated pulmonary leukocyte sequestration and peripheral blood leukopenia induced by lipopolysaccharides. Lipopolysaccharide-induced proinflammatory cytokines were significantly increased in plasma after RBC-MV injection. These effects were not seen in C5aR-deficient mice. In vitro, RBC-MV bound C3 fragments after incubation with plasma but failed to bind immunoglobulins, C1q, or mannose-binding lectin. Preventing thrombin generation inhibited complement activation in vitro and in vivo and reversed the proinflammatory effects of RBC-MV in lipopolysaccharide-primed mice. Finally, the RBC-MV–induced phenotype was recapitulated using phosphatidylserine-expressing liposomes, suggesting that surface expression of phosphatidylserine by RBC-MV was mechanistically involved.

Conclusions—These results point toward a thrombin-dependent mechanism of complement activation by RBC-MV independent of the classical, lectin, or alternative pathway. Besides identifying RBC-MV as potential mediators of transfusion-related morbidity, our findings may be relevant for other inflammatory disorders involving intravascular microvesicle release, for example, sickle cell disease or thrombotic microangiopathy. **(*Arterioscler Thromb Vasc Biol*. 2014;34:313-320.)**

Key Words: blood coagulation ■ blood component transfusion ■ cell-derived microparticles ■ complement system proteins ■ inflammation

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Transfusion of stored erythrocytes is one of the most common in-hospital procedures with ≈14 million transfusions performed in the United States every year.[1] Up to 40% of intensive care patients receive blood products during their hospital stay with a mean of 5 units per patient.[2] International blood bank policies allow storage for ≤42 days between erythrocyte collection and transfusion. During the past decades, there has been substantial controversy over the question whether an increased storage time of blood products before transfusion is associated with higher patient morbidity and mortality.[3] Whereas some studies were negative,[4,5] various other studies found increased overall mortality rates,[6–9] a higher incidence of postoperative infections,[10] renal failure,[7] and a higher frequency of clotting disorders[11,12] in patients who received aged blood products compared with those who received fresh blood products and identified critically ill individuals, that is, patients after trauma or cardiac surgery, to be the most vulnerable patient population.[8,13]

During aging, erythrocytes undergo a series of biochemical and physical changes known as the storage lesion. These changes include lipid and protein oxidation as well as a reduction in deformability and osmotic stability, the latter resulting in considerable hemolysis with release of free hemoglobin and iron.[14] Notably, erythrocytes lose ≈20% of hemoglobin and membrane surface area over time by the release of microvesicles. Erythrocyte-derived microvesicles increase significantly in number during storage[15,16] and therefore large amounts are given to patients at the time of transfusion. Microvesicle release is a coordinate and active process coined ectocytosis to account for the fact that these vesicles (ectosomes) bud directly from the cell membrane.[17] This is contrary to exocytosis that describes the release of vesicles (exosomes) after fusion of intracellular multivesicular bodies with the cell membrane. Irrespective of their cellular origin, ectosomes harbor phosphatidylserine (PS) in their membranes and were shown to have procoagulant activity in vitro with PS serving as a platform for thrombin generation.[18] Also, human erythrocyte-derived ectosomes (red blood cell–derived microvesicles [RBC-MV]) were found to bind complement fragments in vitro,[19] suggesting that they might be proinflammatory via activation of the complement system.

Given their potential proinflammatory and procoagulant properties, we established a murine transfusion model to test

Received on: August 14, 2013; final version accepted on: November 20, 2013.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302378/-/DC1.

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Nonstandard Abbreviations and Acronyms

IL	interleukin
KC	Keratinocyte-derived chemoattractant
LPS	lipopolysaccharides
MCP-1	monocyte chemoattractant protein-1
PMN	polymorphonuclear cell
PS	phosphatidylserine
RBC-MV	red blood cell–derived microvesicles

the hypothesis that RBC-MV derived from aged mouse erythrocytes mediate pulmonary and extrapulmonary pathology in mice independent of the 2 major constituents of blood transfusions (ie, erythrocytes and hemoglobin).

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Characterization of RBC-MV

We first aimed at reproducing the storage lesion of human erythrocytes (RBC) in mice. After 18 days of storage, RBC-MV were isolated from the supernatant of stored RBC by sequential centrifugation and characterized by flow cytometry. Forward- and sideward-scatter analysis revealed a homogenous population that was >95% positive for Ter119, indicating their RBC origin. Consistent with previous reports in humans,[20] between 25% and 45% of all microvesicles stained positive for annexin V, indicating surface expression of PS (Figure 1A). Electron microscopy revealed round-shaped vesicles with a size ≈200 nm (Figure 1B), a finding that was confirmed by nanoparticle tracking analysis (Figure 1C). We next compared surface expression of various complement regulatory proteins, as well as the integrin-associated protein CD47 on RBC-MV to that of equally aged RBC. Whereas CD47 and Crry were highly expressed on both, DAF and CD59a showed only dim expression on RBC-MV compared with RBC, consistent with a specific sorting process of proteins underlying microvesicle shedding (Figure 1D). Finally, we determined both kinetics and magnitude of RBC-MV release during storage over time. RBC-MV could be identified at low quantities in the supernatant of freshly harvested blood (not shown), but dramatically increased in number after 12 days of storage. After 18 days, an average of 1×10^9 annexin V–positive microvesicles could be isolated from 1 mL of stored blood (Figure 1E).

Administration of RBC-MV Has No Effect in Healthy Mice

Our initial hypothesis was that systemic administration of RBC-MV causes acute lung injury in mice analogous to what has been reported after injection of anti–major histocompatibility complex (MHC)-I antibodies.[21–23] We injected 5×10^8 RBC-MV intravenously and analyzed lung histology, lung water weight as an indicator of pulmonary edema and pulmonary leukocyte infiltration by flow cytometry 4 hours later. Histological analysis of lung sections showed no pathology. Also, total leukocyte as well as polymorphonuclear cell (PMN) counts were similar after injection of RBC-MV compared with PBS-treated controls (Figure 2A–2C). There were no signs of systemic inflammation as the proinflammatory cytokines interleukin (IL)-6, keratinocyte-derived chemoattractant (KC), and monocyte chemoattractant protein-1 (MCP-1) in serum were undetectable (Figure 3). The latter findings also ruled out relevant contamination of RBC-MV during storage. These data indicated that RBC-MV cause neither lung pathology nor systemic inflammation in healthy mice.

RBC-MV Prolong Pulmonary Neutrophil Sequestration and Peripheral Blood Leukopenia in Lipopolysaccharide-Primed Mice

We next asked whether priming of mice with lipopolysaccharides (LPS) before injection of RBC-MV resulted in lung injury analogous to the 2-hit models of aged blood supernatant–induced and antibody-induced lung injury.[21,22,24,25] Two hours after intraperitoneal injection of LPS, RBC-MV were administered intravenously. Analysis of lung histology 4 hours later revealed increased leukocyte sequestration in small peripheral vessels compared with LPS-primed animals that had received PBS instead of RBC-MV (Figure 2A). However, histological changes consistent with parenchymal lung injury were not observed in any of the groups. There was an increase in lung water in all treated mice compared with PBS-treated controls. Compared with controls, lung water was significantly higher only in LPS-primed mice after RBC-MV injection (Figure 2B). However, values obtained for all groups were below those reported to represent overt pulmonary edema.[23] We next quantified and phenotyped pulmonary leukocytes by flow cytometry. Consistent with published reports,[26] LPS-primed mice showed an increase in pulmonary leukocyte counts compared with unprimed mice with the majority of these leukocytes being PMN. Administration of RBC-MV at 2 increasing concentrations resulted in a further significant increase in pulmonary PMN sequestration (Figure 2C) 4 hours later. Notably, analysis of peripheral blood leukocytes at that time revealed neutrophilia in LPS-primed mice, which was significantly reduced after addition of RBC-MV. Kinetic studies revealed a pronounced early pulmonary recruitment of PMN in response to LPS. Administration of RBC-MV resulted in a sustained pulmonary PMN sequestration during the next 2 to 4 hours in these mice, whereas PMN counts decreased more rapidly without infusion of RBC-MV (Figure 2D). The influx of $CD11b^+$ $Ly6C^{hi}$ inflammatory monocytes followed different kinetics. Whereas administration of RBC-MV had no impact on their rapid increase after priming with LPS, pulmonary monocyte sequestration was prolonged as evidenced by higher numbers 4 hours after RBC-MV injection (Figure 2D). In sum, administration of RBC-MV in LPS-primed mice resulted in increased pulmonary PMN sequestration without evidence of invasive lung injury.

RBC-MV Amplify Systemic Inflammation

We next asked whether administration of RBC-MV in LPS-primed mice would amplify systemic inflammation. We observed a dose-dependent increase in serum levels of the proinflammatory cytokines IL-6, KC, and MCP-1 after RBC-MV injection (Figure 3).

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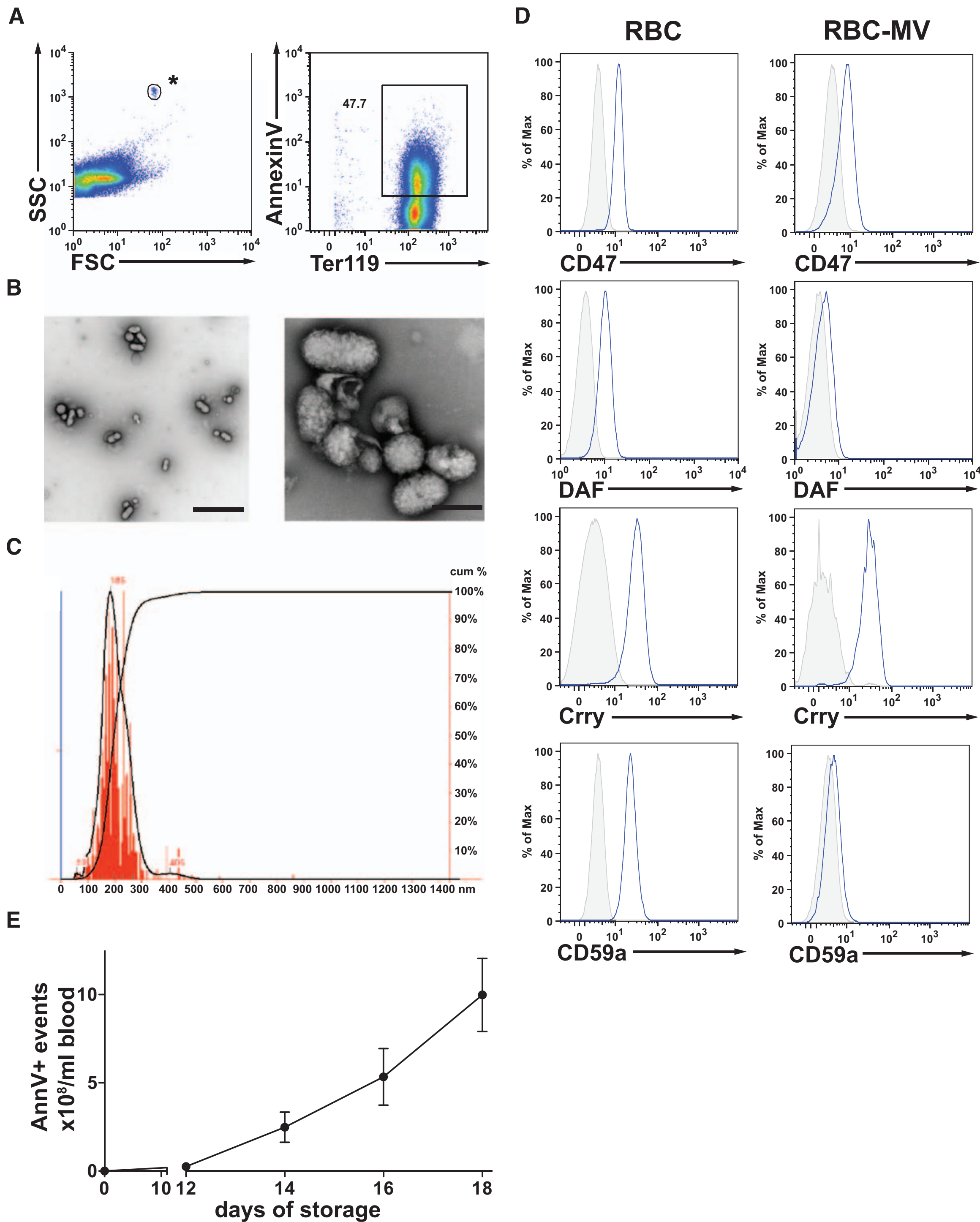

Figure 1. Characterization of red blood cell–derived microvesicles (RBC-MV). Flow cytometric analysis of MV after 18 days of storage (**A** and **D**). Forward/sideward-scatter characteristics compared with 4.2 μm control beads (*) and surface staining for Ter119 and annexin V (**A**). Differential surface expression of the indicated molecules on RBC-MV compared with red blood cells (RBC) both aged for 18 days (**D**). Transmission electron microscopy of RBC-MV reveals round-shaped vesicles with a size of ≈200 nm. Left side bar, 1 μm; right side bar, 200 nm (**B**). Nanoparticle tracking analysis of RBC-MV reveals a homogenous population with a mean diameter of 200 nm (**C**). Kinetics of RBC-MV release during storage. Data are mean±SEM from 3 to 8 independent experiments per time point (**E**).

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Role of Hemoglobin and Surface-Expressed PS

Free hemoglobin has been linked to the complications after transfusion of aged blood.[27,28] Given that RBC-MV contain significant amounts of hemoglobin wrapped inside the vesicles during budding from RBC, we wanted to know whether the phenotype after RBC-MV injection was specific to RBC-MV or whether it could also be elicited by the main content of RBC-MV (ie, hemoglobin). We therefore injected 2 different concentrations of free hemoglobin from lysed aged erythrocytes into LPS-primed mice and analyzed

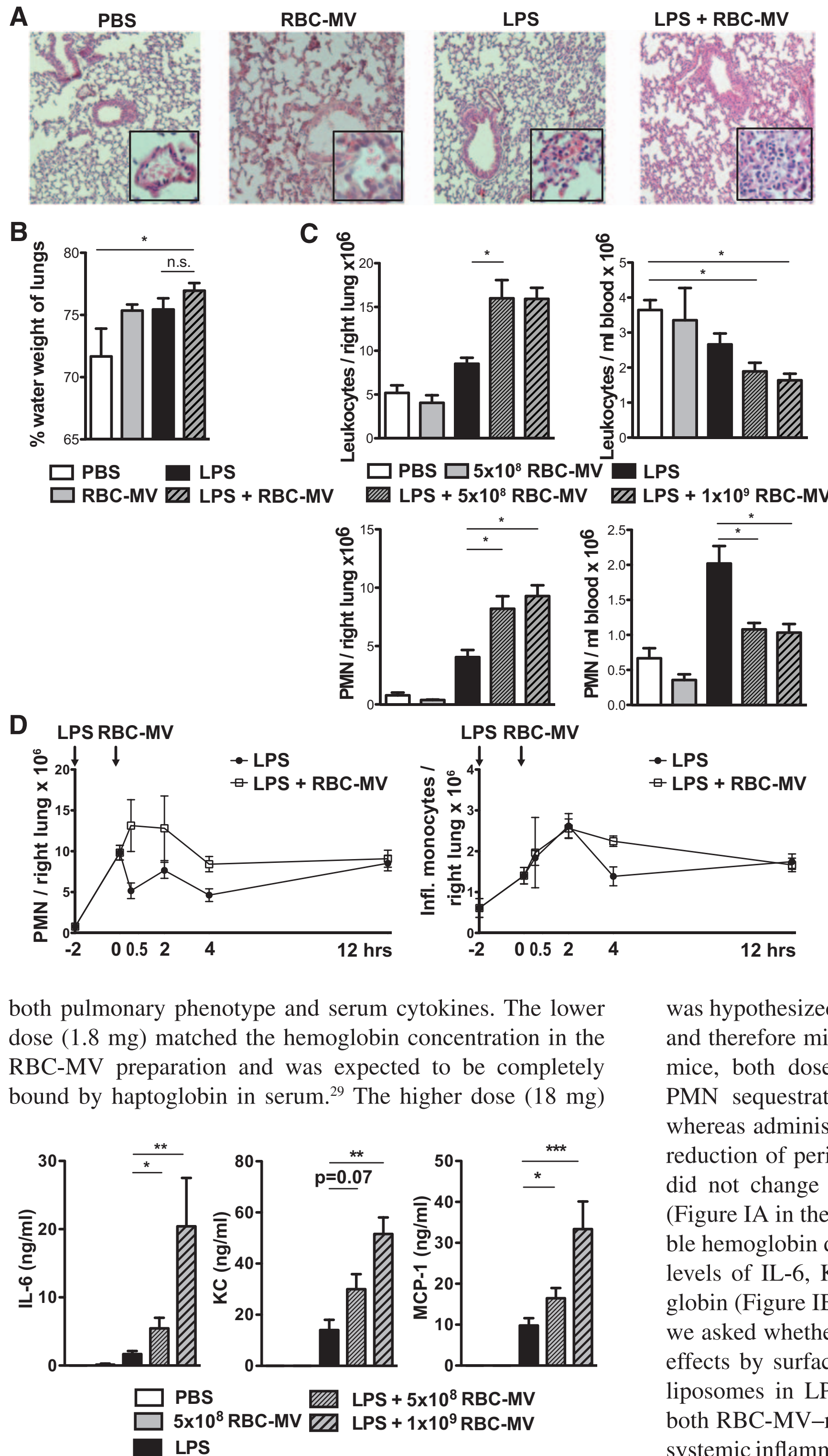

Figure 2. Systemic administration of red blood cell–derived microvesicles (RBC-MV) amplifies pulmonary neutrophil sequestration and peripheral blood leukopenia in lipopolysaccharide (LPS)-primed mice. B6 mice were primed with an intraperitoneal injection of LPS followed by the intravenous injection of 5×10^8 or 1×10^9 RBC-MV (LPS+RBC-MV) 2 hours later. Alternatively, mice were given LPS intraperitoneally followed by PBS intravenously (LPS). Control groups received PBS instead of LPS for priming followed by 5×10^8 RBC-MV intravenously (RBC-MV) or were given PBS intraperitoneally and intravenously (PBS). Four hours later, lungs were harvested and lung sections stained by hematoxylin and eosin. Magnification, ×100; inset, ×600. Pictures are representative of ≥4 mice analyzed per group (**A**). Percentage water weight of lungs of the indicated groups determined as outlined in the Methods in the online-only Data Supplement. n=3 (PBS, RBC-MV only), 12 (LPS), and 15 (LPS+RBC-MV) pooled from several independent experiments (**B**). Accumulation of $CD45^+$ leukocytes and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ polymorphonuclear cells (PMN) in right lungs as well as peripheral blood leukocyte and PMN counts of the indicated groups as determined by flow cytometry. n=4 to 6 per group (lung) and 5 to 7 per group (blood) pooled from 2 representative experiments (**C**). Kinetics of pulmonary $CD45^+$ leukocyte, PMN, and $CD11b^+$ $Ly6C^{hi}$ $Ly6G^{neg}$ inflammatory monocyte sequestration in LPS vs LPS+5×10^8 RBC-MV treated mice. n=4 to 6 per group and time point (**D**). *$P<0.05$ using 1-way ANOVA and Bonferroni post test. Mean±SEM is shown.

both pulmonary phenotype and serum cytokines. The lower dose (1.8 mg) matched the hemoglobin concentration in the RBC-MV preparation and was expected to be completely bound by haptoglobin in serum.[29] The higher dose (18 mg) was hypothesized to exceed the haptoglobin-binding capacity and therefore mimic intravascular hemolysis. In LPS-primed mice, both doses of free hemoglobin induced pulmonary PMN sequestration comparable with RBC-MV. However, whereas administration of RBC-MV resulted in a significant reduction of peripheral blood PMN counts, free hemoglobin did not change LPS-induced peripheral blood neutrophilia (Figure IA in the online-only Data Supplement). At comparable hemoglobin doses, RBC-MV induced significantly higher levels of IL-6, KC, and MCP-1 compared with free hemoglobin (Figure IB in the online-only Data Supplement). Next, we asked whether RBC-MV mediated their proinflammatory effects by surface-expressed PS. We injected PS-containing liposomes in LPS-primed mice. This treatment reproduced both RBC-MV–mediated pulmonary PMN sequestration and systemic inflammation (Figure IIA and IIB, respectively, in the online-only Data Supplement), which was not the case when LPS-primed mice received phosphatidlycholine-containing control liposomes. In addition, we performed experiments blocking surface PS on RBC-MV by preincubating RBC-MV with saturating amounts of annexin V before intravenous injection in LPS-primed mice. This approach did not have a significant effect on pulmonary PMN sequestration or peripheral blood neutropenia (Figure IIC in the online-only Data Supplement). Moreover, IL-6 levels were highly variable after

Figure 3. Dose-dependent amplification of lipopolysaccharide (LPS)-induced systemic inflammation by red blood cell–derived microvesicles (RBC-MV). Serum levels of interleukin (IL)-6, keratinocyte-derived chemoattractant (KC), and monocyte chemoattractant protein (MCP)-1 4 hours after intravenous injection of PBS or RBC-MV in unprimed (PBS and RBC-MV, respectively) or LPS-primed B6 mice (LPS, LPS+5×10^8 RBC-MV, LPS+1×10^9 RBC-MV, respectively) as determined by ELISA. n=6 to 16 per group pooled from ≥3 independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using a nonparametric Mann–Whitney test. Mean±SEM is shown.

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blocking with some values being above and some below those measured in control animals. Importantly, IL-6 levels were increased after injection of free annexin V into LPS-primed mice even in the absence of RBC-MV (Figure IID in the online-only Data Supplement). Further in vitro experiments revealed that incubation of RBC-MV with annexin V in the presence of physiological-free calcium concentrations (1.25 mmol/L as compared with the commonly used 2.5 mmol/L) resulted in a significant reduction of annexin V binding (18.8% versus 51.2%), suggesting that binding of annexin V to PS might not be stable in vivo. Taken together, these results indicated a specific effect of RBC-MV independent of hemoglobin wrapped inside the vesicles or free hemoglobin contaminating the RBC-MV preparations. Studies using liposomes further argued against a role for hemoglobin but suggested a role for surface-expressed PS in mediating the RBC-MV–induced phenotype in LPS-primed mice.

RBC-MV Bind Complement Fragments In Vitro Independent of the Classical or Alternative Pathway

It has been speculated that some of the negative effects of stored red blood cells might relate to the ability of RBC-MV to activate complement via binding of natural antibodies.[30] We therefore investigated classical pathway activation by RBC-MV in vitro. After incubation with plasma, we were unable to detect binding of IgM (Figure 4A) or IgG (not shown) on RBC-MV using flow cytometry. Also, there was no binding of mannose-binding lectin (not shown) or C1q (Figure 5B). That the latter result was not because of technical reasons became evident when we incubated RBC-MV with an antibody against the RBC-MV surface antigen Ter119 before adding plasma. This time, subsequent incubation with an anti-C1q antibody indicated binding of C1q, likely via binding to the anti-Ter119 antibody (Figure 4B). Notably, we could detect binding of C3 fragments using an anti-iC3b antibody (Figure 4C). That this was independent of classical pathway activation was further supported by the fact that C3 fragment binding was revealed after incubation with both wild-type and $Rag^{-/-}$ plasma, the latter being devoid of antibodies. Finally, chelation of calcium by EGTA prevented C3 fragment binding. This effect could not be reversed after addition of magnesium, arguing against involvement of the alternative pathway (Figure 4D).

C5aR Is Critical for the RBC-MV–Induced Phenotype In Vivo

We next assessed plasma levels of C5a in our in vivo model and found a rapid rise in C5a after injection of RBC-MV in LPS-primed mice compared with LPS-treated controls (Figure 4E). To test whether C5a was mechanistically involved, we applied our in vivo protocol to Balb/c mice comparing pulmonary PMN sequestration and serum cytokines between wild-type mice and mice unable to respond to the complement fragment C5a because of a targeted mutation in the gene encoding the C5a receptor ($C5aR^{-/-}$). We observed the same phenotype in Balb/c wild-type compared with B6 mice, indicating a strain-independent phenomenon (Figure 4F and 4G). Strikingly, we found that both the RBC-MV–induced increase in pulmonary PMN sequestration and the increase in proinflammatory cytokines were completely abolished in LPS-primed mice in the absence of C5aR (Figure 4F and 4G), suggesting that the presence of C5aR is critical for the proinflammatory effects mediated by RBC-MV in vivo.

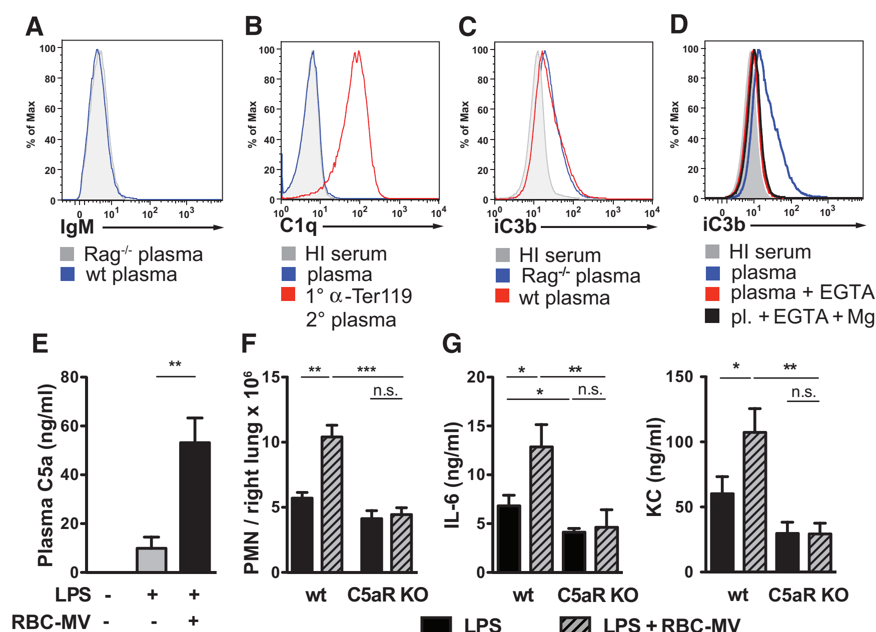

Figure 4. Involvement of complement. Red blood cell–derived microvesicles (RBC-MV) bind C3 fragments independent of classical or alternative pathway activation in vitro. B6 RBC-MV were incubated with wild-type (wt) or $Rag^{-/-}$ plasma or heat-inactivated (HI) control wt serum in vitro. Binding of IgM (**A**), C1q (**B**), or iC3b fragments (**C**) was subsequently revealed by flow cytometry after incubation with fluorochrome-tagged anti-IgM, anti-C1q, or anti-C3b mAbs, respectively. **B**, RBC-MV were incubated with an antibody specifically binding to surface glycophorin-A (Ter119) before incubation with plasma. Binding of C1q was then revealed after incubation with anti-C1q mAb (1° α-Ter119, 2° plasma). To test for alternative pathway activation in vitro, RBC-MV were incubated with plasma in the presence or absence of EGTA, the latter with (EGTA+Mg) or without (EGTA) addition of magnesium. iC3b fragment binding was revealed as in **C** and **D**. Results are representative of >2 independent experiments (**A** to **D**). Increased levels of C5a in LPS-primed B6 mice 15 minutes after intravenous injection of RBC-MV (n=5–6; **E**). Amplification of pulmonary phenotype and serum cytokines by RBC-MV in LPS-primed mice depends on complement receptor C5aR. Balb/c wt and C5aR-deficient mice were treated as described in Figure 2. Four hours later, pulmonary polymorphonuclear cell (PMN) sequestration (n=7–8; **F**) as well as serum cytokines (n=7–11; **G**) were determined. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ using 1-way ANOVA and Bonferroni post test (pulmonary PMN counts) or a nonparametric Mann–Whitney test (cytokines). Mean±SEM is shown. KC indicates keratinocyte-derived chemoattractant; and n.s., nonsignificant.

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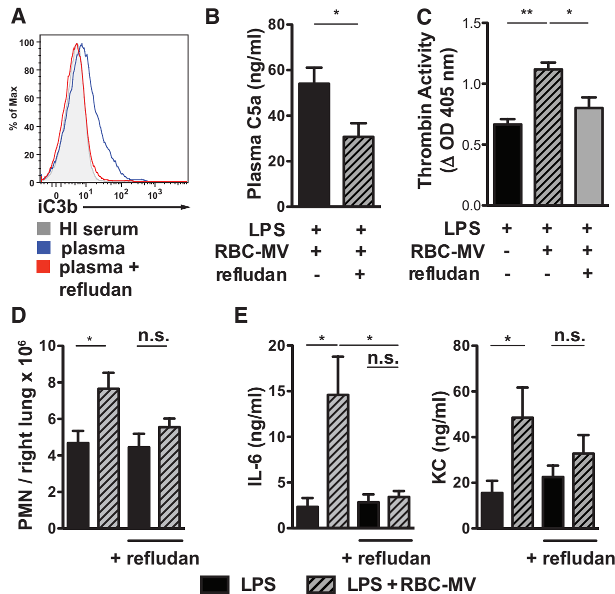

Figure 5. Red blood cell–derived microvesicle (RBC-MV)–induced effects can be reversed by inhibition of thrombin in vitro and in vivo. iC3b binding to RBC-MV in vitro after incubation with plasma can be reversed by refludan (**A**). B6 RBC-MV were incubated with plasma, heat-inactivated (HI) control serum, or plasma from mice that had been anticoagulated with refludan before euthanization. iC3b binding was revealed after incubation with an anti-iC3b mAb by flow cytometry (**A**). Plasma levels of C5a and thrombin are reduced in the presence of refludan. Lipopolysaccharide (LPS)-primed B6 mice anticoagulated with refludan (+) or not (–) were given 1×10^9 RBC-MV intravenously 15 minutes later, plasma was obtained, and C5a levels (**B**) and thrombin activity (**C**) were determined by ELISA and a thrombin assay, respectively, as indicated in Methods in the online-only Data Supplement. Amplification of pulmonary polymorphonuclear cells (PMN) sequestration (**D**) and systemic inflammation (**E**) in LPS-primed B6 mice 4 hours after injection of RBC-MV can be reversed by refludan. n=6 to 8 per group pooled from ≥3 independent experiments (**B** to **E**). *P<0.05 and **P<0.01 using 1-way ANOVA and Bonferroni's post-test (**C** and **D**) or a nonparametric Mann–Whitney test (**B** and **E**). Mean±SEM is shown. IL indicates interleukin; KC, keratinocyte-derived chemoattractant; and n.s., not significant.

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Inhibition of Thrombin Prevents Complement Activation In Vitro and In Vivo and Reverses the RBC-MV–Induced Proinflammatory Phenotype in LPS-Primed Mice

Microvesicles from platelets but also erythrocytes were shown to have procoagulant properties in vitro with surface expression of PS serving as a platform for thrombin generation.[18] Moreover, thrombin can function both as a C5 and C3 convertase[31–33] and therefore can activate complement independent of the classical or alternative pathway. Consistent with these studies, C3 fragment binding after incubation of RBC-MV with plasma in vitro could be prevented completely when plasma was used from mice anticoagulated with either heparin (not shown) or refludan, a specific thrombin inhibitor (Figure 5A). As mentioned earlier, binding of C3 fragments to RBC-MV was also inhibited in the presence of EGTA (Figure 4D). Given the effect of thrombin inhibition on C3 fragment binding, this finding was consistent with calcium being a necessary cofactor for thrombin activation. In vivo, injection of RBC-MV resulted in increased serum levels of thrombin compared with LPS-treated controls (Figure 5C). Refludan prevented RBC-MV–induced pulmonary PMN sequestration (Figure 5D), reversed peripheral blood neutropenia (not shown), and abolished RBC-MV–induced proinflammatory cytokines, most notably IL-6 (Figure 5E). Importantly, refludan also reduced plasma levels of C5a, directly linking thrombin generation to complement activation (Figure 5B).

Discussion

In this study, we established a murine transfusion model to ask whether RBC-MV derived from the supernatant of aged erythrocytes have proinflammatory properties when injected intravenously into mice. We found that infusion of RBC-MV into healthy mice did not result in any pathology. When we extended our studies to LPS-primed mice (mimicking the clinical condition of sepsis), we found that under inflammatory conditions, RBC-MV amplified both pulmonary neutrophil sequestration and peripheral blood neutropenia and induced higher serum levels of proinflammatory cytokines. Mechanistic in vitro and in vivo studies revealed that these effects were due to activation of both the complement and the coagulation system. RBC-MV induced thrombin-dependent complement activation in vitro and in vivo. Moreover, RBC-MV lost their proinflammatory effects in mice unresponsive to the anaphylatoxin C5a and in mice that had received the specific thrombin-inhibitor refludan. Studies using liposomes suggested that the proinflammatory effects of RBC-MV involve surface expression of PS. Taken together, these findings suggest that thrombin can function as a C3 and/or C5 convertase in vivo independent of the classical, lectin, or alternative complement pathway.

The impetus for this work was the clinical observation that transfusion of aged erythrocytes is associated with increased morbidity and mortality in the critically ill, the pathophysiology and triggering factors of this phenomenon still being incompletely understood. The results of our study are in accordance with several clinical observations. First, aged blood is more harmful in critically ill patients compared with healthy individuals, consistent with the observation that RBC-MV in our model were only harmful in endotoxemic mice, whereas healthy mice withstood the harmful effects. Second, studies exploring the pathophysiology of anti-human leukocyte antigen (HLA) antibody–induced transfusion-related lung injury (TRALI)[21–23,34] do not explain why aged blood carries a higher risk of adverse reactions than younger blood, given that the amount of antibody in the transfusion bags is not expected to change over time. Moreover, these studies do not explain the nonpulmonary complications observed in these patients, notably higher rates of multi-organ failure, infections, or thromboembolic complications.[10,11,13] The findings presented here offer a possible antibody-independent mechanism of transfusion-related pathology.

For the generation of mouse RBC-MV, we aimed at mimicking human RBC purification and storage conditions. However, several factors in our protocol differed from human blood banking conditions. This included dextran sedimentation for RBC enrichment and MV purification from RBC aged longer than what is thought to be equivalent to the human maximum storage time of 42 days.[35] These differences might

affect both quality and quantity of mouse RBC-MV. Although these differences should not preclude the mechanistic findings of our article, a direct translation of our findings to human RBC transfusions warrants caution.

Previous animal studies have already suggested that the supernatant of aged erythrocytes can mediate transfusion-related complications.[24,25,36] Hod et al[37] recently found free iron released from damaged stored RBC to amplify LPS-induced inflammation in a murine transfusion model. Despite differences in RBC purification and storage conditions between our study and the work by Hod and co-workers,[35,37] several findings are consistent. First, the amount of stored RBC we used to prepare RBC-MV was comparable with that used by Hod et al for injection of stored RBC supernatants or hemolysates. Second, in the absence of LPS priming, neither RBC-MV in our study nor the supernatant (containing RBC-MV) or hemolysate of stored RBC used by Hod et al induced inflammation. These results also support that our method to generate RBC-MV did not result in a more proinflammatory effect per se. In the presence of LPS priming, infusion of free hemoglobin from aged erythrocytes (likely containing iron) did amplify pulmonary PMN sequestration and resulted in an increase in proinflammatory cytokines in our model. However, when equal doses of hemoglobin were infused, the proinflammatory effects of RBC-MV were significantly more pronounced. Together with the data obtained using PS-positive liposomes (that lack hemoglobin), these results suggested that there is a hemoglobin-independent proinflammatory effect of RBC-MV, likely via surface expression of PS. It is important to note, however, that the evidence using PS-positive liposomes is associative. To test for causality, we performed blocking experiments preincubating RBC-MV with annexin V before intravenous injection and found no significant effect on pulmonary phenotype and proinflammatory cytokines (Figure IIC and IID in the online-only Data Supplement). Given that injection of annexin V in LPS-primed mice increased inflammation in the absence of RBC-MV and considering that blocking surface PS with annexin V might not be stable in vivo, the results from these experiments do not allow a final conclusion on the role of PS on RBC-MV in our model. Further experiments will have to be done to establish causality.

A crosstalk between the complement and the coagulation system has long been suggested, although in vivo evidence is limited. In vitro studies found complement activation products, that is, C3a and C5a, after incubation of serum with clotting factors, notably thrombin.[32,33] Recently, Huber-Lang et al[31] found evidence for thrombin-mediated cleavage of C5 in a mouse model of immune-complex–mediated lung injury, linking coagulation to complement activation and systemic inflammation in vivo. The procoagulant properties of erythrocyte- and platelet-derived microvesicles are well described in the literature and are mainly based on solid-phase in vitro assays of thrombin generation.[18,38] As in our model, the specific thrombin-inhibitor refludan was able to prevent both RBC-MV–induced complement activation in vitro (assessed by binding of C3 fragments to RBC-MV) and in vivo (evidenced by reduced C5a levels in the presence of refludan), our findings support the concept of thrombin-induced complement activation.

Outside the context of RBC transfusions, various other diseases, such as thrombotic microangiopathy and sickle cell anemia, are characterized by hypercoagulability,[39] systemic complement activation,[40] and increased blood levels of microparticles.[41–43] Ståhl et al recently reported the presence of complement fragments on platelet and monocyte-derived microparticles in patients with shigatoxin-induced hemolytic uremic syndrome, a form of thrombotic microangiopathy,[42] suggesting a pathogenetic role of microvesicles in these conditions.

Taken together, we have identified RBC-MV to mediate transfusion-related pathology by thrombin-dependent activation of the complement system in a mouse transfusion model. These findings might not only be relevant for RBC storage and transfusion allocation strategies but describe a proinflammatory amplification loop with potential relevance for other conditions characterized by intravascular microvesicle release.

Acknowledgments

We thank D. Tsakiris and A. Buser (both Department of Hematology, Basel University Hospital) for providing reagents and helpful discussions; M. Courtet and Estelle Gerossier for excellent technical assistance, B. Schneider for performing histology, and V. Olivieri from the Microscopy Core Facility at Biozentrum, Basel University, for performing electron microscopy.

Sources of Funding

Dr Zecher was supported by grants from Forschungsfonds of Basel University and Fondation Machaon, Geneva, Switzerland. Dr Schifferli was funded by grant 32000-116839 from the Swiss National Foundation.

Disclosures

None.

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Significance

Transfusion of aged erythrocytes is associated with increased morbidity and mortality in critically ill patients. Experimental evidence suggests that there are unknown factors in the supernatant of stored blood that mediate pathology. During aging, erythrocytes release cell surface–derived microvesicles. Red blood cell–derived microvesicles accumulate during storage and are given in high numbers to patients during transfusion. We established a murine transfusion model and found that microvesicles derived from aged mouse erythrocytes amplify both endotoxin-induced pulmonary leukocyte sequestration and systemic inflammation. Mechanistic studies revealed that red blood cell–derived microvesicles activate the complement system independent of the classical, lectin, or alternative pathway but via activation of the coagulation factor thrombin. Our study identifies a possible mechanism of transfusion-related pathology. Moreover, microvesicle-induced complement activation might constitute an amplification loop of inflammation in other diseases with intravascular release of microvesicles, such as sickle cell disease or thrombotic microangiopathy.

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