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

Daniel Zecher, Arun Cumpelik, Jürg 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-MVs]). We hypothesized that RBC-MVs mediate some of the deleterious effects of aged blood transfusions.

**Approach and Results**—We established a murine transfusion model using RBC-MVs purified from aged mouse erythrocytes. Injection of RBC-MVs 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 CsaR-deficient mice. In vitro, RBC-MVs bound C3 fragments after incubation with plasma but failed to bind immunoglobulins, C1q, or MBL. Preventing thrombin generation inhibited complement activation in vitro and in vivo and reversed the proinflammatory effects of RBC-MVs in lipopolysaccharide-primed mice. Finally, the RBC-MV–induced phenotype was recapitulated using phosphatidylserine-expressing liposomes, suggesting that surface expression of phosphatidylserine by RBC-MVs was mechanistically involved.

**Conclusions**—These results point toward a thrombin-dependent mechanism of complement activation by RBC-MVs independent of the classical, lectin, or alternative pathway. Besides identifying RBC-MVs 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;34:00-00.*)

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

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 disorders11,12 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 in number, in particular during storage15,16 and therefore large amounts are given to patients at the time of transfusion. Microvesicle release is a coordinate and active process mediated via activation of the complement system.

Expressed on the vesicle membrane is phosphatidylserine (PS) serving as a platform for thrombin generation.15 Also, human erythrocyte-derived ectosomes (red blood cell–derived microvesicles [RBC-MVs]) were found to bind complement fragments in vitro,15 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 the hypothesis that RBC-MVs 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-MVs
We first aimed at reproducing the storage lesion of human erythrocytes (RBC) in mice. After 18 days of storage, RBC-MVs 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, 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 \( \approx 200 \) 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-MVs to that of equally aged RBC. Whereas CD47 and Crry were highly expressed on both, DAF and CD59a showed only dim expression on RBC-MVs 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-MVs release during storage over time. RBC-MVs could be identified at low quantities in the supernatant of stored blood (Figure 1E).

Administration of RBC-MVs Has No Effect in Healthy Mice
Our initial hypothesis was that systemic administration of RBC-MVs causes acute lung injury in mice analogous to what has been reported after injection of anti–MHC-I antibodies.\(^21\)–\(^23\) We injected \( 5 \times 10^8 \) RBC-MVs 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 neutrophil (PMN) counts were similar after injection of RBC-MVs compared with PBS-treated controls (Figure 2A–2C). There were no signs of systemic inflammation as the proinflammatory cytokines interleukin (IL)-6, KC, and MCP-1 in serum were undetectable (Figure 3). The latter findings also ruled out relevant contamination of RBC-MVs during storage. These data indicated that RBC-MVs cause neither lung pathology nor systemic inflammation in healthy mice.

RBC-MVs 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-MVs 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-MVs were administered intravenously. Analysis of lung histology 4 hours later revealed increased neutrophile sequestration in small peripheral vessels compared with LPS-primed animals that had received PBS instead of RBC-MVs (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 higher significantly 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-MVs 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-MVs. Kinetic studies revealed a pronounced early pulmonary recruitment of PMN in response to LPS. Administration of RBC-MVs 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-MVs (Figure 2D). The influx of CD11b\(^+\) Ly6C\(^+\) inflammatory monocytes followed different kinetics. Whereas administration of RBC-MVs 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-MVs in LPS-primed mice resulted in increased pulmonary PMN sequestration without evidence of invasive lung injury.

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

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-MVs 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-MVs or whether it could also be elicited by the main content of RBC-MVs (ie, hemoglobin). We therefore injected 2 different concentrations of free hemoglobin from lysed aged erythrocytes into LPS-primed mice and analyzed 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.  

Figure 1. Characterization of red blood cell–derived microvesicles (RBC-MVs). 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).
(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-MVs. However, whereas administration of RBC-MVs 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-MVs 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-MVs 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 phosphatidylcholine-containing control liposomes. In addition, we performed experiments blocking surface PS on RBC-MVs by preincubating RBC-MVs 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 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-MVs (Figure IID in the online-only Data Supplement). Further
in vitro experiments revealed that incubation of RBC-MVs 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-MVs 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-MVs 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-MVs to activate complement via binding of natural antibodies. We therefore investigated classical pathway activation by RBC-MVs in vitro. After incubation with plasma, we were unable to detect binding of IgM (Figure 4A) or IgG (not shown) on RBC-MVs using flow cytometry. Also, there was no binding of MBL (not shown) or C1q (Figure 5B). That the latter result was not because of technical reasons became evident when we incubated RBC-MVs with an antibody against the RBC-MVs 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-MVs 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 abolished completely 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-MVs in vivo.

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

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Involvement of complement. Red blood cell–derived microvesicles (RBC-MVs) bind C3 fragments independent of classical or alternative pathway activation in vitro. B6 RBC-MVs were incubated with wild-type (wt) or Rag−/− plasma or heat-inactivated (HI) control 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-MVs were incubated with an antibody specifically binding to surface glycoprotein-A (Ter119) before incubation with plasma. Binding of C1q was then revealed after incubation with anti-C1q mAb (1° anti-Ter119, 2° plasma). To test for alternative pathway activation in vitro, RBC-MVs 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 mice were measured 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).

**B6 mice 15 minutes after intravenous injection of RBC-MV (n=5–6; E).** Amplification of pulmonary phenotype and cytokine production by RBC-MV in LPS-primed mice depends on complement receptor C5aR. Balb/c wild-type and C5aR-deficient mice were treated as described in Figure 2. Four hours later, pulmonary 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). Means±SEM is shown. n.s. indicates nonsignificant.
expression of PS serving as a platform for thrombin generation.\textsuperscript{18} Moreover, thrombin can function both as a C5 and C3 convertase\textsuperscript{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-MVs with plasma in vitro could be prevented completely when plasma was used from mice anticoagulated with refludan before euthanization. C3b binding was revealed after incubation with an anti-C3b 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\textsuperscript{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 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; and n.s. indicates not significant.\textsuperscript{19}\textsuperscript{20}\textsuperscript{54}
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-MVs 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-MVs in our study nor the supernatant (containing RBC-MVs) or hemolysate of stored RBC used by Hod et al induced inflammation. These results also support that our method to generate RBC-MVs 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-MVs 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-MVs, 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-MVs 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-MVs 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-MVs 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 al31 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.18,30 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-MVs) 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.51–53 Stähli 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-MVs 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.

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Disclosures

None.

References


**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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Methods

Mice

C57BL/6 (B6), Balb/c and B6 Rag-/- mice were bred at the institution’s animal facility or purchased from Charles River (Sulzfeld, Germany). C.129S4(B6)-C5ar1<sup>tm1Cge/J</sup> (C5aR<sup>−/−</sup>) on a Balb/c background were from the Jackson Laboratories (Bar Harbor, USA). All mice were housed in a specific pathogen-free environment and were used between 7 and 12 weeks of age. Animal care and experimentation were performed in accordance with national guidelines (Federal Veterinary Office) and had been approved by the local government authorities.

Reagents and monoclonal antibodies

Fluorochrome-tagged mAbs against CD11b (clone M1/70), CD45 (30-F11), CD47 (miap301), CD59a (mCD59.3), Ly6C (AL-21), Ly6G (1A8), Ter119 (Ly-76), DAF (RIKO-3), Crry (512), IgG (Poly4053), IgM (RMM-1) and purified anti-CD16/32 (2.4G2) were from Biolegend (San Diego, USA), eBioscience (Vienna, Austria) or BD Pharmingen (Allschwil, Switzerland). Anti-iC3b (3.26) and anti-MBL-C (8G6) were from Hycult Biotech (Uden, the Netherlands). FITC-conjugated anti-C1q (RmC7H8) was from Cedarlane (Burlington, Canada), AnnexinV-APC from Immunotools (Friesoyte, Germany) and 7-AAD from Invitrogen (Zug, Switzerland).

Generation and analysis of red blood cell-derived microvesicles (RBC-MV)

Mouse RBC-MV were generated by aging. Briefly, blood was obtained by cardiac puncture following sacrifice. Erythrocytes (RBC) were purified by dextran sedimentation. RBC were stored between 16 and 18 days at a ratio of 2:1 in the human erythrocyte-storage solution PAGGSM at 4°C in polystyrene tubes (BD). Storage time was chosen empirically to maximize yield of RBC-MV and simultaneously minimize concurrent hemolysis. Preliminary experiments had shown significant hemolysis (hematocrit < 75% from baseline) in samples stored for longer than 18 days. Following storage, supernatants were diluted with PBS and sequentially centrifuged at 250g, 1210g and 13000g. The final pellet containing RBC-MV was diluted in PBS, stored at 4°C and used within 24 hours. RBC-MV were analyzed by flow cytometry in 0.22 µm-filtered buffers. Trucount beads (BD) were used for quantitation. RBC-MV were gated on logarithmic FSC/SSC with an arbitrary threshold set on SSC. RBC-MV were further identified as double positive for Ter119 and AnnexinV. The number of AnnexinV-positive RBC-MV/ml was calculated as follows: (number of Trucount beads x % RBC-MV of total events (FSC/SSC) x % AnnexinV+ of total Ter119+) / % Trucount beads. Flow cytometry was performed using a CyanADP cytometer (Beckman Coulter, Nyon, Switzerland). Data were analyzed using FlowJo Software (TreeStar, San Jose, USA).

Generation of liposomes

Unilamellar liposomes, composed of either L-α-phosphatidylcholine (PC) alone or PC together with equimolar concentrations of L-α-phosphaditylserine (PSPC, both Avanti Polar Lipids, Alabaster, USA) were prepared according to the repeated freeze–thawing method<sup>1</sup> and extruded using nucleopore polycarbonate filters (VWR, San Diego, USA). Final liposome concentration was 16 mM as measured by a standard
phosphate assay. Liposome diameter was approximately 200 nm as determined by electron microscopy.

Two-hit experimental protocol

Mice were primed with an intraperitoneal (i.p.) injection of Lipopolysaccharides (LPS from Escherichia coli 055:B5 used at 1 mg/kg, Cat. Nr. L2880, Sigma, Buchs, Switzerland) followed two hours later by intravenous (i.v.) injection of 5x10^8 or 1x10^9 RBC-MV in a total volume of 200 µl PBS via the tail vain. At the indicated times, mice were sacrificed with thiopental and organs were harvested. Where indicated, LPS-primed mice received 200 µl of hemoglobin (Hb) at 9 mg/ml or 90 mg/ml i.v. prepared from stored erythrocytes instead of RBC-MV. Hb was obtained by freeze-thawing 18 day-old RBC followed by centrifugation at 13000g for 1 hour. Supernatants were analyzed for Hb concentration measuring absorbance at 405 nm with known concentrations of human Hb (Sigma) as standard. Hb content of RBC-MV was determined accordingly. Alternatively, LPS-primed mice received equimolar amounts of PC- or PSPC-liposomes i.v. instead of RBC-MV. Where indicated, the specific thrombin inhibitor refludan (Celgene, Boudry, Switzerland) was given i.p. at 2 mg/kg 15 min prior to and again two and four hours following injection of LPS, respectively.

Electron microscopy

RBC-MV were fixed in 1% glutaraldehyde and adsorbed to parlodion-coated copper grids. After washing, samples were stained with 2% uranylacetate and analyzed using a Philips Morgani 268D transmission electron microscope operated at 80 kV.

Lung histology

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin. 10 µm sections were stained with H&E and analyzed on an Olympus BX61 microscope.

Determination of lung water

Left lungs were removed intact, weighed and dried for > 24 hours. Following drying, lungs were weighed again. The percentage of lung weight accounted for by water was determined as follows: (([pre-drying weight] - [post-drying weight]) / [pre-drying weight]) x100.

Analysis of blood and tissue leukocytes

Peripheral blood was obtained by cardiac puncture. To obtain lung-infiltrating leukocytes, right lungs were excised without prior perfusion and digested for 30 min at 37°C in RPMI-2.5% FCS + 2 mg/ml Collagenase IV + 0.1 mg/ml DNAse I (both Sigma). RBC were removed by hypotonic lysis. Blood leukocytes were counted using GLASSTIC® slides (Hycor, USA), the total cell number of the pulmonary homogenate was counted with an automated cell counter (Beckman Coulter). Pulmonary neutrophil numbers were calculated by multiplying the total cell number of the right lung homogenate by % CD45^+ CD11b^+ Ly6C^+ Ly6G^+ cells following exclusion of doublets and dead (7-AAD^+) cells as determined by flow cytometry. Pulmonary monocyte numbers were calculated accordingly with monocytes being CD45^+ CD11b^+ Ly6C<sub>high</sub> Ly5G^+.

ELISA and thrombin assay
Serum concentrations for IL-6, KC and MCP-1 were analyzed by ELISA following the manufacturers instructions (IL-6 and MCP-1 BD OptEIA, KC R&D Systems). For plasma C5a levels and thrombin activity, blood was drawn into tubes containing 10 mM EDTA. For C5a, ELISA plates were coated with purified monoclonal mouse C5a-specific IgG (5 µg/ml, BD). Following blocking with 10% FCS in PBS, plasma was added. C5a was revealed using a biotinylated monoclonal mouse C5a-specific antibody (0.5 µl/ml, BD) and SA-HRP. Recombinant human C5a (BD) was used as a standard. Thrombin activity was determined using fresh plasma and TH8198 (Pefachrome®, DSM, Basel, Switzerland) as substrate. After thrombin-mediated cleavage of the substrate, the amount of p-nitroaniline dihydrochloride formed was measured by absorbance at 405 nm. The assay was performed in the presence of aprotinin (Sigma). Unspecific absorbance was corrected for by subtracting absorbance in the absence of TH8198 from absorbance in the presence of TH8198 for each individual plasma sample (ΔOD).

In vitro complement binding studies

Blood from Rag⁻/⁻ or wild-type mice was obtained by cardiac puncture and immediately put on ice. Plasma was harvested following centrifugation (10 min, 3000g). Anticoagulated plasma was obtained from mice injected 100µg refludan i.p. 5 min prior to cardiac puncture the blood being aspirated into syringes containing 100µg refludan. 5 µl RBC-MV were incubated with 10% plasma for 30 min at 37°C. Where indicated, 5 mM EGTA with or without 1 mM Mg were added to RBC-MV during incubation. Samples were then washed at 13000g for 10 min. To reveal binding of IgG, IgM, C1q or iC3b, RBC-MV were incubated with the respective fluorochrome-tagged mAbs, washed and analyzed by flow cytometry.

Statistical analysis

All data are presented as mean ± SEM. Depending on data distribution, comparisons between treatment groups were performed using one-way ANOVA with Bonferroni’s multiple comparison post-testing or using a two-tailed Mann-Whitney U test as indicated (Prism; GraphPad Software). Significance was set at p < 0.05.

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Supplemental Figures

Figure I. Effect of free hemoglobin on pulmonary phenotype and serum cytokines in LPS-primed mice. LPS-primed B6 mice received an i.v. injection of PBS (LPS), 1.8 mg hemoglobin (LPS+1.8 mg Hb), 18 mg hemoglobin, or 1x10^9 RBC-MV containing 1.8 mg hemoglobin. 4 hours later, pulmonary and peripheral blood PMN counts (A) as well as serum cytokines (B) were determined. N=5-6/group pooled from >2 independent experiments each. * P < 0.05, ** P < 0.01 using one-way ANOVA and Bonferroni’s post-test (A). N=5-7/group pooled from at least three independent experiments. * P < 0.05, ** P < 0.01 using a nonparametric Mann Whitney test (B). n.s. = non-significant. Mean±SEM is shown.

Figure II. The RBC-MV-induced effects can be mimicked by phosphatidylserine-positive liposomes. RBC-MV-induced pulmonary phenotype (A) and amplification of proinflammatory cytokines (B) in LPS-primed B6 mice can be mimicked by i.v. injection of liposomes containing phosphatidylcholine (PC) and phosphatidylserine (PS) at a 1:1 ratio (L+PSPC). Injection of PSPC in the absence of LPS (PSPC) or control PC-liposomes in LPS-primed mice (L+PC) had no effect. N=7-9/group from at least three independent experiments (A and B). Blocking of PS on RBC-MV by preincubation with saturating amounts of AnnexinV prior to i.v. injection in LPS-primed mice does not reverse the RBC-MV-induced pulmonary phenotype (C) and results in highly variable serum levels of IL-6 (D). N=3-5/group from two independent experiments (C and D). * P < 0.05, ** P < 0.01 using one-way ANOVA and Bonferroni’s post-test (A) or a nonparametric Mann-Whitney test (B). n.s. = not significant. Mean±SEM is shown.
**Fig. 1**

**A**

- **PMN / right lung x 10^6**
  - LPS
  - LPS + 1.8 mg Hb
  - LPS + 18 mg Hb
  - LPS + 1x10^9 RBC-MV + 1.8 mg Hb

**B**

- **IL-6 (ng/ml)**
  - LPS
  - LPS + 1.8 mg Hb
  - LPS + 18 mg Hb
  - LPS + 1x10^9 RBC-MV + 1.8 mg Hb

- **KC (ng/ml)**
  - LPS
  - LPS + 1.8 mg Hb
  - LPS + 18 mg Hb
  - LPS + 1x10^9 RBC-MV + 1.8 mg Hb

- **MCP-1 (ng/ml)**
  - LPS
  - LPS + 1.8 mg Hb
  - LPS + 18 mg Hb
  - LPS + 1x10^9 RBC-MV + 1.8 mg Hb
Fig. II

A

PMN / right lung x10^6

B

IL-6 (ng/ml)

C

PMN / right lung x10^6

D

IL-6 (ng/ml)

- LPS
- LPS + AnnV
- LPS + 5x10^8 RBC-MV
- LPS + (5x10^8 RBC-MV + AnnV)

LPS + 5x10^8 RBC-MV
LPS + (5x10^8 RBC-MV + AnnV)